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Orientation of Substrate and Two Conformations of Lactose Permease†

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ABSTRACT: The accessibility of substrate bound to lactose permease of *Escherichia coli* was investigated by using the fluorescent substrate dansyl galactoside and a membrane-impermeable fluorescence quencher. To determine the orientation of bound substrate, both cells and inside-out vesicles were used. The substrate is oriented with the dansyl group toward the cytoplasm and the galactoside group toward the periplasm. Only half of the dansyl groups are accessible to quencher, irrespective of their orientation. This is interpreted as evidence for two different conformations of lactose permease, one with the binding site open to the cytoplasm and closed to the periplasm and vice versa for the other state.

Lactose permease (LP)[1] of *Escherichia coli* is an integral protein of the cytoplasmic membrane which uses the protonmotive force to accumulate galactosides against their concentration gradient in the cytoplasm [for reviews, see Kaback (1986) and Wright et al. (1986)]. The primary structure is known and consists of 417 amino acid residues (Büchel et al., 1980; Ehring et al., 1980). The protein was shown to be a monomer, at least in the absence of an electrochemical potential (Goldkorn et al., 1984; Dornmair et al., 1985). The secondary structure has been investigated both by circular dichroism and by Raman spectroscopy and was found to be predominantly α-helical (Foster et al., 1983; Vogel et al., 1985). Upon combination of these experimental results with structural predictions, a model for the folding of the LP within the membrane was proposed: Ten membrane-spanning α-helices form a ring whose interior is filled with relatively hydrophilic amino acid residues suited to provide the sugar binding site (Overath & Wright, 1983; Vogel et al., 1985).

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[1] Abbreviations: LP, lactose permease; ESR, electron spin resonance; CPM vesicles, cytoplasmic membrane vesicles; ISO vesicles, inside-out vesicles; DMPC, dimyristoylphosphatidylcholine; GalSGal, β-D-galactosyl-1-thio-β-D-galactoside; DnsEtOGal, 2-(*N*-dansylamino)ethyl β-D-galactoside; NpαGal, *p*-nitrophenyl α-D-galactoside; lactose, 4-*O*-β-D-galactosyl-α-D-glucose; TEMPOamine, 2,2,6,6-tetramethyl-4-aminopiperidine 1-oxide; TMA-TEMPO, 2,2,6,6-tetramethyl-4-(trimethylammonio)piperidine 1-oxide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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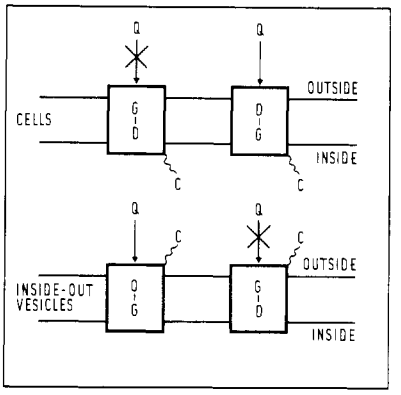

FIGURE 1: Schematic representation of the orientation of lactose permease [specified by the C terminus (C)] in the case of cells and ISO vesicles and two possible orientations of bound dansyl galactoside (D-G). The accessibility of the dansyl group to quencher (Q) in the aqueous phase is also indicated.

Previously, it had been shown that each LP molecule has only one galactoside binding site (Overath et al., 1979; Teather et al., 1980; Wright & Overath, 1984; Seckler, 1986), and evidence has been presented that the binding site is located in the interior of the protein (Mitaku et al., 1984). Transport requires that the binding site is alternatively accessible from either side of the membrane as observed experimentally (Kaczorowski et al., 1979; Wright, 1986b).

An obvious question then is to ask for the orientation of bound substrate relative to the orientation of the protein which is known to be incorporated asymmetrically with the C terminus on the cytoplasmic side (Seckler & Wright, 1984). To answer this question, we employed a substrate with a fluorescent group, dansyl galactoside (Schuldiner et al., 1975a; Overath et al., 1979), together with a membrane-impermeable fluorescence quencher and performed quenching experiments on cells and inside-out vesicles. The dansyl group of bound dansyl galactoside was shown previously to be accessible to water-soluble quenchers (Mitaku et al., 1984). The strategy of the experiment is illustrated in Figure 1. If the substrate is oriented with the dansyl group toward the side of the C terminus and the galactoside group toward the other side, fluorescence quenching should occur for inside-out vesicles and not for cells, and vice versa for opposite orientation of the substrate. Here, we have assumed that the substrate is oriented parallel to the membrane normal in any case. Orientation perpendicular to the membrane normal would lead to the same degree of quenching for cells and inside-out vesicles.

Further structural information on the protein/substrate complex is provided by the limiting value of the fluorescence intensity at high quencher concentration. If quenching occurs with the quencher present on the side of the dansyl groups, quenching may be incomplete, because part of the dansyl groups may be inaccessible, as shown schematically in Figure 2. This would be consistent with the common four-state model used to describe the kinetics of transport (Patlak, 1957). Here, the protein can adopt two different conformations, one with the binding site open to one side of the membrane and closed to the other, and vice versa for the other conformation. Both conformations exist with and without substrate, leading to altogether four different states of the protein. All kinetic data are compatible with this model (Wright, 1986a,b), but structural data in support of the two different conformations have not yet been provided. By performing time-resolved fluorescence measurements up to high quencher concentrations, we were able to demonstrate that LP exists in at least two different conformations.

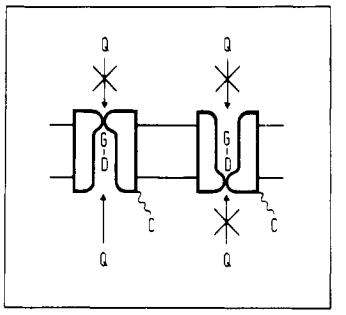

FIGURE 2: Schematic representation of the two different conformations of lactose permease and the accessibility of bound DnsEtOGal to quencher.

MATERIALS AND METHODS

Chemicals. Dimyristoylphosphatidylcholine (DMPC) was purchased from Fluka (Neu-Ulm, FRG) and β-D-galactosyl 1-thio-β-D-galactoside (GalSGal) from Sigma (München, FRG). 2,5-bis(4-biphenylyl)oxazole was obtained from EGA-Chemie (Steinheim, FRG). *p*-Nitrophenyl α-D-[6-^{3}H]galactoside ([6-^{3}H]NpαGal) and 2-(*N*-dansylamino)ethyl β-D-galactoside (DnsEtOGal) were gifts of P. Overath. The spin-labels 2,2,6,6-tetramethyl-4-aminopiperidine 1-oxide (TEMPOamine) and 2,2,6,6-tetramethyl-4-(trimethylammonio)piperidine 1-oxide (TMA-TEMPO) were from Molecular Probes (Junction City, OR).

Sample Preparation. We used the permease-overproducing strain *E. coli* T206, which was shown to bind DnsEtOGal and to perform active transport (Teather et al., 1980). *E. coli* T206 was grown and cytoplasmic membrane (CPM) vesicles were prepared according to Osborn et al. (1972) with slight modifications as described by Wright et al. (1983). In our hands, these CPM vesicles were rather leaky. Another type of CPM vesicles was prepared by high-pressure lysis as described by Seckler and Wright (1984). These authors showed that in such vesicles 95% of the LP molecules are oriented inside-out and, therefore, they will be denoted inside-out (ISO) vesicles.

To prepare cells lacking an electrochemical potential, cells were washed twice in 0.2 M Tris-HCl buffer, pH 8.0, and 10 mM NaN_3 by centrifugation and kept on ice for 15 h under nitrogen. They were resuspended to A_{420} = 130 and treated with 1 mM NaEDTA for 2 min in order to ensure that the outer membrane is permeable (Leive, 1965). The treatment was stopped by addition of 4 mM $MgSO_4$. After centrifugation (5 min, 13000*g*), cells were resuspended in the same buffer. Permease concentrations used for quenching experiments were 20–30 μM for all preparations. Cells, CPM vesicles, and ISO vesicles were incubated with 50 μM tetraphenylphosphonium bromide and 50 μM DnsEtOGal for at least 2 h before measurement, protected from light and oxygen. For each of the preparations, reference samples were prepared containing 5 mM of the competitive inhibitor GalSGal in order to separate the signal of DnsEtOGal bound specifically to LP from signals arising from DnsEtOGal dissolved in water, bound to the membrane, or attached unspecifically to other proteins (Mitaku et al., 1984). To correct for scattered light and intrinsic fluorescence, cells, CPM vesicles, and ISO vesicles were prepared in the same way without DnsEtOGal.

Cells, CPM vesicles, and ISO vesicles were titrated with TMA-TEMPO up to a concentration of 12.5 mM. For each quencher concentration, a new set of samples was prepared consisting of one without DnsEtOGal, one with DnsEtOGal, and one with DnsEtOGal and GalSGal. The samples were measured, quencher was added, and the measurement was repeated within 4 min. They were stirred continuously. CPM

vesicles were sonicated with the given amount of TMA-TEMPO for 5 min in order to let the quencher equilibrate between the external and internal space of the vesicles.

Flow Dialysis Experiments. Permease concentrations were determined by flow dialysis (Wright et al., 1983). Addition of 10 mM TMA-TEMPO did not affect the binding activity of the permease.

To investigate whether substrate is accumulated in the cells, 1 mL of EDTA-treated cells containing 11 μM permease was incubated with 10 μM [6-^{3}H]NpαGal (2.7 TBq/mol) and 50 μM tetraphenylphosphonium bromide in 0.2 M Tris-HCl buffer, pH 8.0, and 10 mM NaN_3 for 2 h, protected from light and oxygen. Cells were loaded into the upper compartment of the flow dialysis chamber. The lower compartment was flushed with degassed buffer, and fractions of 1.1 mL/min were collected. In order to detect accumulated [6-^{3}H]NpαGal, either 1 mM lactose was added or cells were sonicated repeatedly for some seconds on ice with a Branson B12 sonifier equipped with a micro tip at a power setting of ~40 W, until the suspension became opalescent. In both experiments, bound [6-^{3}H]NpαGal was released by adding 5 mM GalSGal.

ESR Measurements. Electron spin resonance (ESR) spectra were recorded on a Varian E-line century series spectrometer with a Bruker ER 140 data system. The spectrometer was operating at 9.5 GHz, modulation 2.5 mT, and microwave power 20 mW.

To examine the membrane impermeability of the spin-label TMA-TEMPO, a Ni^{2+}/Tris complex was used. Measurements were performed in 0.2 M Tris-HCl, pH 8.0, 10 mM NaN_3, 120 mM Ni^{2+}/Tris, and 10 mM TMA-TEMPO on cells (A_{420} = 50) or ISO vesicles (16 mg/mL protein). Control experiments were performed with the membrane-permeable TEMPOamine (1 mM) instead of TMA-TEMPO. Internal volumes were calculated by comparing the spectra of spin-label in the inner compartment with spectra of spin-label in buffer without Ni^{2+}/Tris (Berg et al., 1978).

Steady-State Fluorescence Measurements. A Perkin-Elmer MPF3 fluorometer was used to measure steady-state fluorescence. The excitation wavelength was 335 nm, and the emission was detected between 390 and 600 nm. The direction of the detected light was perpendicular to the exciting light, and the 1 × 1 cm cuvette was oriented to make an angle of 35° with the exciting light. By this surface-front technique, it was possible to reduce the background of scattered light to 20% for vesicles and to 35% for cells of the fluorescence at 500 nm.

The spectrum of DnsEtOGal bound to LP was obtained by subtracting from the spectrum of membranes with DnsEtOGal the spectrum of membranes with DnsEtOGal and GalSGal (Mitaku et al., 1984). The spectrum of DnsEtOGal bound unspecifically to membranes was obtained by subtracting from the spectrum of membranes with DnsEtOGal the spectra of DnsEtOGal bound to lactose permease, of DnsEtOGal dissolved in water, and of membranes without DnsEtOGal. All spectra were corrected for dilution. The fluorescence of DnsEtOGal bound to lactose permease was ~15% of the fluorescence at 500 nm.

Absorption of the spin-label TMA-TEMPO used as quencher was negligible compared to the dansyl fluorescence at the wavelengths and concentrations used. This is partially due to the surface-front technique, where the pathways of exciting and emitted light are short, causing considerable reduction of inner filter effects.

Time-Resolved Fluorescence Measurements. The apparatus used for these measurements has been described previously (Best et al., 1987). It consists of a pulsed laser and single-photon counting electronics. The dansyl fluorescence was excited at 320 nm and the emitted light detected at 480 nm. Data analysis was performed by fitting double-exponential decay curves to the experimental data (Best et al., 1987). For deconvolution, the fluorescence standard 2,5-bis(4-biphenylyl)oxazole in cyclohexane was employed.

Samples used for the measurements contained CPM vesicles (5 μM permease) in 0.2 M Tris-HCl buffer, pH 8.0, 10 mM NaN_3, and 50 μM DnsEtOGal. In order to separate the signal of specifically bound DnsEtOGal from the signals of DnsEtOGal bound unspecifically and dissolved in water, a reference sample containing 5 mM GalSGal was measured. The signal of the reference sample was subtracted from that of the sample before data analysis. Samples and reference samples were measured at 0, 0.5, 1, 2.5, and 5 mM TMA-TEMPO. Before the measurements, they were sonicated each for 5 min in a bath sonicator.

RESULTS

Three points are crucial for the fluorescence quenching experiments to give meaningful results: First, the quencher has to be membrane-impermeable; second, quenching must be of the collisional type; and third, the electrochemical potential of the cells has to be approximately zero for the following reason (Overath et al., 1979). The fluorescence of DnsEtOGal bound specifically to permease is determined by subtracting from the total fluorescence the contribution of substrate in water and bound unspecifically to membranes. The latter is obtained after replacing specifically bound DnsEtOGal by GalSGal. An electrochemical potential would give rise to accumulation of substrate and, concomitantly, to an increase of DnsEtOGal bound unspecifically inside the cells. Addition of GalSGal not only would replace the specifically bound DnsEtOGal but also would lead to a loss of most of the unspecifically bound DnsEtOGal. Therefore, the subtraction to determine the fluorescence of specifically bound DnsEtOGal would lead to an erroneous result, if accumulation were to occur.

Membrane Impermeability of the Quencher. The spin-label TMA-TEMPO was used as fluorescence quencher. Its membrane impermeability was examined by adding TMA-TEMPO both to ISO vesicles and to cells and trying to detect an ESR signal from label inside the vesicles or cells. For this purpose, a Ni^{2+}/Tris complex was added, which leads to line broadening of the ESR signal as shown in Figure 3A,B. Because this complex is membrane-impermeable (Keith & Snipes, 1974), only signals of spin-label outside the vesicles or cells are broadened. Signals from spin-label inside the vesicles or cells (if there are any) are obtained as the difference between the spectra of spin-label together with Ni^{2+}/Tris complex in the presence and absence of vesicles or cells. The difference spectra for TMA-TEMPO and, for comparison, TEMPOamine are shown in Figure 3C,D. The difference spectrum of TMA-TEMPO is a superposition of background and the broadened spectrum of spin-label in the presence of Ni^{2+}/Tris complex which results from nonperfect cancellation of the signals from spin-label in the outer volume. By contrast, the difference spectrum for TEMPOamine resembles the spectrum of spin-label in buffer without Ni^{2+}/Tris complex (Figure 3A). Thus, we conclude that TMA-TEMPO is membrane-impermeable. This finding is supported by the observation that the ESR signal of TEMPOamine added to cells decreases with a half-time of ~4 min, presumably due to reduction of TEMPOamine by metabolic processes within the cells, whereas TMA-TEMPO signals are stable for at least 20 min.

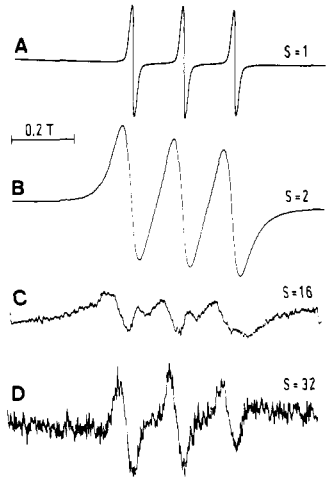

FIGURE 3: ESR spectra of TMA-TEMPO in buffer without (A) and with (B) Ni^{2+}/Tris complex and ESR signal of TMA-TEMPO (C) and TEMPOamine (D) from the inner volume of ISO vesicles (see Materials and Methods). At the right of each spectrum, the spectrometer sensitivity (S) is denoted.

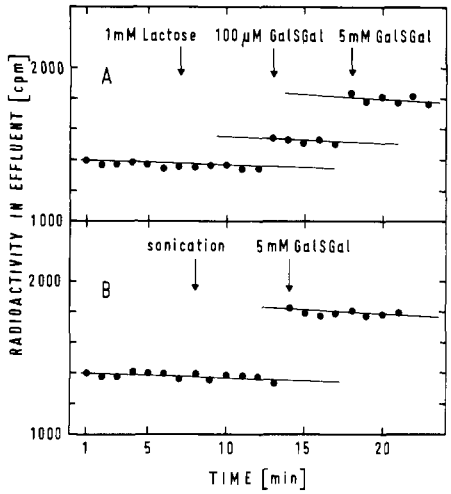

FIGURE 4: Flow dialysis of cells incubated with 10 μM [6-^{3}H]NpαGal. At the times indicated by arrows, either substrate is added to the sample (A), or the sample is sonicated (B).

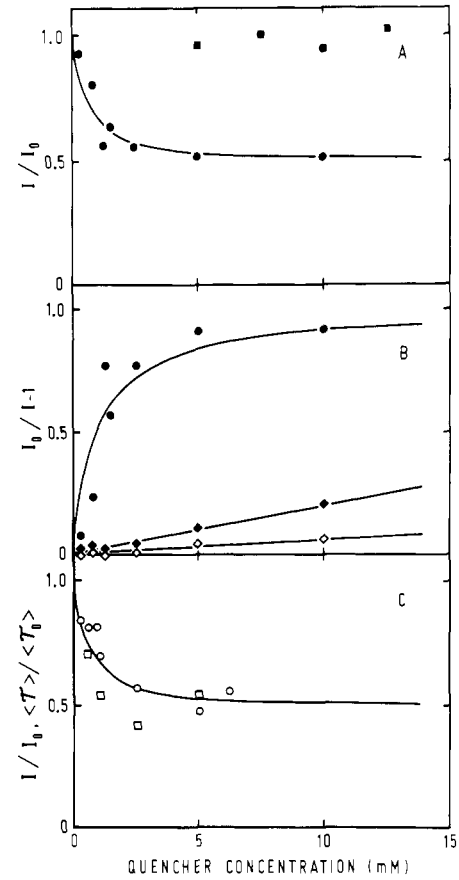

FIGURE 5: (A) Dependence of the relative fluorescence intensity, I/I_0, of DnsEtOGal bound to lactose permease on the concentration of the quencher TMA-TEMPO, for the cases of cells (■) and ISO vesicles (●). The line is a fit using eq 1 with $K_s = 2 \times 10^3$ M^{-1}. (B) Stern–Volmer plot of the quenching of DnsEtOGal bound to lactose permease for ISO vesicles as in (A) (●), DnsEtOGal bound unspecifically to membranes (♦), and DnsEtOGal dissolved in water (◊). The straight lines are fits using eq 2 with $K_s = 20$ M^{-1} for (♦) and $K_s = 6.3$ M^{-1} for (◊). (C) Dependence on quencher concentration of the relative intensity, I/I_0 (○), and of the relative mean lifetime, $\langle\tau\rangle/\langle\tau_0\rangle$ (□), of DnsEtOGal bound to lactose permease for the case of sonicated CPM vesicles. The line is the same as in (A).

It is worth mentioning that from the ESR spectra of TEMPOamine (as in Figure 3D) the inner volumes of cells and ISO vesicles were determined as 2.7 and 2.4 μL/mg of protein, respectively. These numbers are comparable to values obtained with other methods (Reenstra et al., 1980; Zilberstein et al., 1979).

Absence of an Electrochemical Potential. Figure 4 shows the results of two flow dialysis experiments both indicating that DnsEtOGal is not accumulated in cells under the given conditions. Lactose is known to have a dissociation constant of $K_D = 16$ mM for pH 5.0–8.0 but a half-saturation constant for active transport of $K_T = 73$ μM (Wright et al., 1981). Thus, if 1 mM lactose is added to cells incubated with 10 μM [6-^{3}H]NpαGal of $K_D = 24$ μM, radioactivity should be released only under conditions where transport occurs, i.e., in the presence of a finite electrochemical potential. Figure 4A shows that the release of [6-^{3}H]NpαGal is unaltered, whereas 100 μM GalSGal with $K_D = 69$ μM (Wright, 1986b) releases part and 5 mM GalSGal all of the bound [6-^{3}H]NpαGal. From a complete titration, the half-saturation constant for displacement of NpαGal by lactose is obtained as 14 mM. This number agrees with the binding constant for lactose measured with vesicles which lack an electrochemical potential (Wright et al., 1981). Therefore, the cells used in our experiment also had a negligibly small electrochemical potential. Figure 4B shows that sonication of cells does not lead to a release of [6-^{3}H]NpαGal, which implies that the internal and external concentrations of substrate were the same and no accumulation had occurred. Furthermore, cells exhibit the same amount of specific fluorescence as vesicles, for the same concentration of LP (data not shown). If DnsEtOGal would have been accumulated, the "specific fluorescence" would have been overestimated (Overath et al., 1979). Thus, three experiments independently provide evidence that under the given conditions no accumulation of substrate occurs, which is equivalent to the absence of an electrochemical potential.

Orientation of Bound Substrate. The data for the fluorescence quenching of DnsEtOGal bound to LP in cells and ISO vesicles are presented in Figure 5A. The outer membrane of the cells was made permeable (see Materials and Methods) to assure that the quencher can reach the cytoplasmic membrane. In the case of cells, no quenching is observed, while in the case of ISO vesicles quenching is observed. Because LP has only one sugar binding site which must be accessible from both sides of the membrane, this result means that DnsEtOGal is bound to lactose permease with the dansyl group oriented toward the inner side of the cytoplasmic membrane and the galactoside group oriented toward the outer side. A random orientation or an orientation parallel to the

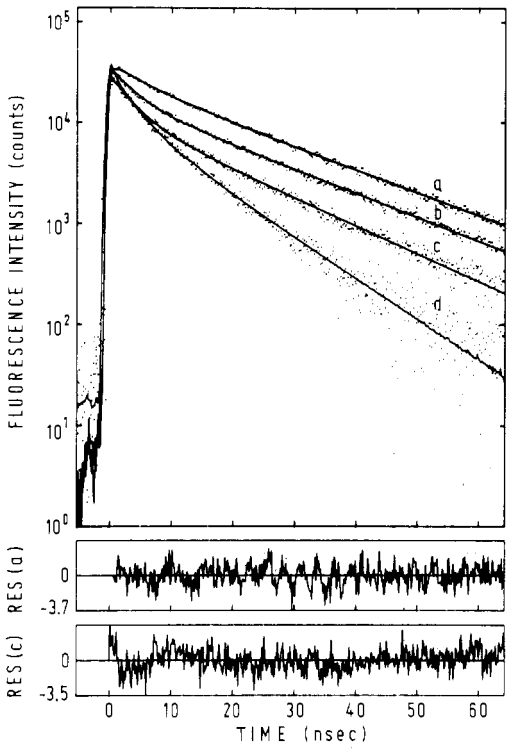

FIGURE 6: Temporal decay of the fluorescence intensity of DnsEtOGal bound to lactose permease for sonicated CPM vesicles at different concentrations of the quencher TMA-TEMPO: (a) 0; (b) 0.5 mM; (c) 1.0 mM; (d) 5.0 mM. The lines are least-squares fits to the data assuming a double-exponential decay. The residuals of the fits for cases a and c are included. The results for the relaxation times and relative amplitudes in case a without quencher were $\tau_0^1 = 5.9$ ns, $b_0^1 = 0.3$, $\tau_0^2 = 19.2$ ns, and $b_0^2 = 0.7$, yielding a mean lifetime of $\langle\tau_0\rangle = 15.1$ ns. These results agree with those of Schuldiner et al. (1975b). The results for the other cases are documented in Figure 5C.

membrane plane is excluded, since no quenching is observed with cells.

As a control, we performed measurements on sonicated CPM vesicles, where the quencher is present on both sides of the membrane. For these measurements to be consistent with the previous ones, the sum of the quenching found with cells and ISO vesicles should be observed. This was indeed the case as shown in Figure 5C.

Two Species of Bound Substrate. It is obvious from Figure 5A,C that the fluorescence intensity of DnsEtOGal bound to LP in ISO vesicles or CPM vesicles does not approach zero at high quencher concentration but levels off at about $I_\infty = I_0/2$. This finding can be interpreted in two ways (Eftink & Ghiron, 1981). Under the assumption of a collisional quenching mechanism, a nonquenchable contribution to the fluorescence implies a nonaccessible species of bound substrate. Because LP has only one sugar binding site (Overath et al., 1979; Teather et al., 1980; Wright & Overath, 1984; Seckler, 1986), the accessible and nonaccessible species of substrate must correspond to two different conformations of the protein/substrate complex. Assuming a static quenching mechanism, the same observation may be interpreted as binding of the quencher to the protein/substrate complex with incomplete quenching of the fluorescence.

To distinguish between these two possibilities, we performed time-resolved fluorescence measurements on CPM vesicles. The results are shown in Figure 6. Upon addition of quencher, the fluorescence intensity at time zero, i.e., at the time of excitation by a light pulse, remains constant, whereas the fluorescence decay becomes faster. The decrease of the mean fluorescence lifetime, $\langle\tau\rangle$, with quencher concentration agrees with the decrease of the steady-state fluorescence intensity (I) (Figure 5C). Such behavior is characteristic for a collisional quenching mechanism (Eftink & Ghiron, 1981). Hence, LP exists in two different conformations: one with the bound substrate accessible from the inner side of the membrane and not from the outer side, and vice versa for the other conformation (Figure 2). The population of the two conformations is determined by the level of quenching at high quencher concentration. The result $I_\infty = I_0/2$ indicates that the two conformations are equally populated under our experimental conditions.

The collisional quenching effect observed either in the steady state or in time-resolved measurements may be described by the relation (Eftink & Ghiron, 1981)

$$I = (I_0/2)\left(1 + \frac{1}{1 + K_d c}\right) \quad (1)$$

Here, c denotes the quencher concentration and K_d the quenching constant. A fit to the experimental data of Figure 5A yields $K_d = 2 \times 10^3$ M^{-1}. For comparison, the quenching of the fluorescence of DnsEtOGal bound unspecifically to membranes or dissolved in water can be described by the relation

$$I = I_0 \frac{1}{1 + K_d c} \quad (2)$$

From a Stern–Volmer plot of the data (Figure 5B), one obtained $K_d = 20$ M^{-1} and $K_d = 6.3$ M^{-1} for unspecifically bound and water-dissolved DnsEtOGal, respectively.[2]

Discussion

LP is known to have only one binding site for galactosides. This has been shown by different types of experiments, such as double labeling (Overath et al., 1979), measurement of binding stoichiometry (Wright & Overath, 1984), or quantitative immunoprecipitation (Seckler, 1986). A further argument in support of a single binding site is that every substrate is able to inhibit competitively the binding and transport of all other galactosides (Wright et al., 1979). The quenching data obtained by us indicate that DnsEtOGal bound to LP is oriented with the dansyl group toward the cytoplasm and the galactoside group toward the periplasm. Evidence has already been presented that the binding site is located in the interior of the protein (Mitaku et al., 1984). Thus, for a DnsEtOGal molecule to be transported into the cell, the dansyl group has to be first to enter the channel formed by the LP. To exit from the cell, the reverse is true, and the galactoside group enters the channel first. At first glance, this mechanism might seem strange, as LP is designed to recognize the galactose moiety selectively and transport lactose into the *E. coli* cell. Thus, one would expect the galactoside moiety to enter the protein first during transport into the cell. It was shown, however, that the kinetic constants for influx and efflux are the same in the absence of a membrane potential, providing evidence that LP is functionally symmetric (Wright, 1986b;

[2] The value for the quenching constant (K_d) of DnsEtOGal bound to lactose permease is high compared to the quenching constant of unspecifically bound and water-dissolved DnsEtOGal. This difference may be explained by considering the orientation and accessibility of DnsEtOGal molecules in the different cases. In water and bound unspecifically, the DnsEtOGal molecules are oriented randomly while hit by quencher molecules, whereas DnsEtOGal molecules bound to lactose permease are oriented always in the same way in the collision process. If this orientation is optimal for energy transfer from DnsEtOGal to quencher, the quenching constant here is larger than in the case of random orientation.

Kaczorowski et al., 1979; Seckler & Wright, 1984).

Our quenching experiments, furthermore, indicate that the DnsEtOGal molecules bound specifically to LP fall into two species. How can this observation be reconciled with the existence of only one binding site? Obviously, the channel for galactoside transport cannot merely be a membrane-spanning hole, because all molecules of the size of a sugar molecule would be able to cross the membrane through this pore. When the channel is open to one side, i.e., the sugar binding site is accessible from this side, it should be closed to the other side by a barrier. After binding of substrate, the protein undergoes a change to a conformation in which the channel is open to the other side of the membrane and closed to the side where the substrate came from. Finally, the substrate leaves the binding site to the other side of the membrane, and the protein undergoes a conformational change back into the initial conformation (Figure 2). Hence, this model involves one binding site, but two species of bound substrate molecules: one species accessible from one side of the membrane and not from the opposite side, and vice versa for the other species. This is what we observed experimentally, so that our result finds a very simple interpretation.

Two conformations of a transport protein with the binding site open to one or the other side of the membrane have been introduced in all common models of transport (Patlak, 1957; Sanders et al., 1984; Wright, 1986a), and evidence for their existence has been deduced from kinetic measurements. In the present paper, the two different conformations are distinguished for the first time by structural investigation. Their populations were found to be equal in number under our experimental conditions, i.e., in the absence of an electrochemical potential. This result is in agreement with a detailed analysis of kinetic data for sugar transport by LP (Wright, 1986b). This analysis led to the conclusion that the two conformations of the protein exposing the binding site to either side of the membrane are equally populated in the absence of membrane potential.

Active transport involving accumulation of substrate in the cell would occur if the two states were populated unequally, the one with the binding site open to the cytoplasmic side being populated more strongly. For the reasons given under Results, the technique used in this study is not suitable for studying the distribution of DnsEtOGal molecules in the presence of a membrane potential. However, it would be interesting to investigate the effect of an electrochemical potential on the distribution of the two conformations of LP by an appropriate technique.

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