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Dansyl-Galactoside, a Fluorescent Probe of Active Transport in Bacterial Membrane Vesicles*

(β-galactoside/carrier/fluorescence/wavelength shift)

JOHN P. REEVES, EMANUEL SHECHTER†, RUDOLF WEIL‡, AND H. R. KABACK

Roche Institute of Molecular Biology, Nutley, New Jersey 07110; †C.N.R.S., Centre de Genetique Moleculaire, Gif-sur-Yvette, France, and Laboratoire des Biomembranes, Universite de Paris, XI, Orsay, France; and ‡ Sandoz Forschungsinstitut, Wein, A 1235, Vienna, Austria

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ABSTRACT **A fluorescent galactoside, 2-(*N*-dansyl)-aminoethyl β-D-thiogalactoside (dansyl-galactoside), competitively inhibits lactose transport by membrane vesicles of *Escherichia coli*, but is not actively transported. An increase in dansyl-galactoside fluorescence is observed upon addition of D-lactate. The fluorescence increase is not observed in membrane vesicles lacking the β-galactoside transport system, and is blocked or rapidly reversed by addition of β-galactosides, sulfhydryl reagents, inhibitors of D-lactate oxidation, or uncoupling agents. The fluorescence increase exhibits an emission maximum at 500 nm and excitation maxima at 345 nm and at 292 nm. The latter excitation maximum is absent unless D-lactate is added, indicating that the bound dansyl-galactoside molecules are excited by energy transfer from the membrane proteins. Titration of vesicles with dansyl-galactoside in the presence of D-lactate demonstrates that the β-galactoside carrier protein represents about 3.3% of the total membrane protein. The data indicate that D-lactate oxidation leads to binding of the fluorescent galactoside to the β-galactoside carrier protein in such a manner that the dansyl group is transferred to a hydrophobic environment within the membrane.**

Transport of β-galactosides and various other metabolites by membrane vesicles of *Escherichia coli* is coupled primarily to oxidation of D-lactate (1–12) or reduced phenazine methosulfate (13). Other oxidizable substrates, such as succinate, L-lactate, α-hydroxybutyrate, and NADH, also support transport, but not nearly as effectively as D-lactate or reduced phenazine methosulfate. D-Lactate oxidation is catalyzed by a membrane-bound, flavin-linked D-lactate dehydrogenase which has been purified to homogeneity by Kohn and Kaback (14) and independently by Futai (15). Studies of the effects of electron-transfer inhibitors and various electron donors on transport of lactose (3, 4) and amino acids (6) indicate that the energy-coupling site for transport lies within a segment of the respiratory chain between D-lactate dehydrogenase and cytochrome b_1. Recent genetic evidence suggests that an electron-transfer-coupling component mediates energy transfer between the respiratory chain and carriers (16).

Fluorescent compounds that exhibit polarity-dependent fluorescence properties have been used to investigate the structure of biological membranes (17). Two such compounds, 1-anilino-8-naphthalene sulfonate (18) and dansyl phosphatidyl ethanolamine (19) have been used to study structural changes associated with transport in membrane vesicles of *E. coli*.

This report describes studies with a fluorescent β-galactoside analogue, 2-(*N*-dansyl)-aminoethyl β-D-thiogalactoside (dansyl-galactoside). Although this compound is not transported, it competitively inhibits lactose uptake by membrane vesicles. During D-lactate oxidation the fluorescent galactoside binds to the β-galactoside carrier protein and is transferred into a nonpolar environment within the membrane. The results indicate that D-lactate oxidation is coupled to one of the initial steps in transport.

METHODS

Growth of Bacteria and Preparation of Membrane Vesicles. Cells of *E. coli* ML 308-225 ($i^-z^-y^+a^+$), ML 30 ($i^+z^+y^+a^-$), or ML 3 ($i^+z^-y^-a^+$) were grown in minimal medium A containing 1% succinate as the sole source of carbon. Membrane vesicles were prepared as described (20).

Synthesis of Dansyl-Galactoside. 2-Aminoethyl tetra-*O*-acetyl-β-D-thiogalactoside hydrobromide was prepared by reaction of tetra-*O*-acetyl-1-thio-β-D-galactose with 2-bromoethylamine hydrobromide. The product was purified by recrystallization and dansylated by reaction with dansyl chloride for 2 hr at room temperature (20°C, pH 9.5). The dansylated product was purified by silicic acid chromatography and recrystallization. Acetyl groups were removed by overnight incubation at 4° in absolute methanol saturated with ammonia. The final product, *N*-dansyl-aminoethyl thiogalactoside, was purified by chromatography on Sephadex LH-20 in methanol followed by repeated recrystallization from 2-propanol.

Fluorescence Was Measured at an angle of 90° with an Aminco Bowman spectrofluorometer with 1-cm² cuvettes. The sample chamber was maintained at 23°C with a circulating water bath. Spectra were not corrected for variations in either intensity of the light source or response of the photomultiplier with wavelength. Additions to the cuvette were made with Hamilton microsyringes. Samples were mixed within 2–3 sec with a small plastic stick.

Lactose Transport by membrane vesicles was assayed at a final concentration of 0.4 mM (3, 4, 21).

N-Ethylmaleimide (NEM) Protection Experiments. Membrane vesicles were incubated for 5 min with 1.0 mM *N*-ethylmaleimide in the presence of various concentrations of dansyl-galactoside. After the reactions were stopped by addition of dithiothreitol (10 mM final concentration), the vesicles

Abbreviations: TDG, β-D-galactosyl-1-thio-β-D-galactopyranoside; TMG, methyl-1-thio-β-D-galactopyranoside; NEM, *N*-ethylmaleimide.

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TABLE 1. *Transport of fluorescent galactosides by membrane vesicles*

Galactoside	Relative fluorescence: minus D-lactate, Supernatant	minus D-lactate, Pellet	plus D-lactate, Supernatant	plus D-lactate, Pellet
Dansyl-galactoside	100	10	100	10
Umbelliferyl-galactoside	100	10	68	47

Membrane vesicles (2 mg/ml) were incubated for 10 min at 25°C, with or without 20 mM D-lactate, in 50 μl of 50 mM potassium phosphate (pH 6.6)–10 mM $MgSO_4$ containing the fluorescent galactoside at a concentration of 0.2 mM. Subsequently, the samples were centrifuged at 40,000 × *g* for 5 min in small conical centrifuge tubes. Supernatants and pellets were separated and suspended in 1.5 ml of 1:1 (v/v) ethanol–water. The fluorescence of each sample was determined with the appropriate wavelengths for excitation and emission. Results are expressed in relative values where the control supernatant for each galactoside has been assigned a value of 100. The values given for dansyl-galactoside in the pellets are similar to those observed when identical experiments were done in the presence 0.5 mM *p*-chloromercuribenzenesulfonate. Since this reagent reacts with the lactose carrier to block exchange and efflux, the values for dansyl-galactoside in the pellet probably reflect the amount of dansyl-galactoside trapped within the extravesicular space.

were washed free of dansyl-galactoside and tested for lactose transport activity, with ascorbate-phenazine methosulfate as electron donor (13).

Reagents and Miscellaneous Analytical Methods. Methylumbelliferyl-*O*-β-D-galactoside was generously provided by Dr. W. Boos. All other chemicals were of reagent grade and were obtained from commercial sources. Protein was determined by the method of Lowry *et al.* (22).

RESULTS

Effect of Dansyl-Galactoside on Lactose Transport by Membrane Vesicles of E. coli ML 308–225. Dansyl-galactoside is a competitive inhibitor of lactose transport in membrane vesicles prepared from *E. coli* ML 308–225. The experiment shown in Fig. 1, indicates competitive inhibition (23); the apparent K_i, given by the negative inhibitor concentration at the point of intersection, is 0.032 mM. Dansyl-galactoside has no effect on either the rate or extent of proline uptake by membrane vesicles. These results suggest that dansyl-galactoside interacts with the binding site of the β-galactoside carrier protein. This conclusion is supported by experiments in which the ability of dansyl-galactoside to protect against inhibition of lactose transport by *N*-ethylmaleimide was determined. At concentrations greater than 0.25 mM, dansyl-galactoside reduces *N*-ethylmaleimide inhibition of the initial rate of lactose uptake by 30–35% (data not shown).

Transport of Dansyl-Galactoside. Although dansyl-galactoside apparently binds to the β-galactoside carrier, it is not transported by the vesicles. Membranes were incubated for 10 min in the presence of 0.2 mM dansyl-galactoside with and without 20 mM D-lactate. The samples were then centrifuged, and the supernatants and pellets were assayed for dansyl-galactoside by fluorescence measurements. Addition of D-lactate does not change the concentration of dansyl-galactoside in either the supernatant or the pellet (Table 1). A similar experiment was done with methylumbelliferyl-*O*-β-D-galacto-

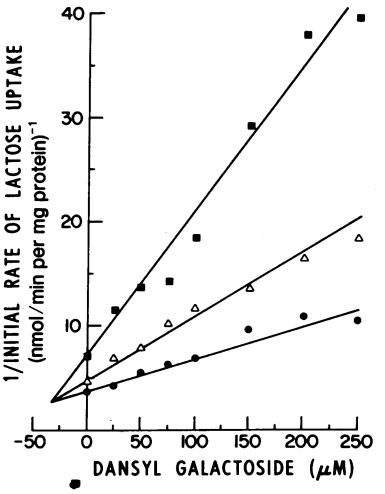

FIG. 1. Inhibition of lactose uptake by dansyl-galactoside. Initial rates (15 sec) of lactose uptake were determined in the presence of 20 mM lithium D-lactate and various concentrations of dansyl-galactoside. ■, 0.1 mM lactose; △, 0.2 mM lactose; ●, 0.4 mM lactose.

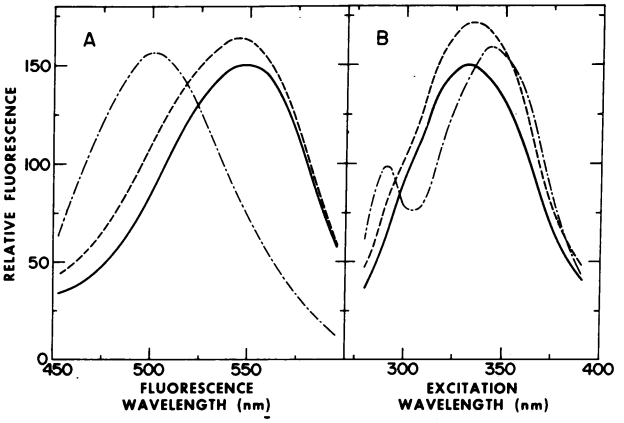

FIG. 2. Emission (*A*) and excitation (*B*) spectra of dansyl-galactoside in the presence of membrane vesicles. Dansyl-galactoside (33 μM) was added to a cuvette containing 50 mM potassium phosphate (pH 6.6), 10 mM $MgSO_4$, and membrane vesicles of *E. coli* ML 308-225 (0.4 mg of protein per ml) in a total volume of 1.5 ml. Spectra were recorded before (——) and 2 min after (– – –) addition of 20 mM lithium D-lactate. Addition of D-lactate entailed diluting the contents of the cuvette by 2%. (·——·), The difference between the spectra with and without D-lactate. Fluorescence intensity is given in relative values, and the difference spectra are plotted on a magnified ordinate scale relative to the spectra with and without D-lactate. For the emission spectra, the exciting wavelength was held constant at 340 nm; for the excitation spectra, fluorescence was monitored at 520 nm. The results are not corrected for the spectral characteristics of the light source or the photomultiplier. The slit leading to the photomultiplier was set at 1.0 mm. Difference spectra were also determined by measuring, at each excitation or emission wavelength, the percentage increase in fluorescence upon addition of D-lactate. Multiplication of the fractional increase in fluorescence at each wavelength by the relative fluorescence intensity at that wavelength generated excitation and emission spectra essentially identical to the difference spectra given above.

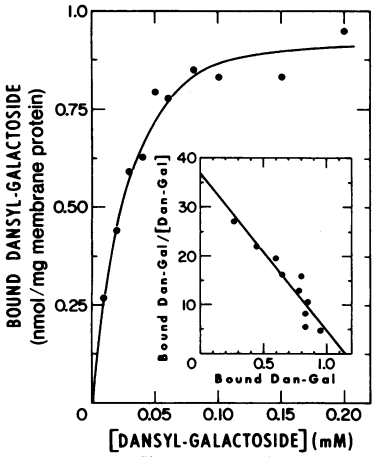

FIG. 3. Effect of dansyl-galactoside concentration on the D-lactate-dependent fluorescence changes. The percentage increase in dansyl-galactoside fluorescence upon D-lactate addition, corrected for the intensity of light scattered by the membranes, was determined at each dansyl-galactoside concentration (excitation, 340 nm; fluorescence 500 nm). The number of bound dansyl-galactoside molecules at each concentration was calculated assuming that each bound galactoside increased its fluorescence by a factor of 55. *Inset:* Data plotted according to the method of Scatchard (26). The regression line was calculated by the method of least squares. K_D (±SEM) for the fluorescence increase is 0.031 ± 0.009 mM; the number of binding sites is 1.14 ± 0.18 nmol/mg of membrane protein. *Dan-Gal*, dansyl-galactoside.

side, another fluorescent galactoside. In contrast to the results obtained with dansyl-galactoside, D-lactate produced a marked increase in fluorescence in the pellet and a corresponding decrease in the concentration of umbelliferyl-galactoside in the supernatant (Table 1). Another indication that dansyl-galactoside is not transported is that no counterflow of lactose was detected in vesicles that had first been incubated with dansyl-galactoside.

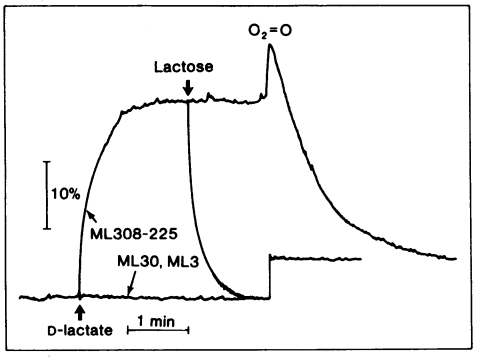

FIG. 4. Time course of changes in dansyl-galactoside fluorescence changes dependent on D-lactate. Lithium D-lactate (20 mM) was added to a cuvette containing dansyl-galactoside (0.033 mM) and membrane vesicles (ML 308-225) under conditions described in Fig. 2, and the fluorescence at 500 nm was recorded (excitation, 340 nm). Similar experiments were conducted with membranes prepared from uninduced *E. coli* ML 30 or from *E. coli* ML 3. In a second experiment with ML 308-225 membranes, 0.1 M lactose was added to a final concentration of 1 mM (*arrow*). In the absence of D-lactate, lactose had little or no effect on dansyl-galactoside fluorescence.

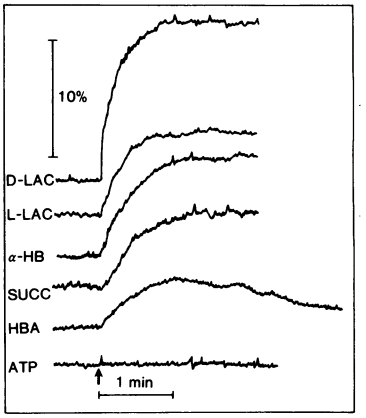

FIG. 5. Effect of electron donors on dansyl-galactoside fluorescence. Conditions were as described in Figs. 2 and 4, except that dansyl-galactoside concentration was 0.085 mM. At the *arrow*, lithium D-lactate (*D-LAC*) (20 mM), lithium L-lactate (*L-LAC*) (20 mM), α-hydroxybutyric acid (α-HB) (20 mM), disodium succinate (*SUCC*) (10 mM), 2-hydroxy-3-butynoic acid (*HBA*) (6.7 mM), or ATP (2 mM) was added. Maximal dilution caused by the additions was 2%; the curve shown for ATP has been corrected for the fluorescence decrease due to dilution.

Fluorescence Properties of Dansyl-Galactoside. Fig. 2*A* and *B* show the emission and excitation spectra, respectively, of 0.03 mM dansyl-galactoside in the presence of membrane vesicles of *E. coli* ML 308-225. Neither spectrum differs significantly from that of dansyl-galactoside alone (data not shown). When 20 mM D-lactate is added to the cuvettes, an increase in fluorescence occurs. This fluorescence increase is not observed with membrane vesicles prepared from either uninduced *E. coli* ML 30 or *E. coli* ML 3, a mutant lacking the β-galactoside transport system. Furthermore, the changes can be completely prevented or reversed by addition of 1 mM lactose (see Fig. 4).

The fluorescence increase (Fig. 2*A*) shows an emission maximum at 500 nm, a region well below the emission maximum of dansyl-galactoside in aqueous solution (550 nm). The excitation spectrum of the fluorescence increase (Fig. 2*B*) exhibits a shift to longer wavelengths at 330–350 nm. In addition, there is a maximum at 292 nm which is absent from the excitation spectrum of dansyl-galactoside in the absence of D-lactate. The maximum at 292 nm coincides with the excitation maximum for membrane-protein fluorescence, and indicates that the dansyl-galactoside molecules responsible for the fluorescence increase can be excited by energy transfer from the tryptophanyl residues of the membrane proteins. This suggestion is confirmed by the finding that the fluorescence of the membrane proteins, measured at 335 nm, decreases when D-lactate is added to membranes in the presence of dansyl-galactoside (data not shown). No such decrease is observed in the absence of dansyl-galactoside (18).

The fluorescence properties of the dansyl group are polarity dependent (19). As the environment becomes progressively less polar, the emission maximum shifts to shorter wavelengths while the excitation maximum shifts to longer wavelengths. In addition, the quantum yield of fluorescence increases markedly in nonpolar media. By comparing the emission spectra of dansyl-galactoside in various mixtures of dioxane and water, it can be shown that the dansyl groups responsible for the fluorescence increase in Fig. 2*A* are in an environ-

ment equivalent in polarity to about 85:15 dioxane–water (data not shown). The results also demonstrate that dansyl-galactoside molecules transferred from water to a medium of this polarity increase their fluorescence at 500 nm by a factor of about 55.

Thus, D-lactate oxidation results in binding of dansyl-galactoside to the membrane in such a way that the dansyl moiety is transferred from a polar to a nonpolar environment. Therefore, the difference spectra in Fig. 2*A* and *B* represent the emission and excitation spectra of the bound dansyl-galactoside.

In Fig. 3, the values for bound dansyl-galactoside were calculated from the magnitude of the fluorescence increase induced by D-lactate at 500 nm, assuming that the fluorescence of bound dansyl-galactoside is increased by a factor of 55§. As shown, the fluorescence increase induced by D-lactate is a saturable function of the dansyl-galactoside concentration. The apparent K_D (±SEM) of the fluorescence increase, calculated from a Scatchard plot (26) (*inset*, Fig. 3), is 0.031 ± 0.009 mM, a value identical to the K_t for dansyl-galactoside inhibition of lactose uptake (0.032 mM). The number of binding sites for dansyl-galactoside is 1.1 ± 0.2 nmol/mg of membrane protein.

Fluorescence Changes of Dansyl-Galactoside Induced by D-Lactate. The time course of the increase in dansyl-galactoside fluorescence induced by D-lactate is shown in Fig. 4. At 0.03 mM dansyl-galactoside, addition of D-lactate produces a 30% increase in fluorescence measured at 500 nm; the fluorescence changes are half complete in 10–13 sec at 23°C. Once the steady state has been attained, the fluorescence remains stable until the cuvette becomes anaerobic. At this point, there is an abrupt increase in fluorescence, due to reduction of an unknown membrane component, followed by a decay of dansyl-galactoside fluorescence to its original level. Membranes prepared from uninduced *E. coli* ML 30, or from *E. coli* ML 3, a mutant lacking the β-galactoside transport system, show no change in dansyl-galactoside fluorescence upon addition of D-lactate (Fig. 4). Upon anaerobiosis, the fluorescence of these membranes increases and thereafter remains steady. The membrane component responsible for the increased fluorescence during anaerobiosis exhibits an emission maximum at 410 nm and excitation maxima at 335 nm and 295 nm. Its exact chemical nature is unknown.

Lactose completely and rapidly reverses the dansyl-galactoside fluorescence changes (Fig. 4). Moreover, addition of lactose before D-lactate practically abolishes the fluorescence changes induced by D-lactate (data not shown). Similar results were obtained with β-D-galactosyl-1-thio-β-D-galactopyranoside (TDG) and methyl-1-thio-β-D-galactopyranoside (TMG).

§ This assumption requires that, before addition of D-lactate, dansyl-galactoside molecules either are not bound to the membrane or are bound in such a way that the dansyl moiety is in an aqueous environment. If binding does occur in the absence of D-lactate, the values in Fig. 3 will be underestimated by an amount proportional to the contribution of such bound molecules to the total fluorescence. Preliminary measurements, based on the decrease in fluorescence observed on the addition of high concentrations of lactose or TDG, indicate that the maximal contribution of bound galactosides to the total fluorescence in the absence of D-lactate is 2–3%.

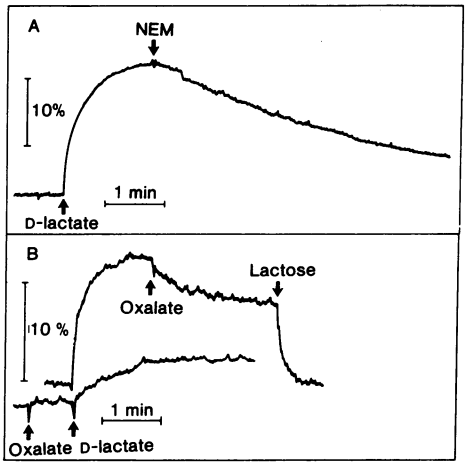

FIG. 6. (*A*) Effect of *N*-ethylmaleimide (*NEM*) on dansyl-galactoside fluorescence increase. Conditions are described in Fig. 4. NEM (1.0 mM) was added at the point indicated and, after the instrument was rapidly readjusted to correct for the effects of NEM absorbance at 340 nm, fluorescence at 500 nm was monitored. In the absence of D-lactate, NEM produced no change in dansyl-galactoside fluorescence other than that due to its own absorbance at 340 nm. (*B*) Effect of dipotassium oxalate (10 mM) on the increase of dansyl-galactoside fluorescence dependent on D-lactate. *Lower curve*, oxalate added before D-lactate; *upper curve*, oxalate added after D-lactate.

The relative effects of various electron donors on the fluorescence of dansyl-galactoside are shown in Fig. 5. D-Lactate is clearly the most effective energy source in stimulating both the rate and extent of the fluorescence changes. L-Lactate, α-hydroxybutyrate, and succinate also stimulate the fluorescence changes, but they are less effective than D-lactate. The fluorescence changes correlate well with the effects of these electron donors on lactose transport (2–4). Ascorbate-phenazine methosulfate could not be tested as an energy source because phenazine methosulfate reduction results in large absorbance changes that interfere with the fluorescence measurements. The "suicide substrate," 2-hydroxy-3-butynoate (7), also stimulates dansyl-galactoside binding to a small extent, but the fluorescence decays to its original level as D-lactate dehydrogenase becomes inactivated (Fig. 5). Neither addition of ATP nor sudden acidification of the medium (ΔpH = 1 unit) results in increased fluorescence of dansyl-galactoside.

Although data will not be presented in detail, the fluorescence increase dependent on D-lactate is blocked by the electron transfer inhibitors 2-heptyl-4-hydroxyquinoline-*N*-oxide (40 μM), amytal (10 mM), and KCN (10 mM). Moreover (Fig. 4) anaerobiosis reverses the fluorescence changes induced by D-lactate. The fluorescence changes are also completely blocked by 5 μM carbonyl cyanide *m*-chlorophenylhydrazone and by treatment of the membranes with mouse duodenal phospholipase AB.

N-Ethylmaleimide also inhibits the increase in fluorescence of dansyl-galactoside induced by D-lactate. When *N*-ethylmaleimide is added to the cuvette after D-lactate, there is a gradual decay of dansyl-galactoside fluorescence to its original level (Fig. 6*A*). This finding represents a striking difference between the properties of the dansyl-galactoside fluorescence changes and lactose transport activity. Sulfhydryl reagents

block lactose uptake in membrane vesicles, but do not cause efflux when added to preloaded vesicles (2, 4). The failure to observe efflux is due in part to the fact that these reagents react with the lactose carrier itself; indeed, lactose exchange in membrane vesicles is blocked by sulfhydryl reagents (4). The difference between the effects of *N*-ethylmaleimide on β-galactoside transport and on the fluorescence behavior of dansyl-galactoside indicates that the fluorescence changes do not reflect events that occur subsequent to transport of the fluorescent galactoside into the vesicle

Potassium oxalate, a competitive inhibitor of D-lactate dehydrogenase, reduces both the rate and extent of the fluorescence increase induced by D-lactate when added before D-lactate (Fig. 6*B*, *lower curve*). When added after D-lactate (*upper curve*, Fig. 6*B*), oxalate reduces the magnitude of the fluorescence change by about 35%. The remainder of the fluorescence increase is reversed by addition of lactose (Fig. 6*B*), TDG, or TMG. Similar results have been obtained with oxamic acid, another competitive inhibitor of D-lactate oxidation. These results correlate well with the effects of oxamate and oxalate on transport of β-galactosides. These inhibitors, at concentrations sufficient to reduce the rate of D-lactate oxidation by more than 90%, markedly reduce the initial rate of lactose uptake by membrane vesicles, but nevertheless allow significant lactose accumulation over prolonged incubation periods (4, 8). Moreover, when added to preloaded vesicles, neither oxalate nor oxamate causes efflux of lactose (4, 8).

DISCUSSION

These results show that D-lactate oxidation causes transfer of dansyl-galactoside into a hydrophobic environment within the membrane. The membrane component that binds the dansyl-galactoside appears to be the β-galactoside carrier itself [i.e., the *M* protein (24)]. This contention is supported by the following observations: (*1*) Dansyl-galactoside binds to the β-galactoside carrier, as shown by its ability to competitively inhibit lactose transport and to protect the carrier partially against inactivation by *N*-ethylmaleimide. The K_i for dansyl-galactoside inhibition of lactose transport and the K_m of the dansyl-galactoside fluorescence changes have the same value. (*2*) The fluorescence changes are not seen in membrane vesicles lacking the β-galactoside transport system. (*3*) The fluorescence changes are rapidly and completely reversed by substances that bind to the β-galactoside carrier (lactose, TMG, and TDG). (*4*) Transport of dansyl-galactoside cannot be detected, making it unlikely that the fluorescence changes reflect binding of dansyl-galactoside to an intravesicular membrane component subsequent to translocation through the membrane. This interpretation is strengthened by the observation that *N*-ethylmaleimide causes a reversal of the fluorescence change induced by D-lactate although it does not cause efflux of accumulated lactose. (*5*) The number of binding sites for dansyl-galactoside (1.1 nmol/mg of membrane protein) is in excellent agreement with the number of β-galactoside carrier proteins (i.e., *M* protein) determined by Jones and Kennedy (25). Since the molecular weight of the *M* protein is 30,000 (25), 1.1 nmol/mg of membrane protein is equivalent to 3.3% of the membrane protein. The value for *M* protein obtained by Jones and Kennedy (25) is 0.35% of the total cell protein or 3.5% of the membrane protein, assuming that the latter constitutes about 10% of the protein in the intact cell (8).

There are at least three possible mechanisms by which D-lactate oxidation might lead to the observed increase in dansyl-galactoside fluorescence. D-Lactate oxidation could (*1*) increase the affinity of the β-galactoside carrier at the external surface of the membrane, (*2*) cause translocation of bound dansyl-galactoside into the hydrophobic interior of the membrane, or (*3*) make the carriers more accessible to the external medium. A detailed discussion of these alternatives will not be presented because the data do not allow a clear choice. Furthermore, the three possibilities are not mutually exclusive. The data strongly suggest that energy is coupled to one of the initial steps in transport and that facilitated diffusion, therefore, cannot be the rate-limiting step for active transport of β-galactosides.

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