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Energy-dependent binding of dansylgalactoside to the *lac* carrier protein: Direct binding measurements*

(active transport/bacterial membrane vesicles/flow dialysis/β-galactoside transport/energy coupling/fluorescence)

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ABSTRACT High specific activity 6′-(*N*-[^{3}H]dansyl)aminohexyl 1-thio-β-D-galactopyranoside (Dns^6-Gal) has been synthesized, and its binding to *Escherichia coli* membrane vesicles measured directly by flow dialysis. With ML 308-225 vesicles containing the *lac* carrier protein, specific binding is not detected in the absence of D-lactate or reduced phenazine methosulfate. In the presence of these electron donors, binding is observed, and the binding constant and number of binding sites are approximately 4 μM and 1.5 nmol/mg of membrane protein, respectively. These values are in excellent agreement with those obtained by fluorescence titration. *p*-Chloromercuribenzenesulfonate, which directly inactivates the *lac* carrier protein, and carbonylcyanide *m*-chlorophenylhydrazone, which collapses the membrane potential, cause release of bound Dns^6-Gal. Moreover, significant binding is not observed with membrane vesicles that are devoid of the *lac* carrier protein. The results provide qualitative and quantitative confirmation of previous studies which indicate that changes in dansylgalactoside fluorescence observed on "energization" of membrane vesicles reflect binding of the probe to the *lac* carrier protein.

Recent studies with (*N*-dansyl)aminoalkyl β-D-galactopyranosides (1–4) and (2-nitro-4-azidophenyl)β-D-galactopyranosides (5, 6) indicate that the *lac* carrier protein in membrane vesicles isolated from *Escherichia coli* does not bind ligand significantly in the absence of a membrane potential (interior negative). As a result of D-lactate or reduced phenazine methosulfate oxidation, or artificially induced ion gradients, an electrical potential is generated across the vesicle membrane (7–10), and changes in dansylgalactoside fluorescence and azidophenylgalactoside-dependent photoinactivation are observed. Alternatively, dilution-induced, carrier-mediated lactose efflux also causes changes in dansylgalactoside fluorescence in a manner which is apparently independent of the membrane potential (2). Based on these observations, it has been postulated (2) that the membrane potential causes the *lac* carrier protein to become accessible to the external medium, to increase its affinity for ligand, or both; and it has been suggested that the *lac* carrier protein or a part of it may be negatively charged.

The strength of these conclusions rests heavily on the contention that the fluorescence changes observed with the dansylgalactosides are due specifically to binding and not to a subsequent translocational event, and this aspect of the problem has been approached in several ways. It has been demonstrated that there is a marked increase in fluorescence anisotropy of 2′-(*N*-dansyl)aminoethyl 1-thio-β-D-galactopyranoside (Dns^2-Gal) on "energization" of membrane vesicles containing the *lac* carrier protein (2, 4), and that the lifetime of its excited-state is increased when the molecule is bound (4). Concurrently, the rotational diffusion of Dns^2-Gal is dramatically decreased (4). In addition, it has been demonstrated by various means that the dansylgalactosides are not transported to any extent (1–3), and finally that *p*-chloromercuribenzenesulfonate (*p*-CMBS) causes rapid reversal of D-lactate-induced dansylgalactoside fluorescence although it does not cause efflux of lactose from the intravesicular pool (3). The latter finding indicates that the changes in dansylgalactoside fluorescence observed on energization cannot be attributed to binding followed by translocation into the hydrophobic milieu of the membrane.

As discussed previously (9), these conclusions represent a significant departure from previous notions regarding the mechanism of active transport. For this reason, documentation of the observations by as many independent means as possible is important. We have now synthesized 6′-(*N*-[^{3}H]dansyl)aminohexyl 1-thio-β-D-galactopyranoside (Dns^6-Gal), the dansylgalactoside analogue exhibiting the highest binding affinity (2), and measured its binding to membrane vesicles by direct means. The results provide definitive evidence in support of the postulate that changes in dansylgalactoside fluorescence observed on energization of membrane vesicles containing the β-galactoside transport system reflect binding of the probe to the *lac* carrier protein. The results also provide quantitative confirmation of data obtained by fluorescence determinations.

METHODS

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 30 ($i^+z^+y^+a^+$) and ML 308-225 ($i^-z^-y^+a^+$) were grown on minimal medium A with 1.0% sodium succinate (hexahydrate), and membrane vesicles were prepared as described (11).

Binding Measurements. Binding of [^{3}H]Dns^6-Gal to membrane vesicles was measured by determining the rate of dialysis essentially as described by Colowick and Womack (12). The apparatus utilized consists of a commercial flow-type dialysis cell (1.0 ml microsize; Bel Art Products) with an additional 2.0-mm hole drilled through the top wall of the upper chamber so that aeration could be maintained. The upper and lower chambers were separated by cellulose dialysis tubing (mean pore size 4.8 nm; Fisher Scientific), and both chambers were stirred continuously by means of small magnetic bars. Membrane vesicles suspended in 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate were added to the upper chamber (total volume

Abbreviations: Dns^2-Gal, 2′-(*N*-dansyl)aminoethyl 1-thio-β-D-galactopyranoside; Dns^6-Gal, 6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside (in previous papers of this series, the abbreviations DG_2 and DG_6 were used); CCCP, carbonylcyanide *m*-chlorophenylhydrazone; *p*-CMBS, *p*-chloromercuribenzenesulfonate; TDG, β--galactosyl 1-thio-β-galactopyranoside.

* This is paper XXXI in the series "Mechanisms of Active Transport in Isolated Bacterial Membrane Vesicles." Paper XXX is given in ref. 4.

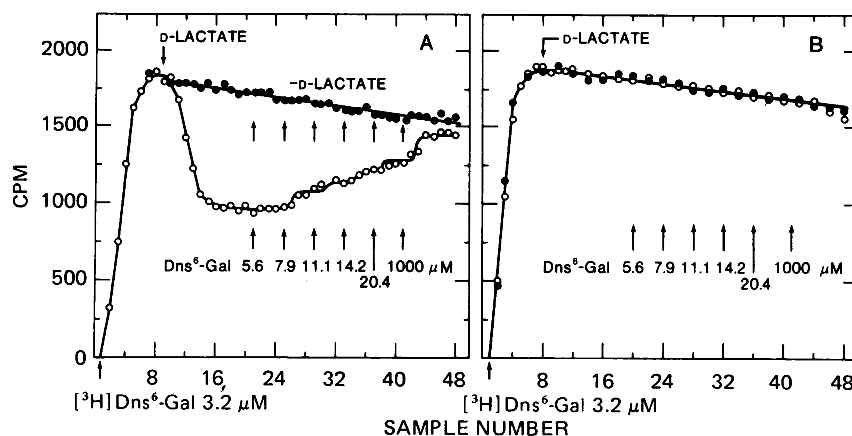

FIG. 1. Binding of [^{3}H]Dns^6-Gal by *E. coli* ML 308-225 (A) and ML 30 (B) membrane vesicles. Binding was assayed by flow dialysis as described in *Methods*. The upper chamber contained 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and the designated membrane vesicles (2.4 mg of membrane protein per ml) in a total volume of 0.8 ml. The experiment was started by addition of [^{3}H]Dns^6-Gal (2500 Ci/mol) at a final concentration of 3.2 μM. When indicated lithium D-lactate was added to the upper chamber (↓) to give a final concentration of 20 mM (O). Nonradioactive Dns^6-Gal (↑) was added with solutions sufficiently concentrated so that the volume of the upper chamber was increased by less than 3%. Fractions of 2.0 ml were collected, and 0.5-ml aliquots were assayed for radioactivity by addition of 10.0 ml of Instabray (Yorktown Research) and counting in a liquid scintillation spectrometer (Beckman LS-250). (●) No D-lactate.

0.8 ml), and at an appropriate time [^{3}H]Dns^6-Gal was added through the aperture in the top wall. Potassium phosphate (0.05 M, pH 6.6) was pumped through the lower chamber at a rate of 4.0 ml/min using a Pharmacia pump (model P3). Fractions of 2.0 ml were collected and sampled for radioactivity by liquid scintillation spectrometry.

Fluorescence Measurements. Fluorescence was measured at an angle of 90° with a Perkin-Elmer Hitachi MPF-4 spectrofluorometer using 1 × 1 cm cuvette as described (1-3). The sample chamber was maintained at 25° with a circulating water bath. The light band pass for excitation and emission was 6 nm. Additions to the cuvette were made with Hamilton microsyringes and mixing was accomplished within 3-5 sec using a small plastic stick.

Preparation of [^{3}H]Dns^6-Gal. 6-Aminohexyl 1-thio-β-D-galactopyranoside (13) was dansylated with [*U*-^{3}H]dansyl chloride (30,000 Ci/mol) using the procedure described for the synthesis of Dns^6-Gal (2). The final product was over 99% pure as judged by previous criteria (2), and the specific activity was diluted appropriately by addition of nonradioactive Dns^6-Gal.

Protein was determined by the method of Lowry *et al.* (14).

Materials. Dns^6-Gal was synthesized by methods described (2). [^{3}H]Dansyl chloride was obtained from The Radiochemical Centre, Amersham, England. All other materials used in these experiments were reagent grade obtained from commercial sources.

RESULTS AND DISCUSSION

The data presented in Fig. 1 represent a typical flow dialysis binding experiment carried out as described in *Methods*. At the inception of the experiment, [^{3}H]Dns^6-Gal (3.2 μM, final concentration) is added to the upper chamber containing ML 308-225 membrane vesicles, and within 30 sec radioactivity appears in the dialysate pumped through the lower chamber (panel A). Subsequently, the concentration of [^{3}H]Dns^6-Gal in the dialysate increases linearly for about 1.5 min, reaching a maximum which then decreases linearly at a slow rate. When D-lactate is added to the upper chamber, the concentration of [^{3}H]Dns^6-Gal appearing in the dialysate decreases dramatically to about 50% of the level observed in the absence of D-lactate. Although not shown, an identical decrease in the concentration of [^{3}H]Dns^6-Gal in the dialysate is observed on addition of ascorbate and phenazine methosulfate (20 mM and 0.1 mM, respectively). Once a new equilibrium is achieved in the presence of D-lactate, stepwise addition of nonradioactive Dns^6-Gal to the upper chamber establishes a series of new equilibria in which an increased fraction of the total ligand becomes diffusible with each addition of nonradioactive ligand. When sufficient nonradioactive Dns^6-Gal is added (i.e., 1000 μM and above), the concentration of [^{3}H]Dns^6-Gal in the dialysate approximates the value observed in the absence of D-lactate. Thus, almost all of the [^{3}H]Dns^6-Gal bound on addition of D-lactate can be displaced by nonradioactive Dns^6-Gal. It is important to note that addition of nonradioactive Dns^6-Gal to the upper chamber in the absence of D-lactate elicits no significant change in the amount of [^{3}H]Dns^6-Gal appearing in the dialysate. This result provides strong confirmation of the previous conclusion (1-4) that significant binding of dansylgalactosides to the *lac* carrier protein is not observed unless the vesicles are energized. The conclusion receives further support from the data presented in panel B, where binding determinations were carried out under identical conditions with membrane vesicles prepared from uninduced *E. coli* ML 30. These vesicles do not catalyze β-galactoside transport (15) and they do not exhibit D-lactate-induced changes in dansylgalactoside fluorescence (1, 2). As shown, there is no difference in the amount of [^{3}H]Dns^6-Gal bound by these vesicles in comparison to ML 308-225 vesicles prior to addition of D-lactate. Moreover, binding is not observed in the presence of D-lactate, and addition of nonradioactive Dns^6-Gal to the upper chamber results in no change in the concentration of [^{3}H]Dns^6-Gal in the dialysate. Because this point is critical and because nonspecific binding of dansylgalactoside can be detected by lifetime and anisotropy measurements (4), [^{3}H]Dns^6-Gal binding to ML 308-225 and ML 30 vesicles was also determined at higher protein concentrations (up to 7.5 mg/ml) in the absence of D-lactate. Data derived from these determinations are shown in Table 1. Approximately 0.1 nmol of Dns^6-Gal is bound per mg of membrane protein at 3.2 μM [^{3}H]Dns^6-Gal, and there is a slight

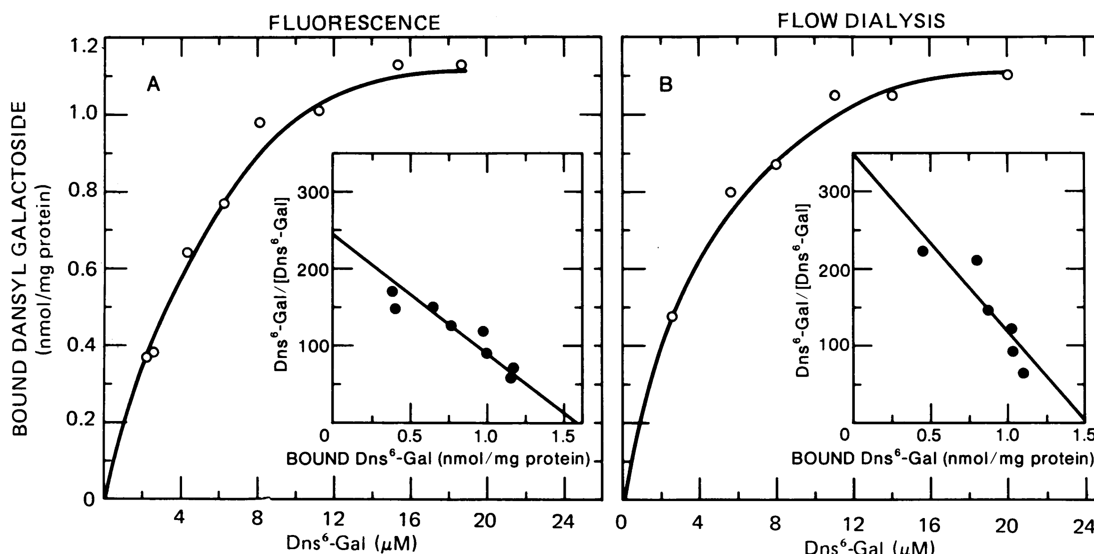

FIG. 2. Effect of Dns^6-Gal concentration on D-lactate-induced Dns^6-Gal binding by ML 308-225 vesicles. (A) Dns^6-Gal was added to a cuvette containing 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and *E. coli* ML 308-225 membrane vesicles (0.4 mg of protein per ml, final concentration) in a total volume of 1.5 ml. The percentage increase in Dns^6-Gal fluorescence upon addition of D-lactate was determined at each Dns^6-Gal concentration as described previously (2) and in *Methods* (excitation, 340 nm; emission, 500 nm). The amount of Dns^6-Gal bound at each concentration was calculated assuming that the fluorescence of the bound molecules increased by a factor of 25 (2). *Inset:* Data plotted according to Scatchard (17). (B) The data from Fig. 1A were treated as determined by Colowick and Womack (12). The equilibrium concentration of [^{3}H]Dns^6-Gal recovered in the dialysate at each Dns^6-Gal concentration is proportional to the free [^{3}H]Dns^6-Gal in the upper chamber. When a large excess of nonradioactive Dns^6-Gal (>1000 μM) is added to the upper chamber, the radioactivity in the dialysate is assumed to represent 100% of the [^{3}H]Dns^6-Gal added. From these two values the fraction of the free [^{3}H]Dns^6-Gal at each Dns^6-Gal concentration was calculated. Values for bound Dns^6-Gal were derived by difference. *Inset:* Data plotted according to Scatchard (17).

but probably insignificant difference between ML 308-225 and ML 30 vesicles. Moreover, binding observed at 7.5 mg of membrane protein per ml in the absence of D-lactate is not diminished by addition of 1.0 mM nonradioactive Dns^6-Gal, 0.1 mM *p*-CMBS, or 10 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (data not shown).

Sequential addition of increasing concentrations of nonradioactive Dns^6-Gal to ML 308-225 vesicles incubated in the presence of D-lactate causes a stepwise displacement of bound ligand (Fig. 1A). Using these data, the amount of Dns^6-Gal bound at each Dns^6-Gal concentration can be calculated (12), and the binding constant (K_D) of the *lac* carrier protein for Dns^6-Gal and the number of Dns^6-Gal binding sites can be determined (Fig. 2B). Clearly, D-lactate-induced Dns^6-Gal binding is a saturable function of the Dns^6-Gal concentration. The apparent K_D calculated from the Scatchard plot presented in the inset is approximately 4.3 μM, and the amount of Dns^6-Gal bound at saturation is about 1.5 nmol/mg of membrane protein. Within experimental error, these values are indistinguishable from those obtained by means of fluorometric titration (Fig. 2A and ref. 2). As determined by the latter method, the K_D is approximately 6.2 μM and the number of binding sites is about 1.5 nmol/mg of membrane protein.

Table 1. Nonspecific binding of Dns^6-Gal by ML 308-225 and ML 30 membrane vesicles

Membrane vesicles	Dns^6-Gal bound (nmol/mg of membrane protein)
ML 308-225	0.108
ML 30	0.085

Binding of Dns^6-Gal was measured in the absence of D-lactate as described in Fig. 1. The upper chamber contained 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, 3.2 μM [^{3}H]Dns^6-Gal (2500 Ci/mol), and ML 308-225 or ML 30 vesicles in a total volume of 0.8 ml. The membrane protein concentrations used were 0, 1.25, 2.5, 5, and 7.5 mg/ml. The concentration of [^{3}H]Dns^6-Gal in the dialysates at equilibrium was plotted as a function of protein, and from the slope of the curve, the amount of Dns^6-Gal bound per mg of protein was calculated.

Previous studies (2, 5, 6, 9, 10) indicate that the ability of membrane vesicles containing the *lac* carrier protein to bind ligand in the external medium depends upon the generation of a membrane potential (interior negative). Thus, agents that collapse the potential prevent D-lactate-induced Dns^6-Gal fluorescence and D-lactate-dependent azidophenylgalactoside photoinactivation. As shown by the experiment presented in Fig. 3A, similar results are obtained when [^{3}H]Dns^6-Gal binding is measured directly by flow dialysis. Addition of the proton conductor CCCP to ML 308-225 vesicles incubated in the presence of [^{3}H]Dns^6-Gal and D-lactate causes a rapid release of bound ligand. Similarly, previous experiments (3) demonstrate that addition of *p*-CMBS to ML 308-225 vesicles that have been incubated with various dansylgalactosides and D-lactate causes a rapid decrease in dansylgalactoside fluorescence. Analogous results are obtained when [^{3}H]Dns^6-Gal binding is measured directly by flow dialysis (Fig. 3B). Shortly after the addition of 0.1 mM *p*-CMBS to ML 308-225 vesicles incubated in the presence of [^{3}H]Dns^6-Gal and D-lactate, there is an increase in the concentration of [^{3}H]Dns^6-Gal in the dialysate. As pointed out previously (3), this finding represents a significant difference between the properties of the dansylgalactosides and lactose transport activity since sulfhydryl reagents do not cause efflux of lactose accumulated by the vesicles (3, 16).

The results presented in this communication provide a

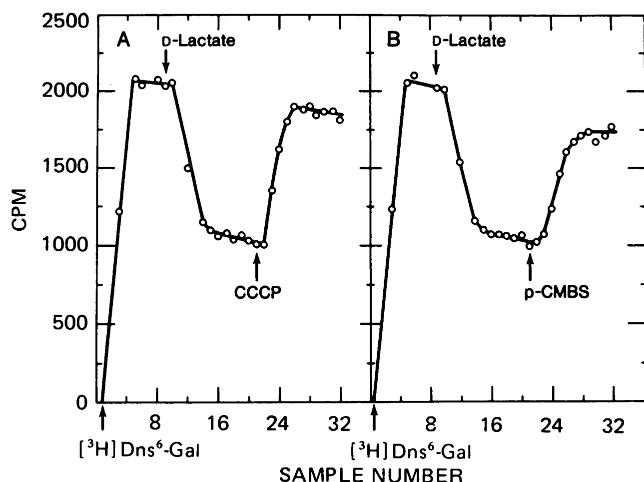

FIG. 3. Effect of CCCP (A) and *p*-CMBS (B) on D-lactate-induced Dns^6-Gal binding by ML 308-225 membrane vesicles. [^{3}H]Dns^6-Gal binding was determined by flow dialysis exactly as described in Fig. 1A, and lithium D-lactate was used at a final concentration of 20 mM. Where indicated, CCCP or *p*-CMBS was added to the upper chamber to yield final concentrations of 10 μM and 0.1 mM, respectively.

clear and direct demonstration that binding of Dns^6-Gal to the *lac* carrier protein in membrane vesicles isolated from *E. coli* ML 308-225 is dependent upon energy coupling. As such, the findings provide strong support for previous conclusions derived from fluorescent studies with the dansylgalactosides (1–4) and photoinactivation studies with azidophenylgalactosides (5, 6). It should be emphasized, however, that none of the techniques utilized allows differentiation between the two alternative models proposed as an explanation of this phenomenon (2). Thus, whether the membrane potential causes an increase in the accessibility of the *lac* carrier protein on the external surface of the membrane or an increase in its affinity for ligand or alters both of these functions simultaneously cannot be answered at the present time.

A final point that is worthy of discussion is the apparent discrepancy between the results presented here and those of Kennedy *et al.* (18), who studied the binding of certain galactosides to membrane particles prepared by ultrasonic disruption. These workers demonstrated binding of β-D-galactopyanosyl-1-thio-β-D-galactopyranoside (TDG) and *p*-nitrophenyl α-D-galactopyranoside which was independent of the presence of an energy source and was not inhibited by the addition of sodium azide. However, as demonstrated by Jones and Kennedy (19), using a double isotope technique, the total amount of M protein (i.e., *lac* carrier protein) in the membrane is approximately 10 to 20 times higher than that detected previously by TDG-protection experiments (20) and subsequently by binding assays (18). In addition, it should be emphasized that the total amount of M protein as determined by Jones and Kennedy (19) is very similar to the number of dansylgalactoside binding sites as determined by fluorescence titration (Fig. 2 and refs. 1 and 2) or by direct binding assays (Fig. 2). Since it is entirely possible that the techniques we have utilized previously (1–6) and in this paper may not be sufficiently sensitive to detect less than 10% of the binding observed under energized conditions, there may be no real discrepancy. In any case, regardless of whether or not there is a small amount of binding in the nonenergized state, it seems quite clear from this and the previous studies (1–6) that the great bulk of the *lac* carrier protein is unable to bind ligand unless the membrane is energized.

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