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FUNCTIONAL SYMMETRY OF THE β-GALACTOSIDE CARRIER IN *ESCHERICHIA COLI*

RON M. TEATHER, ODILE HAMELIN, HEINZ SCHWARZ and PETER OVERATH

Max-Planck-Institut für Biologie, D74 Tübingen (G.F.R.)

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Summary

Cytoplasmic membrane vesicles with either normal or inverted orientation were prepared from *Escherichia coli*. The lactose transport activity of these vesicle preparations was compared. The parameters measured were net efflux, counterflux, and K^+/valinomycin-induced active uptake of lactose. With membrane vesicles derived from both wild-type and cytochrome-deficient strains the right-side-out and inverted membrane preparations showed similar rates of lactose flux in all assays. According to these criteria, the activity of the β-galactoside transport protein is inherently symmetrical.

One major difference was observed between the native and inverted vesicle preparations: the inverted vesicles had approximately twice the specific activity of native vesicles in the counterflux and K^+/valinomycin-induced uptake assays. This difference can be largely ascribed to the presence in the normal vesicle preparation of vesicles with a high passive permeability to lactose. Such vesicles are apparently absent from the inverted vesicle preparations.

Introduction

A considerable body of evidence (see ref. 1 for a recent review) supports the proposal originally made by Mitchell [2] that the β-galactoside carrier, the product of the y gene of the *lac* operon, functions as a proton/galactoside symporter. Thus, the movement of a β-galactoside through the membrane appears to be dependent on the concurrent transport of a proton. In the presence of a proton gradient and/or a membrane potential both whole cells and membrane vesicles accumulate β-galactosides against a concentration gradient [3—9]. Likewise, in both preparations the transport protein catalyses efflux as well as ex-

Abbrevations: ACMA, 9-amino-6-chloro-2-methoxyacridine; PCMBS, *p*-chloromercuribenzene sulfonic acid; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazol.

change diffusion of solute [6,10—13]. In whole cells depleted of endogenous energy reserves the carrier equilibrates β-galactosides [12,14] with the concurrent transport of a proton.

The chemiosmotic hypothesis as originally stated by Mitchell [2] does not require any asymmetry in the lactose transport protein, the asymmetrical behavior of the system arising as a consequence of a proton motive force, acting across the membrane, generated by some other primary transport system. Much of the subsequent kinetic analysis of this and related transport systems has assumed a functionally symmetrical carrier. In fact, an inherently asymmetrical carrier would not be expected to show very different kinetic properties [15—17].

Two kinds of studies have offered experimental evidence that in the presence of an energy supply, i.e. a pH and/or electrical potential difference, the properties of the transport system are changed on one side of the membrane. Using whole cells, Winkler and Wilson [12] showed that upon energization the apparent Michaelis constant of the carrier on the inside of the membrane is decreased while remaining unaffected on the outside. A more recent analysis [18] supports this observation. On the other hand, Kaback and his associates (see ref. 19 for review) recently demonstrated that in membrane vesicles the binding of non-transportable substrates to the carrier is dependent on the availability of an energy supply, and concluded that energization either increases the affinity of the carrier for its substrate at the outer membrane surface or causes the exposure of normally hidden binding sites to the outer surface.

In view of the complexity of this system, it is difficult to make mechanistic interpretations only from kinetic data. To determine whether or not the lactose transport system behaves symmetrically we have taken a more direct approach to the problem and compared lactose fluxes in membrane vesicles with normal and inverted orientations. In this way we are able to circumvent the difficulties normally encountered in comparing influx and efflux rates, particularly under non-energized conditions where influx is impossible to measure accurately. Membrane vesicles prepared according to Kaback [20] are almost all in the native configuration [20—22]. However, these vesicles are readily inverted by brief sonication without being greatly reduced in average size. We compare native and inverted vesicles in terms of net efflux rates, exchange of labelled and unlabelled lactose in the absence of a concentration gradient, counterflux, and active uptake driven by the membrane potential produced by K^+ efflux in the presence of valinomycin.

Materials and Methods

Bacterial strains. The *Escherichia coli* strains ML308-225 (*lac* $i^-z^-y^+a^+$) and K207 (a *hemA* derivative of ML308-225) have been described [23]. K131 was derived from ML308 (*lac* $i^-z^+y^+$) by a single nitrosoguanidine mutagenesis and lacks both β-galactosidase and β-galactoside transport activity.

Growth conditions and membrane preparation. ML308-225, K207, and K131 were grown as previously described [24]. Vesicles with native orientation were prepared as described by Kaback [20]. Inverted vesicles were prepared by brief sonication of the native vesicle preparation. The native vesicles were sus-

pended in 100 mM potassium phosphate (pH 6.6) at a concentration of 1 mg membrane protein/ml. 2.5 of this suspension in a 15 ml conical centrifuge tube was sonicated for 1 min (4 × 15 s, with 1-min pauses) at 4°C using a Branson Sonifier Model B12 (medium probe, 25 W output).

Assays. Protein was assayed by the method of Lowry et al. [25]. Active transport of lactose was assayed as described by Kaback [20], using 50 μM [^{3}H]lactose (20 Ci/mol) and 50 mM lithium D-lactate or LiCl. NADH and D-lactate oxidase activities were measured at 37°C with an oxygen electrode as previously described [24].

Energy-dependent ACMA fluorescence quenching was assayed as described by Vogel and Steinhart [26], with final substrate concentrations of 2.5 mM for ATP and 0.1 mM for NADH and D-lactate. The initial rate of fluorescence quenching was measured by varying the amount of membrane in the reaction mixture and plotting the initial rate observed against the amount of membrane present. The initial rate of quenching given in Table I is based on the initial linear portion of the resulting curves.

For the assay of lactose efflux, membrane vesicles were pelleted at 48 000 × *g*, 10 min, 0°C and resuspended at 10 mg protein/ml in 25 mM potassium phosphate (pH 6.6), 50 μM [^{3}H]lactose (860 Ci/mol). The suspension was then incubated on ice for at least 60 min. Control experiments showed that under these conditions membrane vesicles with or without a functional lactose permease were permeable to lactose at a similar rate ($t_{1/2}$ for equilibration is approx. 3 min in both cases). All subsequent steps were at 25°C. 1 M $MgSO_4$ was added to 10 mM. 25 μl samples (±1 μl 2.5 mM PCMBS) were then incubated at 25°C for at least 5 min before being diluted (zero time) with 5 ml of 25 mM potassium phosphate, 10 mM $MgSO_4$, containing either 50 μM sucrose (net efflux assays), 50 μM lactose (exchange assays), or 50 μM sucrose plus 10 μM valinomycin or 20 μM TTFB (efflux in the presence of uncoupling agents). In the case of PCMBS-treated vesicles, the dilution buffer also contained 0.1 mM PCMBS. PCMBS-treated vesicles serve as a control for lactose movement across the membrane that is not mediated by the transport protein. Efflux was terminated by rapid filtration through a Millipore HAMK filter (0.45 μm pore size) which had been presoaked in the dilution buffer. The filters were washed once with 5 ml of the dilution buffer, dried and counted in a liquid scintillation counter in 5 ml of toluene/omnifluor scintillant (Packard). Counterflux assays were performed essentially as described by Winkler and Wilson [12] for whole cells, except that no inhibitors were used and sucrose was added to the uptake medium to avoid any osmotic shock to the vesicles. Vesicles (5 mg membrane protein) were loaded as described above in 5 ml 25 mM potassium phosphate (pH 6.6), 10 mM $MgSO_4$, containing either 20 mM lactose or 20 mM sucrose (control). After centrifugation the supernatant was removed and the pellet was gently and thoroughly stirred up with a glass rod and warmed to 25°C. At zero time the vesicles were rapidly suspended by vortexing (approx. 3 s) in 1.2 ml 25 mM potassium phosphate, 10 mM $MgSO_4$, 20 mM sucrose, 50 μM [^{3}H]lactose (430 Ci/mol). 100-μl samples were taken at intervals and rapidly filtered and washed with 5 ml 25 mM potassium phosphate, 10 mM $MgSO_4$, 20 mM sucrose as described above.

Active uptake was assayed using the membrane potential produced by K^+

efflux in the presence of valinomycin as the energy source. Membrane vesicles were first washed and resuspended in 0.4 M potassium phosphate (pH 6.6). After incubation on ice for 60 min, $MgSO_4$ was added to 10 mM, the vesicles were centrifuged to form a pellet, and the supernatant was removed. After 5 min at 25°C the tube was rinsed once with 15 ml 0.4 M sodium phosphate (pH 6.6), 10 mM $MgSO_4$, care being taken not to disturb the membrane pellet. The pellet was then resuspended in 3 ml 0.4 M sodium phosphate (pH 6.6), 10 mM $MgSO_4$, 100 μM [^{3}H]lactose (107 Ci/mol). After 3—5 min valinomycin (10 mM in 95% ethanol) was added to a final concentration of 10 μM (zero time). 250 μl samples were taken at intervals, filtered, and washed with 5 ml 0.4 M sodium phosphate (pH 6.6), 10 mM $MgSO_4$ as described above.

Electron microscopy. Samples for freeze-fracture electron microscopy were fixed in 2% glutaraldehyde at 37°C for 30 min followed by overnight incubation at room temperature. Before spinning down the vesicles they were incubated for 30 min at room temperature with a 2-fold molar excess of lysine to inactivate the remaining glutaraldehyde. This treatment facilitates subsequent resuspension of the pellet. The vesicles were pelleted, washed once in 25 mM potassium phosphate (pH 6.6), 10 mM EDTA, 30% glycerol, and resuspended in the same medium in a volume approximately equal to the volume of the pellet. The preparation was processed for electron microscopy by standard freeze-fracture techniques in a Balzers BAF 300 [27], except that cleavage was performed at —120°C and the fracture faces were immediately shadowed.

Results

Characterization of the vesicle preparations

Strain ML308-225 is wild type with respect to lactose transport function. Strain K207 was chosen as a control for this study because it lacks a functional electron transport chain when grown in the absence of δ-amino levulinic acid. Vesicles prepared from this strain are thus incapable of energizing lactose transport using substrates of the respiratory chain, although the lactose transport protein remains fully functional in whole cells [23]. Strain K131 lacks the lactose transport protein and was used as a control for any movement of lactose across the membrane which is independent of the lactose carrier protein. The following experiments using ML308-225 vesicles were performed to determine whether or not the sonicated vesicle preparations were in fact inverted under the conditions used. It is assumed that K207 and K131 membrane vesicles respond to sonication in the same way as ML308-225 vesicles.

Fig. 1 shows lactose transport in the presence and absence of D-lactate by native and sonicated vesicles of ML308-225. The maximum uptake level for native vesicles under these conditions is normally 70—80 nmol/mg membrane protein. Such vesicles are almost all in the native configuration [20—22]. After 1 min sonication the maximum uptake level is reduced to less than 1 nmol/mg membrane protein. Neither native nor sonicated vesicles from K207 or K131 show active uptake under these conditions.

The failure of the sonicated vesicles to accumulate lactose is not due to inactivation of the lactose transport protein since, as shown below, lactose efflux, counterflux, and active uptake with K^+/valinomycin proceed normally (Figs.

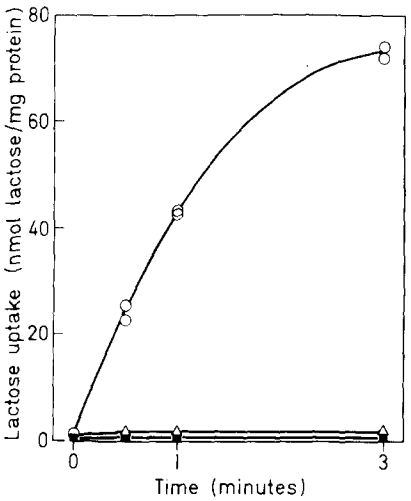

Fig. 1. Active uptake of lactose by native and sonicated vesicles from ML308-225. Lactose uptake was measured as described [20]. △, native vesicles; ○, native vesicles + lithium D-lactate; ■, sonicated vesicles ± lithium-D-lactate.

2, 3 and 4). By the same criteria the vesicles are not abnormally permeable to lactose. Also, the K^+/valinomycin-induced active uptake assays (Fig. 4) show that the sonicated vesicles are able to couple a correctly oriented membrane potential to the active uptake of lactose. We therefore conclude that the soni-

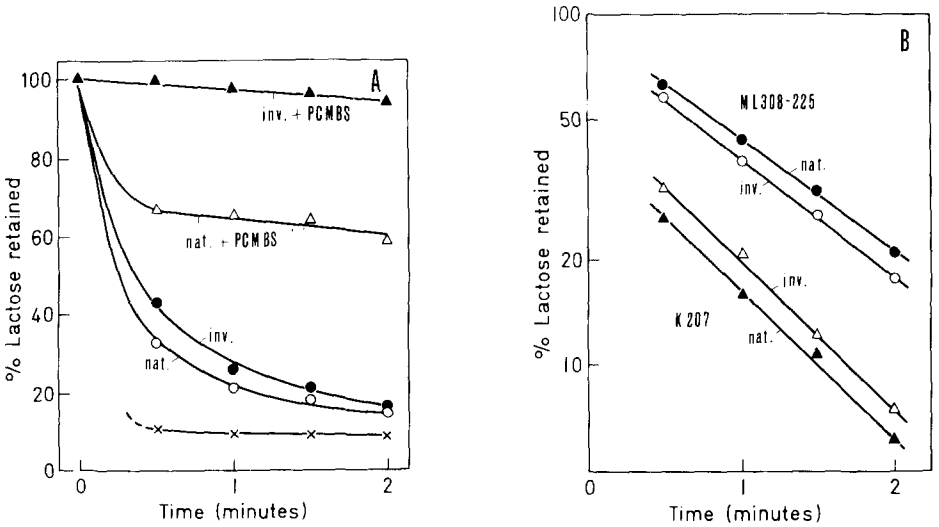

Fig. 2. Lactose efflux from native and sonicated vesicles. (A) Untreated experimental data for lactose efflux from ML308-225 vesicles. ○, native vesicles; △, native vesicles treated with PCMBS; ●, sonicated vesicles; ▲, sonicated vesicles in the presence of PCMBS; ×, native or sonicated vesicles in the presence of 50 μM lactose, 20 μM valinomycin, or 10 μM TTFB. (B) Semilogarithmic plot of lactose efflux from vesicles derived from ML308-225 and K207 after correction for background counts. The 100% lactose level was estimated by extrapolation to zero time of the efflux curves in the presence of PCMBS. ○, native and ●, inverted vesicles from ML308-225; △, native and ▲, inverted vesicles from K207.

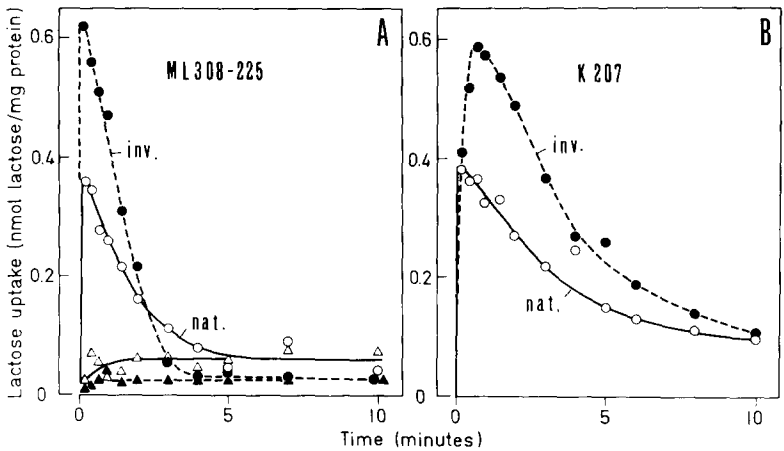

Fig. 3. Counterflux in native and sonicated vesicles from ML308-225 and K207. Counterflux was assayed as described in Materials and Methods, using 50 μM [^{3}H]lactose and either 20 mM sucrose or 20 mM lactose in the assay medium. (A) ML308-225 membrane vesicles. ○, native vesicles in the presence of 20 mM sucrose; △, native vesicles in the presence of 20 mM lactose; ●, inverted vesicles with sucrose; ▲, inverted vesicles with lactose. (B) K207 native (○) and sonicated (●) vesicles in the presence of 20 mM sucrose.

cated vesicles are not correctly energized for lactose uptake by the addition of D-lactate.

The failure of D-lactate to stimulate lactose uptake is not due to any effect of sonication on the lactate oxidase system. As shown in Table I, sonication reduces the rate of D-lactate oxidation by only about one-third. There remain two possible explanations for the failure of the sonicated vesicles to couple D-lactate oxidation to lactose uptake: either the vesicles are inverted by sonication or the membrane orientation is randomized within each vesicle so that no net H^+ gradient is produced. These possibilities can be differentiated by measurement of the rate of energy-dependent ACMA fluorescence quenching. It has been shown that the rate of ACMA fluorescence quenching in inverted vesi-

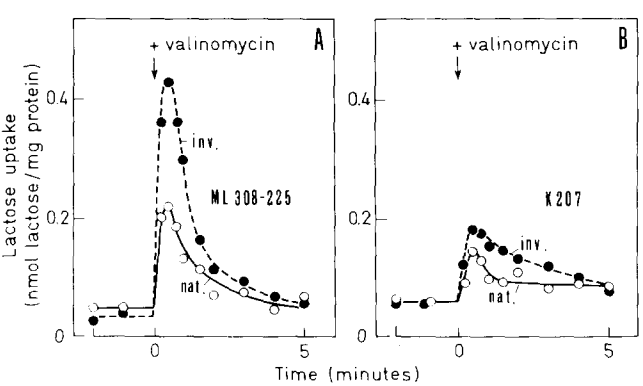

Fig. 4. Active uptake of lactose in native and sonicated vesicles driven by K^+ efflux in the presence of valinomycin. The experiment was performed as described in Materials and Methods. (A) Native (○) and sonicated (●) vesicles from ML308-225. (B) Native (○) and sonicated (●) vesicles from K207.

TABLE I

RESPIRATION RATE AND ACMA FLUORESCENCE QUENCHING OF NATIVE AND SONICATED VESICLES FROM ML308-225

Oxygen consumption and ACMA fluorescence quenching were assayed as described in Materials and Methods.

Substrate	Respiration rate (ng at 0/min per mg protein)			ACMA fluorescence quenching (percent quenching/min per μg protein)		
	native	Inverted	I/N	Native	Inverted	I/N
ATP	—	—	—	1.06	5.33	5.03
NADH	415	936	2.26	1.40	7.86	5.61
D-Lactate	1200	850	0.71	0.24	2.00	8.33

cles from *E. coli* is proportional to the rate of electron flux through the respiratory chain [28].

Native (right side out) vesicles are not expected to show any quenching of ACMA fluorescence. Native vesicles prepared according to Kaback [20] do in fact show some quenching, as previously reported [29]. However, the initial rate of ACMA fluorescence quenching is greatly increased by sonication of the vesicles, as shown in Table I.

The substrates used for these assays fall into two categories: those to which the membrane is essentially impermeable such as NADH and ATP [29,30], and D-lactate which equilibrates freely across the membrane via a specific transport system [31]. With the impermeable substrates the rate of fluorescence quenching observed reflects only the amount of the enzyme system concerned which is exposed on the outer surface of the vesicle. Thus a small degree of fluorescence quenching such as is observed with the native vesicle preparation could be produced either by the presence of a small proportion of inverted vesicles or by a partial randomization of orientation of the enzyme system within the vesicles. The increase observed in the rate of quenching after sonication could be produced either by inversion of more vesicles or by greater randomization of membrane orientation. On the other hand, the rate of quenching observed with a permeant substrate such as D-lactate is more likely to be a measure of the number of inverted vesicles, in that an internally randomized vesicle, oxidizing D-lactate at both the inner and outer membrane surfaces, would not be expected to produce any net H^+ gradient across the membrane and would thus not show any quenching. As shown in Table I, sonication in fact stimulates the rate of D-lactate-dependent ACMA fluorescence quenching to a greater extent than it does the rate of ATP- or NADH-dependent quenching. The rate of D-lactate-dependent ACMA fluorescence quenching in sonicated vesicles is comparable to that observed in vesicles prepared by lysis in a French press. This result clearly suggests that sonication does not tend to randomize membrane orientation but leads mainly to inversion of the membrane vesicles under these conditions. On the basis of the D-lactate-dependent ACMA fluorescence quenching it appears that about 12% of the native vesicle preparation is in fact already inverted, either during vesicle preparation or as a result of freezing the vesicles for storage [21].

A completely independent approach to investigating the orientation of the vesicles is physical characterization by freeze-fracture electron microscopy [20, 21]. In native vesicles the intramembranous particles adhere to the P face, corresponding to the inner half of the bilayer in whole cells. In inverted vesicles the particles adhere to the E (outer) face. When vesicles sonicated under our conditions are examined, the proportion of inverted vesicles observed can be correlated with the loss of D-lactate-dependent transport activity in the preparation. In the native vesicle preparations about 95% of the observed vesicles are of native orientation (calculated on the basis of membrane area). In a preparation sonicated so that the initial rate of D-lactate-dependent lactose transport is reduced by about 75%, 60% of the vesicles observed are inverted. The average diameter of the vesicles observed was reduced by 50% relative to the native vesicles. Thus both functional and structural experiments support the view that brief sonication of native vesicles under our conditions leads to inversion of membrane orientation.

Lactose efflux and exchange

The efflux of lactose from preloaded membrane vesicles in the absence of a metabolizable substrate is a simple measure of lactose permease function. If vesicles loaded with labelled lactose are diluted into a medium containing an equal concentration of unlabelled lactose, efflux of labelled lactose from the vesicle is observed, representing exchange of the labelled internal lactose with unlabelled external lactose, but in this case no net lactose movement occurs.

Fig. 2a shows lactose efflux and exchange from native and inverted membrane vesicles prepared from ML-308-225. Lactose exchange, and efflux in the presence of the uncoupling agents TTFB or valinomycin (in the presence of K^+) are too rapid to be measured accurately under these conditions, but in the absence of uncouplers the rate of net effflux can be readily measured. The relatively slow rate of lactose efflux in the absence of uncouplers can probably be explained by the establishment of a proton motive force across the membrane which opposes further lactose efflux (see ref. 1 for a discussion of this point). The initial rapid loss of part of the internal lactose pool observed with native vesicles in the presence of PCMBS was also observed with native vesicles from strains K207 and K131. Since it is not dependent on the presence of a functional lactose carrier protein it presumably reflects the presence of a population of leaky vesicles. This effect was not observed with inverted vesicles. The presence of leaky vesicles in the native vesicle population is supported by the finding that such preparations contain vesicles permeable to high molecular weight dextrans, while sonicated membrane vesicles are uniformly impermeable (Devor, K., personal communication). The data in Table I is also consistent with the presence of unsealed vesicles. Sonication increased the rate of NADH oxidation only about 2-fold, while the rate of NADH-dependent ACMA quenching increases over 5-fold. This discrepancy can be accounted for by the contribution to the oxygen consumption rate of vesicles permeable to NADH.

Fig. 2b shows lactose efflux plotted semilogarithmically, after correction of the data for non-specific leakage (native vesicles only) and background. The rates of efflux for native and inverted vesicles are essentially identical for both ML308-225 and K207, although there is some difference between vesicles from

the two strains. Initial rates of efflux are in the order of 2 nmol lactose/min per mg protein, compared with about 50 nmol/min per mg protein for active uptake by native vesicles of ML308-225 in the presence of D-lactate.

Counterflux

This assay method [11,12] also allows an estimation of the level of active lactose permease, as the time course of the reaction varies with the permease level [32]. As shown in Fig. 3, the time course for counterflux is identical for native and inverted vesicles from either ML308-225 or K207. K131 vesicles show no counterflux (data not shown). The considerably longer time course for counterflux shown for native and inverted vesicles from K207 can be attributed to the lower level of lactose permease in this strain under the growth conditions used [23]. It can be concluded that the lactose carrier activity in native and inverted vesicles is identical. The difference in the maximum level of [^{3}H]lactose accumulation can be attributed to the presence of leaky vesicles in the native vesicle population, which on the basis of the efflux studies (cf. Fig. 2) account for about 30% of the total vesicle population.

Active uptake induced by K^+ efflux in the presence of valinomycin

In this assay active uptake is driven by the membrane potential produced by unidirectional efflux of K^+ mediated by the K^+ ionophore valinomycin [33–35]. It should thus be possible to drive active transport in any membrane vesicle, irrespective of its orientation, assuming that the transport system can respond. As shown in Fig. 4, active uptake again follows the same time course in both native and inverted vesicles. The very poor uptake shown by K207 vesicles in this assay (maximum concentration effect about 2-fold) is in agreement with the more rapid rate of net efflux shown by this strain (Fig. 2b) in suggesting that membrane vesicles from this strain are abnormally permeable to cations and/or anions. This would not be expected to affect the counterflux assays shown above since the apparent uptake observed in this case is a function of exit competition between labelled and unlabelled substrate molecules [36]. The apparently greater effectiveness of the inverted vesicles in this assay is again assumed to be a function of the presence of leaky vesicles in the native preparation, as discussed above.

Discussion

The results presented in this paper provide evidence for the functional symmetry of the β-galactoside transport system in *E. coli*. The use of membrane vesicles with native and inverted orientation makes the investigation of the possible role in secondary active uptake of inherently asymmetric transport activities relatively simple. In the case of the β-galactoside transport system investigated here, it is clear that the asymmetric behavior of the system in active uptake, which presumably reflects differing substrate affinities or carrier availability on either side of membrane, is solely a function of the direction and magnitude of the proton motive force.

This result places certain restrictions on mechanistic hypotheses regarding the mode of action of a transport system. Although such models are currently

unfashionable [37], functional symmetry is most obviously compatible with models of the rotating carrier type, in which the entire protein molecule is free to rotate in the plane of the membrane, although other classes of models can be made to show functional symmetry as well.

For those who are currently engaged in the isolation and in vitro reconstitution of secondary transport systems of this type, our results offer the encouragement that orientation of protein insertion into the reconstituted membrane should not affect the assay of transport activity.

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