Purified reconstituted lac carrier protein from Escherichia coli is fully functional

(turnover number/biphasic kinetics/symport/proteoliposome/proton electrochemical gradient)

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ABSTRACT Proteoliposomes reconstituted with lac carrier protein purified from the plasma membrane of Escherichia coli catalyze each of the translocation reactions typical of the β-galactoside transport system (i.e., active transport, counterflow, facilitated influx and efflux) with turnover numbers and apparent Km values comparable to those observed in right-side-out membrane vesicles. Furthermore, detailed kinetic studies show that the reconstituted system exhibits properties analogous to those observed in membrane vesicles. Impression of a membrane potential (ΔΨ, interior negative) causes a marked decrease in apparent Km (by a factor of 7 to 10) with a smaller increase in Vmax (=3-fold). At submaximal values of ΔΨ, the reconstituted carrier exhibits biphasic kinetics, with one component manifesting the kinetic parameters of active transport and the other exhibiting the characteristics of facilitated diffusion. Finally, at low lactose concentrations, the initial velocity of influx varies linearly with the square of the proton electrochemical gradient. The results provide quantitative support for the contention that a single polypeptide species, the product of the lac y gene, is responsible for each of the transport reactions typical of the β-galactoside transport system.

β-Galactoside transport across the plasma membrane of Escherichia coli is mediated by the lac carrier protein or lac permease, an intrinsic membrane protein encoded by the lac y gene (see refs. 1 and 2 for recent reviews). This protein catalyzes the coupled translocation of substrate with protons in a symport reaction. Therefore, in the presence of a proton electrochemical gradient (ΔμH+, interior negative and alkaline), downhill transport of protons in response to ΔμH+ drives uphill transport of substrate (i.e., active transport). Downhill transport of substrate drives uphill transport of protons with generation of ΔμH+, the polarity of which reflects the direction of the substrate concentration gradient. The lac carrier protein has been purified to homogeneity in a functional state and shown to be the product of the lac y gene (3, 4), Proteoliposomes reconstituted with this polypeptide catalyze essentially all of the transport activities observed in intact cells and right-side-out membrane vesicles. Thus, in addition to counterflow, proteoliposomes reconstituted with lac carrier protein accumulate lactose against a concentration gradient in the presence of artificially imposed membrane potentials (ΔΨ, interior negative) or pH gradients (ΔpH, interior alkaline). Moreover, it has been shown directly that downhill movements of lactose drive uphill translocation of protons.

Subsequently, other important similarities between "native" and purified lac carrier have been documented. For example, studies with right-side-out membrane vesicles indicate that lactose efflux down a concentration gradient occurs by an ordered process in which lactose is released from the carrier prior to the symported proton, and the carrier appears to recycle in the protonated form during exchange and counterflow (5, 6). The observations on which these conclusions are based have been confirmed and extended recently with the reconstituted system (7, 8). Finally, when proteoliposomes are prepared with purified lac carrier protein and a terminal o-type cytochrome oxidase purified from E. coli, lactose is accumulated against a concentration gradient when ΔμH+ is generated via turnover of the oxidase (9). Parenthetically, it is also noteworthy that a secondary structure model for the lac carrier protein has been proposed (10), monoclonal antibodies against the purified protein have been prepared and characterized (11), and it has been shown directly that the protein spans the bilayer (12).

Taken as a whole, the observations with purified reconstituted lac carrier provide a strong qualitative indication that lactose transport in E. coli requires a single polypeptide species, the product of the lac y gene. In contrast, evidence suggesting that lactose transport may require additional components has been presented (13–16), and most recently, Wright et al. (17), using lac carrier partially purified and reconstituted by different techniques, were able to elicit counterflow activity but were unable to show ΔΨ- or ApH-driven lactose accumulation. The studies described here were undertaken in an effort to resolve this issue on a quantitative basis.

EXPERIMENTAL PROCEDURES

Purification and Reconstitution of lac Carrier Protein. The lac carrier protein was purified from E. coli T206 by the method of Newman et al. (3) using the modifications described by Foster et al. (4). Column fractions containing lac carrier protein were pooled and kept at 4°C until reconstitution into proteoliposomes (generally no more than 1–2 hr after elution from DEAE-Sepharose). Prior to reconstitution, the pooled fractions were diluted with ice-cold column buffer to a concentration of 25 μg per ml of protein.

Reconstitution of the lac carrier into proteoliposomes was carried out with E. coli phospholipids as described by Garcia et al. (7). The final preparation contained 50 mM potassium phosphate, pH 7.5/1 mM dithiothreitol/37.5 mg of phospholipid per ml/56 μg of lac carrier protein per ml. Aliquots were frozen and stored in liquid nitrogen.

Proteoliposomes were thawed at room temperature and sonicated in a bath-type sonicator until the preparation was only slightly opaque (8–15 sec) (4). Where indicated, the proteoliposomes were concentrated by centrifugation for 1 hr at 45,000 rpm in a Beckman type 50 Ti rotor (175,000 gmax). The supernatant was discarded, and the pellet was resuspended in 50 mM potassium phosphate, pH 7.5/1 mM dithiothreitol to a given protein concentration.

Abbreviations: ΔμH+, proton electrochemical gradient; ΔΨ, membrane potential; ΔpH, pH gradient.

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Transport Assays. All assays were conducted at pH 7.5 in a water bath maintained at 25°C. Lactose efflux, counterflow, and ΔΨ-driven active transport were carried out as described (7, 8).

For measurements of lactose-facilitated diffusion, sonicated proteoliposomes were concentrated 3- to 5-fold, and valinomycin and nigericin were added to final concentrations of 20 μM and 2 μM, respectively. An aliquot (1 μl) was then diluted 1:100 into 50 mM potassium phosphate (pH 7.5) containing given concentrations of [1-14C]lactose (6 to 9 mM/ml; 1 Ci = 37 GBq). Reactions were terminated by rapid dilution with 3 ml of ice-cold 50 mM sodium phosphate (pH 7.5), and the samples were filtered immediately through Millipore type GSTF filters (0.2 μm, Millipore) and washed twice with the same volume of cold buffer. Radioactivity retained on the filters was determined by liquid scintillation spectrometry. Experimental values were corrected for zero-time controls obtained by adding an aliquot (1 μl) of proteoliposomes to reaction mixtures that had already been diluted with 3 ml of cold sodium phosphate (pH 7.5), followed by filtration and washing as described (18).

Protein Determinations. Protein was assayed by a modification (3) of the method of Schaffner and Weissmann (19) using bovine serum albumin as standard.

Internal Volume of Proteoliposomes. The internal volume of proteoliposomes reconstituted with purified lac carrier protein was determined from the trapped volume of 86Rb+ and [1-14C]lactose as described (7).

Calculations. The magnitude of the ΔΨ generated theoretically by a given potassium diffusion gradient in the presence of valinomycin was calculated from the following relationship

\[
\Delta \Psi (mV) = -59 \log [K^+]_i/[K^+]_o,
\]

where [K+]i and [K+]o denote internal and external potassium concentrations, respectively, at the time of dilution.

Steady-state levels of lactose accumulation were converted into Δμlac (in mV) using the following relationship

\[
\Delta \mu_{lac} (mV) = -59 \log [\text{lactose}]_i/[\text{lactose}]_o,
\]

where [lactose]i and [lactose]o denote internal and external lactose concentrations, respectively, at the steady-state level of accumulation.

RESULTS

Facilitated Diffusion of Lactose in Proteoliposomes. When lactose is added to a suspension of proteoliposomes reconstituted with purified lac carrier protein in the absence of an imposed ΔμH+, uptake proceeds relatively slowly (Fig. 1), and at least 1 hr is required for internal lactose to equilibrate with the external concentration (not shown). In contrast, addition of valinomycin and nigericin increases the initial rate of uptake 2- to 5-fold, and equilibration is complete within 5–7 min. Because lactose uptake under these conditions is essentially completely blocked by p-chloromercuribenzenesulfonate, it is apparent that the equilibration process in the proteoliposomes is almost entirely carrier mediated and that passive influx of the disaccharide occurs at a very low rate. Thus, elucidation of the initial velocity of facilitated diffusion is particularly clear-cut in this system.

The observations are qualitatively similar to those reported with intact cells (20, 21) and right-side-out membrane vesicles (22) and are consistent with the finding that addition of lactose to proteoliposomes containing purified lac carrier protein under the same conditions leads to alkalinization of the external medium (4). Clearly, therefore, downhill movement of lactose along a concentration gradient drives the uphill translocation of protons, and in the absence of ionophores a ΔμH+ (interior positive and/or acid) is established that acts to slow the symport reaction.

Kinetics of Facilitated Diffusion and ΔΨ-Driven Active Transport. As shown previously (3, 4, 8), proteoliposomes reconstituted with purified lac carrier protein catalyze lactose accumulation against a gradient when a ΔΨ (interior negative) is imposed by means of a potassium diffusion gradient (K+ι → K+o) in the presence of valinomycin. The data presented in Fig. 2 (Inset) contrast the kinetics of facilitated diffusion (ΔΨ = 0 mV) with those of ΔΨ-driven active transport (ΔΨ = −134 mV). In both cases, initial velocities were measured at pH 7.5, and the data are presented in the form of V/5 plots in which Vmax is approximated from the y intercept and apparent Km from the slope of the function. As shown with right-side-out membrane vesicles (5, 6) and intact cells (23), the kinetics of lactose transport in proteoliposomes are dramatically different in the presence and absence of ΔμH+. Thus, in comparison to facilitated diffusion, the apparent Km for ΔΨ-driven active transport is decreased from (3.13 ± 0.66) × 10−3 M to (0.47 ± 0.13) × 10−3 M, while Vmax is increased from 11.43 ± 0.42 to 35 ± 0.9 μmol of lactose per min per mg of lac carrier protein.

In further analogy to the native system (24), when a sub-maximal ΔΨ of about −32 mV is imposed, the system exhibits its biphasic kinetics (Fig. 2). One component of the overall process exhibits a low apparent Km typical of ΔΨ-driven active transport (ca. 0.4 × 10−3 M), while the second exhibits a much higher apparent Km approximating that observed for facilitated diffusion (ca. 1.6 × 10−3 M).

Relationship Between Initial Velocity of Lactose Transport and ΔμH+. In addition to causing a decrease in apparent Km for substrate, ΔμH+ (interior negative and/or alkaline) alters the distribution of the lac carrier between high and low apparent Km pathways as a square function (24). That is, over a relatively low range of lactose concentrations, the "apparent" Vmax varies linearly with the square of ΔΨ (interior negative) or ΔpH (interior alkaline). To a reasonable approximation, a similar relationship is observed in proteoliposomes reconstituted with purified lac carrier protein (Fig. 3). In the experiments shown, potassium diffusion gradients were imposed in the presence of valinomycin to yield theoretical ΔΨ
Values of 0 to -153 mV (interior negative), and initial velocities of lactose transport were measured at a low substrate concentration to minimize the contribution of the high apparent $K_m$. Biphasic kinetics of lactose uptake at a submaximal $\Delta \Psi$ are shown. Proteoliposomes were treated with valinomycin at a final concentration of 20 $\mu$M and aliquots (1 $\mu$L) were diluted 1:100 into 13.8 mM potassium phosphate (pH 7.5) and 36.2 mM sodium phosphate (pH 7.5) containing [1-14C]lactose (6-59 mCi/mmol) at concentrations ranging from 0.04 to 20 mM. At various times, reactions were terminated and assayed. Data were obtained from linear initial portions of the time courses. (Inset) Kinetics of facilitated diffusion ($\bullet$) and $\Delta \Psi$-driven lactose transport ($\circ$). For facilitated diffusion measurements, proteoliposomes were treated with valinomycin and nigericin at final concentrations of 20 $\mu$M and 2 $\mu$M, respectively, and aliquots (1 $\mu$L) were diluted 1:100 into 50 mM potassium phosphate (pH 7.5) containing [1-14C]lactose (6-59 mCi/mmol) at concentrations ranging from 0.27 to 20 mM. At various times, the reactions were terminated and assayed. For $\Delta \Psi$-driven lactose transport, proteoliposomes were treated with valinomycin at a final concentration of 20 $\mu$M, and aliquots (1 $\mu$L) were diluted 1:200 into 50 mM sodium phosphate (pH 7.5) containing [1-14C]lactose (6-59 mCi/mmol) at concentrations ranging from 0.04 to 1.2 mM. At various times, reactions were terminated and assayed.

Fig. 3. Relationship between initial velocity of lactose transport and $\Delta \mu_{liac}$. Proteoliposomes containing purified lac carrier protein were concentrated to 185 $\mu$g of protein per ml in 50 mM potassium phosphate, pH 7.5/1 mM dithiothreitol. Initial rates of lactose transport were then measured under the conditions given below. Data are presented in the form of $V$ vs. $V/S$ plots, in which the intercept with the y axis represents $V_{max}$ and the slope of the function yields the apparent $K_m$. Phosphorylation yields the apparent $V_{max}$ and the slope of the function will be shown. Proteoliposomes were treated with valinomycin and nigericin at final concentrations of 20 $\mu$M and 2 $\mu$M, respectively, and aliquots (1 $\mu$L) were diluted 1:200 into 50 mM phosphate buffer (pH 7.5) that contained various ratios of sodium and potassium as well as [1-14C]lactose (59 mCi/mmol) at a final concentration of 0.075 mM. Reactions were terminated at various times and processed. Regardless of the magnitude of the imposed potassium diffusion potential, maximal levels of [1-14C]lactose accumulation were achieved within 5 min and remained constant between 5 and 15 min. For initial velocity values of lactose uptake, aliquots (1 $\mu$L) of the proteoliposomes were diluted 1:200 to 1:400 into 50 mM phosphate buffer (pH 7.5) that contained various ratios of sodium and potassium but no radioactive lactose. After a 10-min incubation, [1-14C]lactose (59 mCi/mmol) was added to a final concentration of 0.075 mM to initiate transport. Reactions were terminated at times ranging from 0 to 8 sec and processed. (A) Initial velocity vs. $\Delta \mu_{liac}$. Initial velocities of lactose transport and steady-state levels of accumulation were determined at theoretical $\Delta \Psi$ values ranging from 0 to -154 mV. Values of $\Delta \Psi$ and $\Delta \mu_{liac}$ were calculated from Eqs. 1 and 2. Initial velocities at each value of $\Delta \mu_{liac}$ are expressed relative to that observed in the absence of $\Delta \Psi$ (0.5 $\mu$mol of lactose per min per mg of protein). (B) Initial velocity vs. ($\Delta \mu_{liac}$)$^2$. Values plotted were derived from the data in A.

Fig. 2. Kinetics of lactose transport in the presence and absence of $\Delta \Psi$ (interior negative). Proteoliposomes containing purified lac carrier protein were concentrated to 167 $\mu$g of protein per ml in 50 mM potassium phosphate, pH 7.5/1 mM dithiothreitol. Initial rates of lactose transport were then measured under the conditions given below. Data are presented in the form of $V$ vs. $V/S$ plots, in which the intercept with the y axis represents $V_{max}$ and the slope of the function will be shown. Although the magnitude of the $\Delta \Psi$ is difficult to quantitate, it is apparent that it increases with increasing values of the imposed $\Delta \Psi$. As a consequence, $\Delta \mu_{liac}$ is in equilibrium with the theoretical values of $\Delta \Psi$ generated via potassium diffusion gradients up to -60 mV but deviates from $\Delta \Psi$ in a systematic manner at greater imposed potentials. For these reasons, $\Delta \mu_{liac}$ was used to estimate $\Delta \mu_{liac}$. The data in Fig. 3A show that the initial velocity of lactose uptake increases ~8-fold from 0 to about -120 mV as an upward curvilinear function of $\Delta \mu_{liac}$ (i.e., $\Delta \mu_{liac}$). Furthermore, the relationship is linear when relative initial velocity is plotted as a function of ($\Delta \mu_{liac}$)$^2$, indicating that the function is exponential to the second power of $\Delta \mu_{liac}$ (Fig. 3B).
Table 1. Comparison of turnover numbers of the lac carrier protein: ML 308-225 membrane vesicles vs. proteoliposomes reconstituted with purified carrier

<table>
<thead>
<tr>
<th>Reaction*</th>
<th>Membrane vesicles†</th>
<th>Proteoliposomes‡</th>
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<tbody>
<tr>
<td>∆Ψ-driven influx</td>
<td>16 (0.2)</td>
<td>16–21 (0.5 ± 0.1)</td>
</tr>
<tr>
<td>Counterflow</td>
<td>16–39 (0.45)</td>
<td>28 (0.6)</td>
</tr>
<tr>
<td>Facilitated diffusion</td>
<td>8–16 (20)</td>
<td>8–9 (=3.1 ± 0.7)</td>
</tr>
<tr>
<td>Efflux</td>
<td>8 (2.1)</td>
<td>6–9 (2.5 ± 0.5)</td>
</tr>
</tbody>
</table>

*All reactions were carried out at pH 7.5 and 25°C.
†Determination of the amount of lac carrier protein in ML 308-225 membrane vesicles is based on photolabeling experiments with p-nitro[2-LH]phenyl-α-D-galactopyranoside, which indicate that the carrier represents about 0.5% of the membrane protein. Kinetic parameters for ∆Ψ-driven influx, counterflow, facilitated diffusion, and efflux, respectively, were taken from Robertson et al. (24), Padan et al. (27), Garcia et al. (28), and Kaczorowski et al. (6).
‡Values in parentheses indicate apparent Kₘ values × 10⁻² M (±SEM) for determinations conducted on at least two different preparations of reconstituted purified lac carrier.

**DISCUSSION**

Purification of the lac carrier protein as described by Newman et al. (3) and Foster et al. (4) yields a single polypeptide with an amino acid composition closely matching that predicted from the DNA sequence of the lac y gene (33). Moreover, the presence of the polypeptide is dependent on expression of the lac y gene, and at the phenomenological level, the reconstituted protein catalyzes all translocation activities typical of the β-galactoside transport system (3, 4, 7–9). Nevertheless, rigorous proof that the purified lac carrier protein is the only polypeptide responsible for lactose transport is dependent on a demonstration that turnover numbers for the purified lac carrier in proteoliposomes are similar to those obtained for the protein in its native environment.

The data in Table 1 testify to the high degree of functionality retained by the purified reconstituted lac carrier. Clearly, freeze-thaw/sonicated proteoliposomes prepared by octylglucoside dilution exhibit turnover numbers comparable to those obtained for the protein in active transport, counterflow, facilitated influx, and efflux that fall within the range of values reported for ML 308-225 membrane vesicles. In addition, with the possible exception of facilitated diffusion, the apparent Kₘ values obtained with proteoliposomes are similar to those determined with right-side-out membrane vesicles. Taken as a whole, the observations indicate strongly that the lac carrier protein retains much of its activity during purification and reconstitution by the methods described.

Additional kinetic similarities between proteoliposomes containing purified lac carrier and right-side-out membrane vesicles have also been revealed. (i) Monophasic V vs. V/S plots are observed for lactose transport in the absence or presence of a sufficiently large ∆Ψ, and the kinetic parameters obtained are strikingly different. In the presence of ∆Ψ, the apparent Kₘ is decreased by a factor of 7 to 10, while Vₘₐₓ is increased by a factor of about 3. (ii) At a submaximal value of ∆Ψ, biphasic kinetics are observed. One component of the V vs. V/S plot exhibits the kinetic characteristics of ∆Ψ-driven active transport (i.e., low apparent Kₘ), while the other exhibits the high apparent Kₘ typical of facilitated diffusion. The data support the contention (6, 24) that in addition to acting thermodynamically as the driving force for active transport, ∆Ψ₂alters the distribution of the lac carrier between two different kinetic pathways. (iii) The initial velocity of ∆Ψ-driven lactose transport in proteoliposomes varies linearly with the square of ∆Ψ₂(i.e., ∆Ψ₂). As discussed by Robertson et al. (24) with respect to similar observations in right-side-out membrane vesicles, a possible explanation that could account for the phenomenon is that the lac carrier exists in two forms, monomers and dimers, that the monomer catalyzes facilitated diffusion and the dimer catalyzes active transport, and that ∆Ψ₂promotes aggregation of monomers to dimers. Although this notion is by no means proven, it is interesting that studies using radiation inactivation analysis are consistent with the idea (2, 34, 35).

In any event, regardless of mechanistic interpretations, the activity of purified lac carrier protein reconstituted into proteoliposomes is comparable to the activity of the native carrier. Thus, it seems reasonable to conclude that a single polypeptide, the product of the lac y gene, is solely responsible for the reactions catalyzed by the β-galactoside transport system. It also follows that purified lac carrier reconstitutes with a high degree of fidelity. That is, the orientation of the protein in the reconstituted system must be similar to that in the bacterial cytoplasmic membrane. Importantly, this conclusion is entirely consistent with recent studies using monoclonal antibodies prepared against purified lac carrier protein (11). Binding experiments with right-side-out and inside-out membrane vesicles show that the epitope for antibody 4Bl is present virtually exclusively on the plasmal surface of the membrane. Furthermore, the antibody binds to proteoliposomes reconstituted with purified lac carrier with a stoichiometry similar to that observed in right-side-out membrane vesicles (refs. 2 and 36; unpublished observations).

Biochemistry: Viitanen et al.