Tozer, R. G., & Dunn, S. D. (1987) *J. Biol. Chem. 262*, 10706–10711.
Walker, J. E., Saraste, M., & Gay, H. J. (1984) *Biochim. Biophys. Acta 768*, 164–200.
Wise, J. G., Latchney, R. L., & Senior, A. E. (1981) *J. Biol. Chem. 256*, 10383–10389.
Wood, J. M., Wise, J. G., Senior, A. E., Futai, M., & Boyer, P. D. (1987) *J. Biol. Chem. 262*, 2180–2186.

Kinetic Analysis of Lactose Exchange in Proteoliposomes Reconstituted with Purified *lac* Permease

Julius S. Lolkema,[‡] Nancy Carrasco,[§] and H. Ronald Kaback*,[‖]
Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110
Received August 1, 1990; Revised Manuscript Received October 3, 1990

ABSTRACT: Lactose exchange catalyzed by purified *lac* permease reconstituted into proteoliposomes was analyzed with unequal concentrations of lactose on either side of the membrane and at low pH so as to prevent equilibration of the two pools. Exchange with external concentrations below 1.0 mM is a single-exponential process, and the apparent affinity constants for external and internal substrate are close to the apparent K_Ms reported for active transport and efflux, respectively [Viitanen, P. V., Garcia, M. L., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1629]. At external lactose concentrations above 1.0 mM, a second kinetic pathway becomes evident with an apparent affinity constant of about 6 mM which is similar to the apparent K_M for facilitated influx. A second pathway is not observed with respect to internal lactose even when the concentration is increased up to 80 mM. Furthermore, high internal or external lactose concentrations do not inhibit the exchange reaction. Biphasic kinetics with respect to external lactose are retained in a mutant permease that catalyzes exchange but is defective in H^+-coupled lactose transport. It is suggested that *lac* permease has more than one binding site and that this may be the underlying reason for the biphasic kinetics observed for both exchange and H^+-coupled lactose transport.

The β-galactoside transport system of *Escherichia coli* catalyzes cotransport (i.e., symport) of lactose and H^+ across the cytoplasmic membrane with a stoichiometry of unity [for reviews, see Kaback (1983, 1989, 1990)]:

$$\text{lactose}_{out} + H^+_{out} \Leftrightarrow \text{lactose}_{in} + H^+_{in} \qquad (1)$$

lac permease, the product of the *lac Y* gene, is the only gene product required, and the protein has been solubilized from the membrane, purified to homogeneity, and reconstituted into proteoliposomes in a functional state (Newman et al., 1981; Foster et al., 1982). The reconstituted system is ideal for studying the kinetic properties of *lac* permease due mainly to the passive impermeability of the proteoliposomes to ions and lactose (Garcia et al., 1983). Furthermore, both the turnover number of the permease and also its apparent K_M for lactose are similar in proteoliposomes and membrane vesicles with respect to membrane potential driven lactose accumulation, counterflow, facilitated diffusion (i.e., lactose influx under nonenergized conditions), and efflux (Viitanen et al., 1984).

The permease is a hydrophobic polytopic plasma membrane protein, and based on circular dichroic measurements and hydropathy analysis of the primary sequence (Foster et al., 1983), a secondary structure model was proposed in which the protein consists of 12 hydrophobic domains in α-helical conformation that traverse the membrane in zig-zag fashion, connected by hydrophilic segments with the N- and C-termini on the cytoplasmic surface (Kaback, 1983, 1989, 1990). The general features of the model are consistent with other spectroscopic measurements (Vogel et al., 1985),[1] chemical modification (Page & Rosenbusch, 1988), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), and immunological studies (Carrasco et al., 1982, 1984a,b; Seckler et al., 1983, 1984, 1986; Herzlinger et al., 1984). Moreover, recent studies on a large number of *lacY-phoA* (*lac* permease–alkaline phosphatase) fusions have provided strong exclusive support for the 12-helix model (Calamia & Manoil, 1990).

Mechanistic studies with right-side-out membrane vesicles (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979) and reconstituted proteoliposomes (Garcia et al., 1983; Viitanen et al., 1983) have led to a kinetic model for efflux, equilibrium exchange, and counterflow. The model is consistent with the effects of D_2O (Viitanen et al., 1983), the behavior of a monoclonal antibody that uncouples lactose from H^+ translocation (Carrasco et al., 1984), and the properties of various uncoupled mutants (Herzlinger et al., 1985; Püttner et al., 1986, 1989; Carrasco et al., 1986, 1989; Menick et al., 1987). Accordingly, efflux down a concentration gradient consists of a minimum of five steps (Figure 1): (1) binding of substrate and H^+ on the inner surface of the membrane (order unspecified); (2)

* To whom correspondence should be addressed.
[‡] Present address: Department of Physical Chemistry, State University of Groningen, Zernikelaan 16, 9716 AH Groningen, The Netherlands.
[§] Present address: Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.
[‖] Present address: Department of Physiology, Howard Hughes Medical Institute, Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA 90024-1570.

[1] In addition to circular dichroic and laser Raman spectroscopy, Fourier-transform infrared studies also show that purified *lac* permease is largely helical (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished work).

0006-2960/91/0430-1284$02.50/0

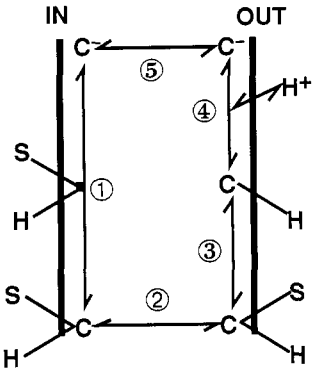

FIGURE 1: Schematic representation of reactions involved in lactose efflux, exchange, and counterflow. C represents *lac* permease. The order of substrate binding at the inner surface of the membrane is not implied.

translocation of the ternary complex to the outer surface; (3) release of substrate; (4) release of H^+; and (5) return of the unloaded permease to the inner surface. Furthermore, release of H^+ (step 4) appears to be the rate-limiting step for efflux (Kaczorowski & Kaback, 1979; Viitanen et al., 1983). Alternatively, exchange and counterflow with external lactose at saturating concentrations involve steps 1–3 only, and the permease recycles in the protonated form:

$$\text{lactose}_{out} + E_{out} \Leftrightarrow \text{lactose}_{in} + E_{in} \qquad (2)$$

where E_{out} and E_{in} refer to states of the protonated permease that bind lactose at the outer or inner surface of the membrane, respectively. In contrast to efflux, this reaction is independent of pH and unaffected by the imposition of pH gradients (ΔpH)[2] or membrane potentials ($\Delta\Psi$; Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Garcia et al., 1983; Viitanen et al., 1983). Although the model is consistent with many observations, it fails to explain the biphasic kinetics of lactose transport observed at intermediate values of the H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$; Robertson et al., 1980; Viitanen et al., 1984; Page, 1987).

In this paper, it is demonstrated that the exchange reaction also exhibits biphasic kinetics and that this property is retained by a mutated permease in which Glu325 is replaced with Ala (Carrasco et al., 1986). These and other considerations suggest that *lac* permease may have more than one binding site for lactose.

Experimental Procedures

Materials

[1-^{14}C]Lactose was purchased from Amersham/Searle, dithiothreitol (DTT) was from Sigma, valinomycin was from CalBioChem, and *E. coli* phospholipids (ether/acetone extract) were from Avanti. All other materials were of reagent grade and were obtained from commercial sources as described (Viitanen et al., 1986).

Methods

Purification and Reconstitution of lac Permease. *lac* permease was solubilized from the membrane fraction of *E. coli* T184 harboring plasmid pGM21, purified, and reconstituted into proteoliposomes as described (Newman et al., 1981; Foster et al., 1982; Viitanen et al., 1986). Proteoliposomes were resuspended in 50 mM potassium phosphate (KP_i, pH 7.5) containing 1.0 mM DTT to a final concentration of 80 μg/mL protein and 37.5 mg/mL phospholipid and stored in liquid nitrogen. Permease with Ala in place of Glu325 was solubilized, purified, and reconstituted in the same way to protein and phospholipid concentrations of 40 μg/mL and 37.5 mg/mL, respectively.

Transport Assays. Proteoliposomes were thawed rapidly at 25 °C and diluted 100-fold in 50 mM KP_i/1.0 mM DTT at given pH values. After equilibration for 15 min at room temperature, the proteoliposomes were collected by centrifugation for 1 h at 45 000 rpm in a Beckman 50Ti rotor at 10 °C and resuspended in the original volume of buffer. To an aliquot of 45 μL was added 5.0 μL of [1-^{14}C]lactose to a given concentration, and valinomycin was added to a final concentration of 10 μM. The sample was then sonified in a bath sonicator until the suspension was clear (usually twice for 5 s each). Large particles were removed by centrifugation for 30 s at maximal speed in a Beckman Microfuge, and the supernatant was used directly for transport assays.

Exchange measurements were performed by dilution of 1 μL of proteoliposomes prepared as described into 2.0 mL of 50 mM KP_i at a given pH with or without unlabeled lactose as follows: A 5.0-μL syringe was used to place a 1.0-μL bead of proteoliposomes on the wall of a test tube above the surface of the dilution buffer, and a syringe containing 50 μL of 200 mM $HgCl_2$ was positioned above the tube. At zero time, which was indicated by a metronome clicking at intervals of 1 s, the tube was placed on a spinning vortex mixer, and at the desired time, the $HgCl_2$ solution was injected rapidly into the vortexing solution. Control experiments show that $HgCl_2$ abolishes all transport activity instantaneously. In this manner, reproducible measurements can be made at time intervals of 1 s. The contents of the tube were then filtered immediately as described (Garcia et al., 1983), and the filter was washed twice with 2.0 mL of 50 mM KP_i (pH 5.5)/5.0 mM $HgCl_2$ and assayed for radioactivity by liquid scintillation spectrometry. Zero-time points were obtained by adding $HgCl_2$ prior to the proteoliposomes. Background radioactivity was determined by allowing reactions to proceed until the samples reached a constant minimal value (about 1 h). All experiments were performed at 25 °C, and the data were processed by correcting for background radioactivity and plotting on a logarithmic scale. Back-extrapolation of a straight line through the data points accounted for 80–90% of the measured zero-time value, and the extrapolated value was taken as the true zero time. This value was also used to calculate the internal volume of the proteoliposomes (Garcia et al., 1983).

Protein Determinations. Protein was measured as described by Schaffner and Weissmann (1973).

Results

Exchange under Nonequilibrium Conditions. Kinetic analysis of lactose exchange requires measurement of exchange rates when the lactose concentration is not equal on both sides of the membrane. Under these conditions, the system is not in thermodynamic equilibrium, and net transport of lactose and H^+ may occur which would interfere with the exchange measurements. However, lactose efflux down a concentration gradient is pH dependent, and at pH 5.5, the rate of exchange is about 100 times faster than the rate of net lactose transport (Garcia et al., 1983; Viitanen et al., 1983). Thus, when proteoliposomes are loaded with 3.0 mM [1-^{14}C]lactose and diluted into buffer at pH 5.5, no significant efflux is observed over 15 s [Figure 2A (●)]. As discussed previously (Kaczorowski & Kaback, 1979), external pH might influence the rate of efflux either by affecting the rate of H^+ transfer to solvent

[2] Abbreviations: ΔpH, pH gradient across the membrane; $\Delta\Psi$, electrical potential across the membrane; $\Delta\bar{\mu}_{H^+}$, H^+ electrochemical gradient; DTT, dithiothreitol; KP_i, potassium phosphate.

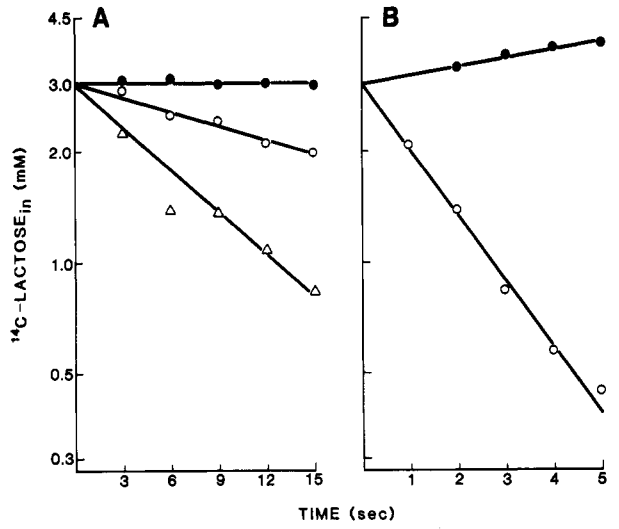

FIGURE 2: Relative rates of net lactose transport and exchange at pH 5.5. (A) Proteoliposomes loaded with 3.0 mM [1-^{14}C]lactose were diluted 2000-fold in buffer containing 0 (●), 100 (○), or 700 μM (△) unlabeled lactose. (B) Proteoliposomes loaded with 3.0 mM [1-^{14}C]lactose were diluted 2000-fold in buffer containing 25 mM unlabeled lactose (○) or 50-fold in 25 mM [1-^{14}C]lactose (●).

or by altering the distribution between protonated and unprotonated forms of the permease. In any case, addition of unlabeled lactose to the dilution buffer leads to loss of internal [^{14}C]lactose as a single-exponential process with a slope that is dependent on the external lactose concentration [Figure 2A (○, △); in addition, cf. Appendix]. Under these conditions, the protonated carrier translocates [^{14}C]lactose from the internal pool to the outer surface of the membrane where it exchanges with unlabeled lactose in the medium (Figure 1). Subsequently, the ternary complex returns to the inner surface of the membrane, and unlabeled lactose from the external medium is exchanged for [^{14}C]lactose in the internal pool. The cycle is then repeated until the distribution of the isotopically labeled sugar is random.

Clearly, low pH is suitable for exchange measurements under nonequilibrium conditions when the internal lactose concentration (S_i) is higher than the external concentration (S_o). In the reverse situation, when the external concentration is higher than internal, conditions are less ideal, and significant net influx is observed with S_o = 25 mM and S_i = 3 mM [Figure 2B (●)]. Since exchange under the same conditions is much faster [Figure 2B (○)], this effect has been ignored; however, it should be noted that the exchange rates are somewhat underestimated when $S_o > S_i$.

Exchange Kinetics at Low External Lactose Concentrations. The exchange reaction can be described by the equation:

$$\frac{S_o}{S_i}\frac{1}{k_iE_i} = \frac{1}{k_o\mathcal{E}}\left[1 + \frac{S_o}{K_{Mo}(S_i)}\right] \quad (3)$$

with

$$K_{Mo}(S_i) = K_{Mo}\frac{S_i}{S_i + K_{Mi}} \quad (4)$$

where k_iE_i is the measured rate constant for exchange and K_{Mo} and K_{Mi} have the same meaning as the Michaelis constants derived from initial rate measurements (for a derivation, cf. Appendix).

Proteoliposomes loaded with 1.0 mM [1-^{14}C]lactose were diluted 2000-fold into buffer containing unlabeled lactose at concentrations ranging from 0.1 to 0.9 mM. The experiment was repeated with internal [1-^{14}C]lactose at 3.0 and at 10 mM,

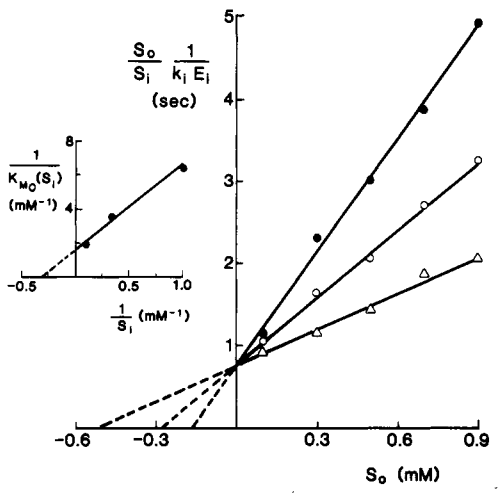

FIGURE 3: Kinetic analysis of the lactose exchange reaction. Proteoliposomes loaded with 1.0 (●), 3.0 (○), or 10 mM (△) [1-^{14}C]-lactose were diluted 2000-fold into buffer containing the indicated unlabeled lactose concentrations. The data were analyzed according to eq 3.

Table I: Comparison of Apparent Affinities (K_M) and Turnover Numbers (TN) of Reactions Catalyzed by Reconstituted *lac* Permease

	K_M (mM)	K_{Mi} (mM)	TN (s^{-1})
active transport[a] (pH 7.5, $\Delta\bar{\mu}_{H^+}$ = −100 mV)	0.5		16–21
facilitated influx[a] (pH 7.5)	3.1		8–9
efflux[a] (pH 7.5)		2.5	6–9
exchange (pH 5.5)	0.6 and 6	3.0	14 and 50

[a] Data from Viitanen et al. (1984).

and the rate constants were plotted according to eq 3 (Figure 3). The data are consistent with the exchange process described in eq 2. Each function yields the dependence of the exchange rate on the external lactose concentration at a given internal lactose concentration. It is evident that the apparent affinity constant for external lactose (intercept with the x axis) is a function of S_i (eq 4). Extrapolation to infinite internal concentration yields a K_{Mo} value of 0.6 mM (Figure 3, secondary plot). In the same way, a K_{Mi} value of 3 mM is calculated to be the affinity constant for internal lactose at saturating concentrations of external lactose. The common y intercept represents the second-order rate constant for exchange at infinitely low external lactose concentration, and the maximum rate of exchange is computed to be 0.85 mM/s ($V_{max} = K_{Mo}k_o\mathcal{E} = K_{Mi}k_i\mathcal{E}$) or a turnover number of 14 s^{-1}. The data are summarized in Table I.

Exchange Exhibits Biphasic Kinetics. The rate constant for loss of internal [^{14}C]lactose during exchange increases with external lactose concentration until saturation is achieved. Figure 3 shows that an external lactose concentration of about 0.3 mM gives half the maximal rate constant when the internal concentration is 3.0 mM. However, when the external concentration is increased above the level expected to give a maximal exchange rate, a second kinetic pathway becomes apparent (Figure 4A). In the experiment shown, the external lactose concentration was varied from 0.1 to 25 mM, the measured rate constants were converted to initial rates by multiplying by the internal concentration ($v = k_iE_iS_i$), and the data are plotted according to Eadie and Hofstee. Clearly, the kinetics are biphasic with respect to external lactose concentration, and the two apparent affinity constants, K_{Mo} (S_i = 3.0 mM), are about 0.3 and 2.5 mM.

A possible explanation for the biphasic kinetics is that two populations of permease are present as a result of random

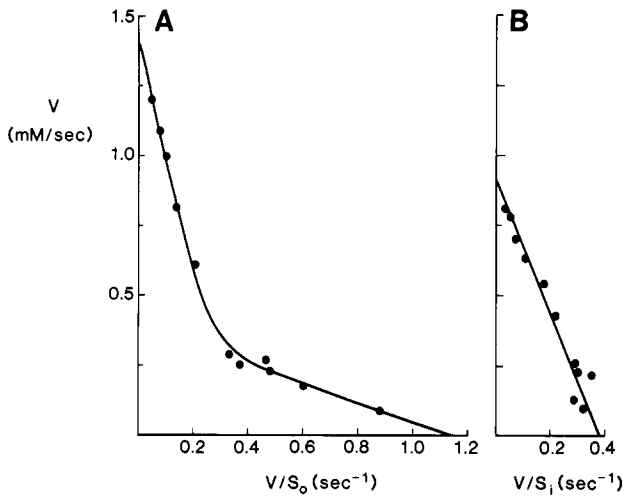

FIGURE 4: Biphasic kinetics of lactose exchange. (A) Proteoliposomes loaded with 3.0 mM [1-^{14}C]lactose were diluted 2000-fold into buffer containing unlabeled lactose concentrations ranging from 0.1 to 25 mM. Initial rates were plotted in an Eadie–Hofstee plot. (B) Proteoliposomes loaded with [1-^{14}C]lactose at concentrations ranging from 0.3 to 25 mM were diluted into buffer containing 3.0 mM unlabeled lactose.

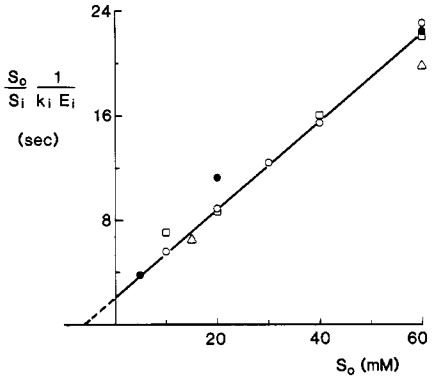

FIGURE 5: Lactose exchange at high internal lactose concentrations and at pH 5.5. Proteoliposomes loaded with 20 (●), 40 (○), 60 (△), or 80 mM (□) [1-^{14}C]lactose were diluted into indicated concentrations of unlabeled lactose. The rate constants were plotted according to eq 3.

insertion during reconstitution, particularly because one apparent affinity constant for external lactose (K_{Mo} = 2.5 mM) is similar to that obtained for internal lactose in the experiments presented in Figure 3 (K_{Mi} = 3.0 mM). Although studies with two monoclonal antibodies directed against external epitopes in the permease indicate that the orientation of reconstituted permease is largely the same as in the cell membrane (Carrasco et al., 1984), the reverse kinetic experiment was performed to test this possibility. Thus, exchange was measured with a constant external lactose concentration of 3.0 mM and internal concentrations ranging from 0.3 to 25 mM (Figure 4B). As shown, an Eadie–Hofstee plot of the data reveals a single phase with no indication of an apparent affinity constant of less than 3.0 mM. The observation provides additional support for the argument that the permease probably reconstitutes with a high degree of fidelity.

In order to detect a second, lower affinity kinetic pathway with respect to internal concentration, exchange was also measured at internal lactose concentrations ranging from 20 to 80 mM (Figure 5). Clearly, the rate is saturated over this range of internal lactose concentrations, indicating that there is only one apparent affinity constant with respect to internal lactose (K_{Mi} = 3.0 mM). The data also show that the low-affinity K_{Mo} at saturating internal lactose concentration is

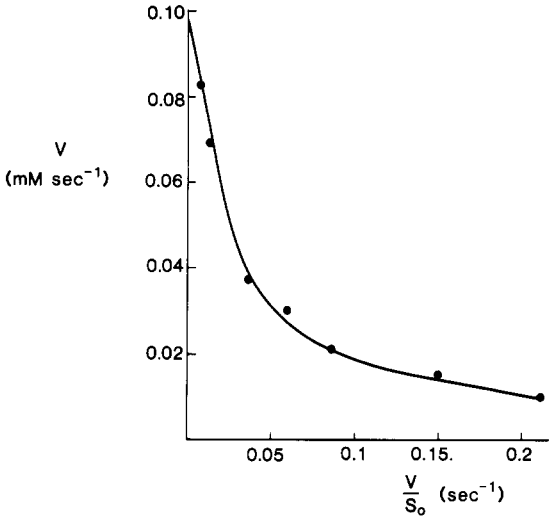

FIGURE 6: Lactose exchange catalyzed by E325A permease. Proteoliposomes reconstituted with E325A permease and equilibrated at pH 7.2 were loaded with 1.0 mM [1-^{14}C]lactose and diluted into 50 mM KP$_i$, pH 7.2, containing 0.5–10 mM unlabeled lactose, as indicated. The data are plotted according to Eadie–Hofstee.

about 6.0 mM. The maximum exchange rate via the low-affinity pathway is 3 mM/s (turnover number = 50 s^{-1}) which is 3–4 times higher than that of the high-affinity pathway. In addition, it is noteworthy that high concentrations of lactose on either side of the membrane do not inhibit the exchange reaction.

In summary, lactose exchange kinetics are characterized by two K_Ms with respect to external substrate and only one K_M with respect to internal substrate. In this respect, the permease is asymmetric.

Exchange by E325A Permease. Exchange kinetics were also examined in a mutant permease that contains Ala in place of Glu325 (i.e., E325A; Carrasco et al., 1986, 1989). The mutant is completely defective in the H^+/lactose symport but catalyzes equilibrium exchange and counterflow at normal rates and binds *p*-nitrophenyl α-D-galactopyranoside almost as well as wild-type permease. Experiments with purified, reconstituted E325A permease not only confirm the previous observations but also lead to even more clear-cut results due to the inability of the mutant permease to catalyze efflux. Thus, hardly any efflux of lactose whatsoever is observed with E325A proteoliposomes at pH 7.5 for up to 8 min (Carrasco et al., 1989), while wild-type permease catalyzes efflux under the same conditions with a half-time of about 1 min (Viitanen et al., 1983). Therefore, exchange measurements with E325A permease under conditions where $S_i > S_o$ are not restricted to low pH values because no net transport occurs. On the other hand, E325A permease catalyzes facilitated influx of lactose at a rate comparable to the wild type, but importantly, the reaction is uncoupled from H^+ influx (Carrasco et al., 1989). Thus, as in the case of wild-type permease, the rate constants for exchange are slightly underestimated when $S_o > S_i$. Figure 6 shows the rate of exchange in proteoliposomes reconstituted with E325A permease as a function of the external concentration with S_i at 1.0 mM. Biphasic kinetics with respect to external lactose are clearly retained in the mutant. Two factors contribute to the lower rates observed with E325A permease relative to the wild type (Figure 4): (i) the internal lactose concentration is 3 times lower; (ii) the protein to lipid ratio is 2 times lower. Other experiments [cf. Carrasco et al. (1986, 1989)] indicate that the exchange activity of E325A permease is comparable to the wild type. In addition, it is noteworthy that the data are not significantly different when the assays

are carried out at pH 6–8 (not shown).

Discussion

Net Flux versus Exchange. lac permease activity can be measured by using different experimental regimes, thereby allowing assay of net flux through different kinetic routes. $\Delta\bar{\mu}_{H^+}$-driven lactose influx and downhill lactose influx involve translocation of the ternary complex between H^+, lactose, and the permease from the periplasmic to the cytoplasmic side of the membrane and translocation of the unloaded permease in the reverse direction. The K_Ms for external lactose in these two reactions differ by about 1 order of magnitude (cf. Table I). Efflux, on the other hand, represents the overall rate of net downhill flux in the opposite direction: binding of H^+ and lactose to an internally exposed site(s), translocation of the ternary complex, ordered dissociation of lactose and H^+ at the periplasmic side of the membrane, and translocation of the empty site(s) back to the cytoplasmic side (Figure 1). The K_M for internal lactose in this process is about the same as that for downhill influx of lactose.

In the present study, we measured lactose exchange under nonequilibrium conditions where the net transport of lactose and H^+ is much slower than exchange. Therefore, this method involves only translocation of the ternary complex in both directions across the membrane. Surprisingly, the exchange measurements exhibit all the K_Ms observed in the other reactions (Table I). With respect to external lactose, exchange is a biphasic process with one affinity constant similar to the K_M for active transport and a second K_M that is of the same order of magnitude as the K_M for facilitated influx. With respect to internal lactose, a single apparent affinity constant is detected with a value similar to the K_M for net efflux.

The primary difference between exchange and net flux is whether or not the kinetic step corresponding to return of the permease occurs in the loaded or unloaded state. During exchange, the permease returns as a ternary complex followed by dissociation of lactose. In contrast, during net flux, the carrier returns in the empty state. Apparently, the measured K_Ms are not sensitive to these different routes. Thus, either the rate constants for return of the permease via the two pathways are similar or they do not contribute significantly to the affinity constants.

Results obtained from exchange experiments should be independent of whether the internal or external pool contains the labeled substrate. In this study, we chose to label the internal lactose pool. In the converse situation, when the external pool is labeled, the phenomenon is called entrance counterflow. Under these conditions, Viitanen et al. (1984) reported a K_M of 0.6 mM for external lactose with an internal lactose concentration of 10 mM. This value is in good agreement with the results presented here. $K_M(S_o)$ is calculated to be 0.46 mM by using the kinetic parameters given in Table I. Importantly, however, the present results also indicate that K_Ms and turnover numbers measured for counterflow are apparent kinetic parameters that depend on the internal lactose concentration or on the ratio of internal to external lactose concentrations.

Mechanism of Exchange. The kinetics of exchange in the "high affinity domain" (Figure 4A) are in good agreement with eq 3 which was derived for the following reaction sequence (cf. Appendix):

$$\text{lactose}_{out} + E_{out} \Leftrightarrow \text{lactose}\cdot E_{out} \Leftrightarrow \text{lactose}\cdot E_{in} \Leftrightarrow E_{in} + \text{lactose}_{in} \quad (5)$$

The main feature of the mechanism is that exchange of one external lactose molecule for one internal molecule proceeds in two separate sets of reactions. In the first set of reactions, the permease binds lactose on the outer surface of the membrane, for instance, translocates the molecule to the inside and releases the lactose. The sequence is then repeated from inside to out in the second set of reactions. Importantly, only one lactose molecule is bound at the same time. This mechanism is opposed to one in which both internal and external lactose bind simultaneously to the permease and are translocated in the same step.

The simplest way to explain exchange as depicted in eq 5 is to envisage a transporter with a single binding site that is exposed alternatively to either side of the membrane. Under properly chosen conditions for exchange, the binding site changes its orientation only when lactose is bound, while under conditions of net transport, the unloaded permease isomerizes spontaneously. The different conditions reflect the protonated and unprotonated carrier, respectively.

In contradistinction, the permease may have two binding sites, one on each side of the membrane, that may or may not have different affinities for lactose. In this case, the equilibrium between lactose·E_{out} and lactose·E_{in} (eq 5) would reflect "movement" of lactose within the protein from one site to the other. Consequently, exchange should be inhibited when lactose at the opposite side of the membrane occupies the putative empty binding site (i.e., trans inhibition). However, the experimental evidence presented in Figure 5 does not indicate any degree of inhibition at internal lactose concentrations of up to 80 mM or external concentrations of up to 60 mM. Therefore, a model with a single translocation site is more consistent with the data.

Biphasic Kinetics. A permease that catalyzes symport of lactose and H^+ should catalyze $\Delta\bar{\mu}_{H^+}$-driven lactose uptake as well as $\Delta\mu_{lac}$-driven H^+ uptake utilizing the same kinetic route. Both activities have been demonstrated, and each is characterized by a different K_M for lactose. Facilitated lactose influx in the absence of $\Delta\bar{\mu}_{H^+}$ exhibits a low apparent affinity for lactose (K_M = 3.1 mM in proteoliposomes reconstituted with purified permease), whereas imposition of a $\Delta\Psi$ (interior negative) results in an increase in apparent affinity (K_M = 0.5 mM; Viitanen et al., 1984). When lactose uptake is measured at intermediate values of $\Delta\bar{\mu}_{H^+}$, the kinetics are biphasic and very similar to the data presented for exchange (Figure 4).

A number of explanations for this phenomenon have been suggested: (i) Robertson et al. (1980) and Goldkorn et al. (1984) suggested that $\Delta\bar{\mu}_{H^+}$ induces dimerization of the permease with different kinetic features for monomer and dimer which is unlikely in view of more recent experiments (Dornmair et al., 1985; Costello et al., 1987). (ii) Wright et al. (1985) explained the K_M shift by postulating a $\Delta\bar{\mu}_{H^+}$-induced conformational change at the lactose binding site resulting in a lower K_D, a suggestion made earlier by Schuldiner et al. (1975). (iii) More recently, Page (1987) rationalized the biphasic kinetics by proposing random binding of lactose and H^+ with different dissociation constants for binding to the free permease or the binary complex. The two phases would reflect the two different orders of binding at the external face of the membrane.

It is likely that the mechanism underlying the biphasic kinetics observed for active transport and exchange is the same, and if so, the present study is inconsistent with the explanations put forward thus far. The exchange measurements documented here were carried out in the absence of $\Delta\bar{\mu}_{H^+}$, and conditions were chosen such that the permease remains fully protonated (cf. Figure 1). Thus, it seems unlikely that either $\Delta\bar{\mu}_{H^+}$ or ordered binding with lactose preceding H^+ at the outer

face of the membrane can be responsible for the results.

The conclusion that the biphasic kinetics are independent of $\Delta\bar{\mu}_{H^+}$ or the protonated/deprotonated state of the permease is supported by results with E325A permease which exhibit similar biphasic kinetics for exchange (Figure 6). According to the kinetic scheme presented in Figure 1, the mutant is defective in all reactions that involve deprotonation of the permease, and its behavior is consistent with the conclusion that it is unable to deprotonate at the outer surface of the membrane (Carrasco et al., 1986, 1989). Therefore, it is evident that neither $\Delta\bar{\mu}_{H^+}$ nor H^+ binding per se is responsible for the two kinetic phases. Moreover, similar results are obtained when the experiments are carried out between pH 6.0 and 8.0.

Two Binding Sites. Equilibrium exchange and binding are similar in that the distribution of the enzyme over all possible states is the same. Exchange measures the flux through these states, while binding measures the fraction of the enzyme in the substrate-complexed states. However, the saturation behavior of both parameters is the same. Analytically, it can be demonstrated that the affinity constant for an equilibrium exchange reaction such as that depicted in eq 5 is identical with the phenomenological binding constant for lactose in this system (cf. Appendix). In this regard, it is important to note that binding of lactose to *lac* permease in the equilibria of eq 5 is characterized by only one binding constant that is comprised of all the rate constants in the system. On the basis of these considerations and the observation that the exchange measurements are biphasic (i.e., two K_Ms are observed), it is reasonable to suggest that *lac* permease may have two distinct physical binding sites for lactose and that this is the underlying mechanism of the biphasic kinetics. Independent support for the contention that the permease contains two binding sites has been obtained recently from binding studies with the high-affinity lactose analogue *p*-nitrophenyl α-D-galactopyranoside (Lolkema & Walz, 1990).

Both sites could be catalytic, or one could be a regulatory site. In the first case, there would be two distinct kinetic cycles by which lactose is transported across the membrane, and each cycle would have a set of equilibria as described in eq 5. The affinity for internal lactose (K_{Mi}) would have to be the same for exchange through the two cycles, since monophasic kinetics are observed with respect to internal lactose concentrations. The phenomenological binding constants for lactose calculated from the measured K_Ms are 3.6 and 9 mM. In the second case, *lac* permease would have a regulatory site at the periplasmic side of the membrane with relatively low affinity. The high-affinity exchange pathway (Figure 4A) would reflect exchange through the catalytic cycle, and binding of external lactose to the regulatory site would increase the turnover rate.

ACKNOWLEDGMENTS

We thank Dieter Walz for many helpful discussions.

APPENDIX

lac permease catalyzes exchange between external (S_o) and internal (S_i) lactose:

$$S_o + E_o \underset{k_i}{\overset{k_o}{\rightleftharpoons}} E_i + S_i \qquad (a)$$

where E_o and E_i refer to those states of the permease that bind lactose on the outer and inner side of the membrane, respectively, and k_o and k_i are second-order rate constants in the two directions. Equilibrium a is kinetically analyzed by labeling one lactose pool and following the appearance of the label in the other pool. Since the enzyme concentration is much lower than the substrate concentrations, (i) the system will reach equilibrium very rapidly, and (ii) the equilibrium concentrations of lactose will equal the initial concentrations. The concentrations of E_o and E_i are set by the lactose concentrations and independent of the distribution of label, simplifying the exchange to a first-order process:

$$S_o \underset{k_iE_i}{\overset{k_oE_o}{\rightleftharpoons}} S_i \qquad (b)$$

with

$$k_oE_o/k_iE_i = S_i/S_o \qquad (c)$$

In a homogeneous system, exchange should follow a single exponential with a slope that equals the sum of the two pseudo-first-order rate constants. However, in this particular case, we have to account for the different internal and external volumes. The fractional internal volume of the proteoliposomes under the conditions employed is typically 1.4×10^{-5}, making the "back flow" of label from the external to the internal compartment negligible. The decrease of internal [^{14}C]lactose due to exchange is therefore adequately described by

$$[^{14}C]S_i = S_i \exp(-k_iE_it) \qquad (d)$$

Equilibrium a is a simplification in the sense that it does not account for the saturation behavior of the enzyme. The actual concentrations of E_i and E_o are determined by the equilibria:

$$S_i + E_i \overset{K_{Di}}{\Leftrightarrow} S{\cdot}E_i \overset{K_r}{\Leftrightarrow} S{\cdot}E_o \overset{K_{Do}}{\Leftrightarrow} E_o + S_o \qquad (e)$$

where K_{Di} and K_{Do} are dissociation constants for the enzyme–substrate complex on the inner and outer surfaces of the membrane, respectively, and K_r is the equilibrium constant for the translocation of the ternary complex. The concentrations of E_o and E_i are expressed in these equilibrium constants, the total enzyme concentration $\mathcal{E}$, and the concentrations of S_i and S_o.

It follows for the relation between the measured rate constant k_iE_i and the lactose concentrations:

$$\frac{1}{k_iE_i} = \frac{1}{k_i\mathcal{E}}\left(1 + \frac{S_i}{K_{Mi}} + \frac{K_{Mo}}{K_{Mi}}\frac{S_i}{S_o}\right) \qquad (f)$$

where K_{Mi} and K_{Mo} are functions of K_{Di}, K_r, and K_{Do} [see also Lolkema et al. (1985)]. Equation f is equivalent to eq 3 under Results which is a convenient form to describe experiments with a constant internal concentration and variable external concentration. A plot of $S_o/S_ik_iE_i$ as a function of S_o yields a straight line for every S_i. The different lines intersect the y axis at the same value ($1/k_o\mathcal{E}$).

In case of equilibrium exchange ($S_i = S_o = S$), eq f simplifies to

$$\frac{1}{k_iE_i} = \frac{1 + K}{k_i\mathcal{E}}\left(1 + \frac{S}{K_M}\right) \qquad (g)$$

with

$$K = \frac{K_{Mo}}{K_{Mi}} \text{ and } K_M = K_{Mi} + K_{Mo} \qquad (h)$$

where K is the overall equilibrium constant for equilibria e. The rate of exchange saturates with an affinity constant K_M that equals the sum of the individual affinity constants for internal and external substrate.

Algebraic manipulation reveals the relationship between the affinity constants and the equilibrium constants in eq e:

$$K_{Mi} = K_{Di}\frac{1}{1 + K_r} \text{ and } K_{Mo} = K_{Do}\frac{K_r}{1 + K_r} \qquad \text{(i)}$$

In the same way, it can be shown that the fraction of the enzyme in states $S{\cdot}E_i$ and $S{\cdot}E_o$ in equilibria e as a function of the substrate concentration ($S = S_i = S_o$) saturates with an apparent K_D that equals

$$K_D = \frac{K_{Di} + K_r K_{Do}}{1 + K_r} = K_{Mi} + K_{Mo} = K_M \qquad \text{(j)}$$

Consequently, the phenomenological binding constant equals the K_M obtained from equilibrium exchange measurements.

Registry No. H^+, 12408-02-5; lactose, 63-42-3; *lac* permease, 9033-40-3.

References

Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937.

Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6894.

Carrasco, N., Viitanen, P., Herzlinger, D., & Kaback, H. R. (1984a) *Biochemistry 23*, 3681.

Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672.

Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486.

Carrasco, N., Püttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 2533.

Costello, M. J., Escaig, J., Matsushita, K., Viitanen, P. V., Menick, D. R., & Kaback, H. R. (1987) *J. Biol. Chem. 262*, 17072.

Dornmair, K., Corin, A. F., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3633.

Foster, D. L., Garcia, M. L., Newman, M. J., Patel, L., & Kaback, H. R. (1982) *Biochemistry 21*, 5634.

Foster, D. L., Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31.

Garcia, M. L., Viitanen, P. V., Foster, D. L., & Kaback, H. R. (1983) *Biochemistry 22*, 2524.

Goldkorn, T., Rimon, G., & Kaback, H. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3322.

Goldkorn, T., Rimon, G., Kempner, E. S., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1021.

Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688.

Kaback, H. R. (1983) *J. Membr. Biol. 76*, 95.

Kaback, H. R. (1989) *Harvey Lect. 83*, 77.

Kaback, H. R., Bibi, E., & Roepe, P. D. (1990) *Trends Biochem. Res.* (in press).

Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691.

Kaczorowski, G. J., Robertson, D. E., & Kaback, H. R. (1979) *Biochemistry 18*, 3697.

Lolkema, J. S., & Walz, D. (1990) *Biochemistry 29*, 10120.

Lolkema, J. S., ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1985) *Eur. J. Biochem. 154*, 387.

Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804.

Page, M. G. P. (1987) *Biochim. Biophys. Acta 897*, 112.

Page, M. G. P., & Rosenbusch, J. H. P. (1988) *J. Biol. Chem. 263*, 15906.

Robertson, D. E., Kaczorowski, G. J., Garcia, M. L., & Kaback, H. R. (1980) *Biochemistry 19*, 5692.

Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502.

Schuldiner, S., Kerwar, G. K., Weil, R., & Kaback, H. R. (1975) *J. Biol. Chem. 250*, 1361.

Seckler, R., & Wright, J. K. (1984) *Eur. J. Biochem. 142*, 269.

Seckler, R., Wright, J. K., & Overath, P. (1983) *J. Biol. Chem. 258*, 10817.

Seckler, R., Möröy, T., Wright, J. K., & Overath, P. (1986) *Biochemistry 25*, 2403.

Stochaj, V., Bieseler, B., & Ehring, R. (1986) *Eur. J. Biochem. 158*, 423.

Viitanen, P. V., Garcia, M. L., Forster, D. L., Kaczorowski, G. J., & Kaback, H. R. (1983) *Biochemistry 22*, 2531.

Viitanen, P. V., Garcia, M. L., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1629.

Viitanen, P. V., Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1986) *Methods Enzymol. 125*, 429.

Vogel, H., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3625.

Wright, J. K., Dornmair, K., Midaku, S., Möröy, T., Neuhaus, J. M., Seckler, R., Vogel, H., Weigel, U., Jähnig, F., & Overath, P. (1985) *Ann. N.Y. Acad. Sci. 456*, 326.