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Kinetic Evidence for Two Interconvertible Forms of the Folate Transport Protein from *Lactobacillus casei*†

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Lactobacillus casei **cells contain a folate transport protein which exhibits a high affinity for folate. The dissociation constant (K_D) for folate derived from binding parameters at the steady state (at 0°C) is 0.4 nM at pH 7.5 and 0.1 nM at pH 6.0. In the present study, folate binding to this protein at pH 7.5 (and 0°C) was shown to follow second-order kinetics and to proceed with an association constant (k_{+1}) of 4.9×10^7 liter/mol per min. k_{+1} was not affected by preincubation conditions which alter the energetic state of the cell. Measurements on the extent of binding showed further that (at 0°C) essentially all unoccupied folate-binding sites reside at or are readily accessible to the outer surface of the membrane. In contrast, after saturating the binding site with [^{3}H]folate, the first-order rate constant (k_{-1}) for dissociation of the bound substrate (at 0°C) was found to vary substantially with the conditions employed. k_{-1} was 0.028/min in freshly harvested cells, but it increased by 2.8-fold in cells preincubated at 23°C for 60 min and by 5.4-fold in isolated membranes. In addition, the faster rate observed in preincubated cells (k_{-1} = 0.077/min) returned to a slower rate after brief exposure of the cells to pH 6.0 (k_{-1} = 0.041/min), glucose (k_{-1} = 0.050/min), or both (k_{-1} = 0.012/min). k_{-1} was twofold lower at pH 6.0 than at pH 7.5 and was less dependent on the preincubation conditions, although it also increased substantially (5.5-fold) when the cells were converted to plasma membranes. The proposed explanation for these results is that the folate transport protein of *L. casei* exists in two forms which can be distinguished by the accessibility of the binding site to the external medium and whose amounts are dependent upon the presence of bound folate, the pH, and the energetic state of the cell. It is suggested that these forms are transport proteins with binding sites oriented towards the inner and outer surfaces of the membrane.**

Lactobacillus casei cells contain a single high-affinity transport system which is specific for the uptake of folate compounds (2, 11, 15, 16). The process requires energy for the accumulation of folate, whose free intracellular level can exceed the external concentration by several thousandfold (8). The energy source has not yet been identified, although it appears to be a high-energy phosphate compound rather than the proton motive force (8). The transport system is composed of a binding protein, which has been extracted from the cell membrane and purified to homogeneity (6, 7, 10), and at least one other cellular component (9). The latter is also required for the uptake of thiamine and biotin and has been suggested to be involved in the coupling of energy to these transport processes (9).

The folate transport protein of *L. casei* mediates the translocation of substrate into the cytoplasm at an unusually slow rate. In fully energized cells, a complete transport cycle at 30°C requires approximately 65 s per binding site (8). One advantage of this slow rate is that it has been possible to perform binding measurements without the concurrent transport of folate into the cytoplasm (5, 8). This is particularly important in affinity measurements, since at substrate concentrations near the K_D for folate (ca. 0.4 nM at pH 7.5), binding is slow and a long incubation interval (60 min) is required to reach a steady state (5). Binding measurements have also provided evidence that transport occurs via a folate-cation symport mechanism (3, 5). Evidence favoring this model is that the binding protein requires cations to exhibit a high affinity for folate and that divalent cations fulfill this requirement at 1,000-fold-lower levels than do their monovalent counterparts. The implication of these results is that the transport protein contains a cation-binding site and that the latter preferentially accomodates a cation or cations with a plus two charge. Transport of the folate dianion could then occur in conjunction with cations, which would allow uptake to proceed unopposed by the large membrane potential of these cells (8). In the present study, additional information has been obtained on the binding and translocation steps which constitute the initial phase of the folate transport cycle. The results provide evidence that the orientation of the binding site and its rate of reorientation across the membrane are substantially affected by the presence of bound folate.

MATERIALS AND METHODS

Radiolabeled folate. [3′,5′,7,9-^{3}H]folate was obtained from Amersham Corp. and purified by thin-layer chromatography on cellulose sheets (Eastman 13255) with 0.1 M potassium-HEPES [4-2(2-hydroxyethyl)-1-piperazine-ethanesulfonate (pH 7.5)] as the solvent. Samples were stored (at −20°C) in the presence of 10% ethanol to reduce decomposition. ^{86}Rb (35 Ci/g) was obtained from New England Nuclear Corp.

Growth of cells. *L. casei* subsp. *rhamnosis* (ATCC 7469) cells were grown for 16 h at 30°C in the medium described by Flynn et al. (1) containing limiting amounts (1.0 nM) of folate and 5 g of vitamin-free casein hydrolysate (ICN Pharmaceuticals, Inc.) per liter.

Preincubation of cells. Freshly grown cells were washed with 100 volumes of either HEPES-magnesium buffer (50 mM potassium-HEPES, 5 mM $MgCl_2$, [pH 7.5]) or MES [2-(*N*-morpholino)ethanesulfonate]-magnesium buffer (50 mM potassium-MES, 5 mM $MgCl_2$ [pH 6.0]), suspended to a

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density of 6×10^8/ml (A_{650}, 0.9), and either used directly or incubated at various times at 23°C with the desired additions.

Isolation of plasma membranes. Freshly grown cells (25 g) were washed at 4°C with 100 volumes of 50 mM potassium phosphate (pH 6.5), suspended in 250 ml of the same buffer, and then disrupted by repeated passage through a Manton-Gaulin homogenizer (4,000 lb/in^2). The temperature during the latter procedure was maintained between 10 and 20°C by the addition of ice. After centrifugation at 24,000 × *g* (15 min, 4°C), the particulate fraction was recovered and suspended in 10 volumes of the HEPES-magnesium buffer. Intact cells were removed by centrifugation at 2,000 × *g* (20 min, 4°C), and the plasma membranes in the supernatant fraction were collected by centrifugation at 24,000 × *g* (5 min, 4°C). The concentration of membranes was adjusted to 0.1 mg of protein per ml for exchange studies and to 0.04 mg of protein per ml for determination of K_D values. Protein was measured (in the absence of HEPES) by a modified Lowry procedure with sodium dodecyl sulfate (14) and with bovine serum albumin as the standard. Electron micrographs of the isolated plasma membranes were prepared from gluteraldehyde-fixed samples by the procedure of Linder et al. (13).

Determinations of the time dependence of folate uptake. Folate uptake by cells was determined in an assay mixture consisting of cells (1.2×10^{10}), 10 nmol of [^{3}H]folate (500 dpm/pmol), and buffer in a final volume of 20 ml. After incubation for various times at the desired temperature, cells were removed in 1.0-ml aliquots, collected onto nitrocellulose filters (diameter, 25 mm; pore size, 0.45 μm), washed with 2 ml of ice-cold buffer, and analyzed directly for radioactivity in 10 ml of scintillation fluid (CytoScint; West Chem Products). Control samples were prepared by adding an excess of unlabeled folate (100 μM) before adding the [^{3}H]folate. Results are expressed in nanomoles of [^{3}H]folate bound per 10^{10} cells.

Rate of folate binding to the transport protein. Initial rates of binding were measured in individual assay mixtures containing cells (1.5×10^8), 0.8 pmol of [^{3}H]folate (20,000 dpm/pmol), and buffer at the desired pH in a final volume of 10 ml. After incubation for various times at 0°C, the cells were collected onto prechilled filters (pore size, 0.45 μm) and analyzed for bound radioactivity. The time required to complete the filtration step was less than 20 s. Samples containing excess unlabeled folate (100 μM) served as controls. The second-order rate constant (k_{+1}) for binding was calculated from the equation $k_{+1} = \{2.303/[t \times (A_0 - B_0)]\} \times \log [(A \times B_0)/(A_0 \times B)]$, where A_0 and B_0 are initial concentrations of [^{3}H]folate and folate-binding protein, respectively, and A and B are the corresponding concentrations at time t.

Rate of folate exchange. The transport protein was saturated with folate by combining freshly harvested or pretreated cells (1.2×10^{10}) with 10 nmol of [^{3}H]folate (500 dpm/pmol) and buffer in a final volume of 20 ml and incubating the mixture for 10 min at 0°C. The exchange reaction was then initiated by the addition of 100 μl of 20 mM unlabeled folate. Cells in 1.0-ml aliquots were removed at the desired times, collected onto prechilled filters (pore size, 0.45 μm), washed with 2 ml of ice-cold buffer, and analyzed for bound radioactivity. The time required to filter and wash each sample was less than 30 s. Control values were determined separately in samples in which an excess of unlabeled folate (100 μM) was added before the [^{3}H]folate was added. Exchange rates in isolated plasma membranes were determined similarly, except that the membranes (0.1 mg of protein per ml) were collected onto filters (pore size, 0.65 μm). The recovery of membranes by the filters was 70 ± 10%. Results are expressed in nanomoles of [^{3}H]folate bound per milligram of membrane protein. Dissociation rate constants (k_{-1}) were calculated from the equation $k_{-1} = 0.693/t_{1/2}$, where $t_{1/2}$ is the time at which 50% of the bound [^{3}H]folate had exchanged with the unlabeled folate.

***K_D* determinations.** Dissociation constants (K_D values) were determined in individual assay mixtures containing various concentrations of [^{3}H]folate (0.05 to 20 nM) and cells (5×10^8) in a final volume of 50 ml. After incubation for 60 min at 0°C, the cells were collected onto filters (pore size, 0.45 μm) and analyzed for radioactivity. In control samples, an excess of unlabeled folate (100 μM) was added before the [^{3}H]folate was added. K_D was defined as the concentration of folate required for half saturation of the binding site and was calculated from a double-reciprocal plot of folate bound versus the free folate concentration at the steady state. K_D measurements in isolated plasma membranes were performed similarly, except that the assay volume was reduced to 36 ml and the membranes were collected by centrifugation at 40,000 × *g* (10 min, 4°C).

Other determinations. The membrane transition temperature was determined in cells that had been washed twice with 100 volumes of 50 mM sodium-HEPES (pH 7.5) and suspended to a density of 6×10^8/ml in 50 mM sodium-HEPES containing 1.0 mM glucose. After incubation for 2 min at 30°C, the cells were distributed into 5.93-ml aliquots and equilibrated to various temperatures between 0 and 39°C. Valinomycin (10 μl of a 5.0 μM solution in ethanol) and ^{86}Rb (60 μl of a 100 μM solution in pH 7.5 buffer) were then added to final concentrations of 10 nM and 1.0 μM, respectively, and the incubation was continued at the same temperature. At various times (0.5 to 20 min), cells were removed in 1.0-ml aliquots, collected onto filters (pore size, 0.45 μm), washed with 2 ml of ice-cold buffer, and analyzed for associated radioactivity. Influx was determined from the linear portion of the time profile, using samples lacking valinomycin as the control. Rates are reported in picomoles of ^{86}Rb transported per minute per 10^{10} cells.

Membrane potentials were determined from the distribution of ^{86}Rb across the membrane at the steady state. Cells were prepared as described above for determinations of membrane transition temperature, except that the final valinomycin concentration was increased (by 1,000-fold) to 10 μM. ^{86}Rb uptake reached a maximum under these conditions within 10 min at each temperature, and its intracellular concentration was determined from a cell volume of 2.4×10^{-12} ml (8). Membrane potentials were calculated by the Nernst equation.

RESULTS

Rate of folate binding to the transport protein. Various substrate binding properties of the folate transport protein of *L. casei* have been reported previously (5). In these studies, transport of the substrate into the cytoplasm was minimized by use of a low assay temperature (0°C) and cells that had been exhausted of energy reserves. The binding protein under these conditions was shown to have a high affinity for folate, although a slow binding rate at low concentrations of folate (0.5 to 10 nM) necessitated incubation intervals of up to 60 min (at 0°C) to reach a steady state. At 1.0 μM folate, however, rates of substrate binding were rapid, and maximum amounts of bound folate were achieved within 15 s (Fig. 1A). Uptake did not increase further over a subsequent 10-min incubation period, confirming that transport is very

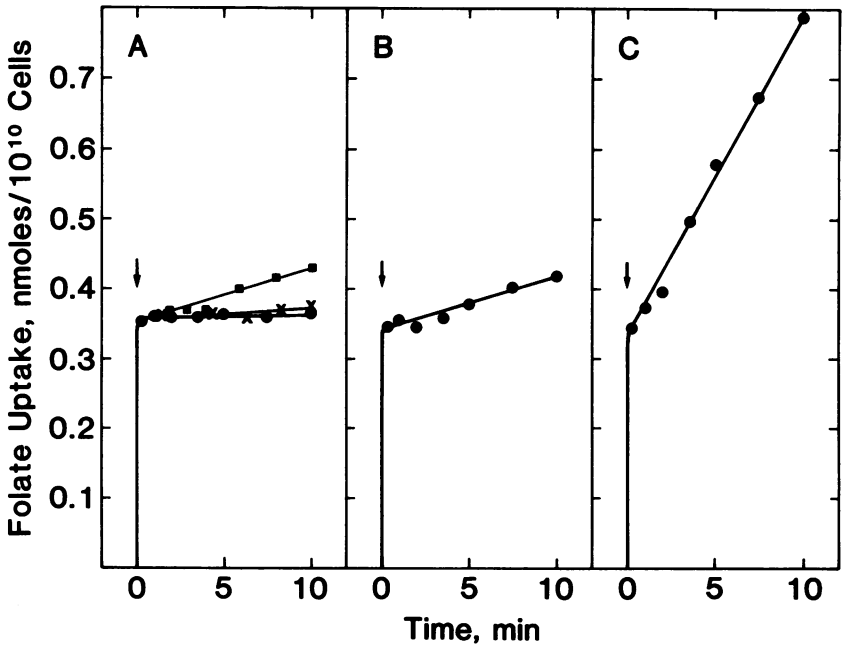

FIG. 1. Uptake of [^{3}H]folate by *L. casei* cells as a function of metabolic state and temperature. Measurements were performed in cells pretreated as indicated in HEPES-magnesium buffer (pH 7.5) at 0°C (A), 23°C (B), and 37°C (C). Arrows indicate the time of addition of [^{3}H]folate. Symbols: X, freshly-harvested cells; ●, cells preincubated in HEPES-magnesium buffer for 60 min at 23°C; and ■, cells preincubated for 2 min at 23°C in HEPES-magnesium buffer containing 1.0 mM glucose.

slow under these conditions. Transport (at 0°C) was also minimal in freshly harvested cells but it increased to measurable levels in cells preincubated with glucose (Fig. 1A). When similar measurements were performed in energy-depleted cells at 23°C (Fig. 1B) and 37°C (Fig. 1C), no change was noted in the magnitude of the initial binding phase, indicating that the binding sites for folate are fully accessible to the external surface of the membrane over the temperature range used (0 to 37°C). A second uptake component, however, was apparent at the higher temperatures; its contributions to total uptake were 22 and 132% of the binding component after 10 min at 23 and 37°C, respectively.

The *L. casei* plasma membrane undergoes a phase transition of 14°C. This value was determined from the temperature dependence of valinomycin-mediated influx of ^{86}Rb by energized cells. Since valinomycin must diffuse through the lipid bilayer to mediate transport (12), influx via this ionophore decreases abruptly at the phase transition temperature of the membrane. The temperature at which this change occurred in our studies (14°C) can be readily observed from an Arrhenius plot of the data (Fig. 2). The membrane potential under these conditions ranged from −140 to −160 mV.

The rate of folate binding to the transport protein can be measured by using low concentrations of [^{3}H]folate (0.1 nM) with a high specific activity (20,000 cpm/pmol). These conditions provide the necessary sensitivity to measure binding when less than 5% of the binding sites have been occupied. The binding rate in freshly harvested cells (Fig. 3, closed circles) was linear for 2 to 5 min at 0°C and then gradually decreased thereafter. The association rate constant (k_{+1}) for this process was 4.9×10^7 liters per mol per min. The same initial rate was observed in cells that had been preincubated for 60 min at 23°C (Fig. 3C) and in cells preincubated for 2 min at 23°C with 1.0 mM glucose (Fig. 3B). The extent of binding, however, was 20% higher after 30 min at 0°C in both fresh cells and cells preincubated with glucose, compared with that in energy-depleted cells. At pH 6.0, k_{+1} was 9.7×10^7 liters per mol per min (Fig. 3).

Exchange of bound folate in untreated cells. Since folate is bound reversibly by the transport protein, the rate of release of bound [^{3}H]folate in the presence of an excess of unlabeled

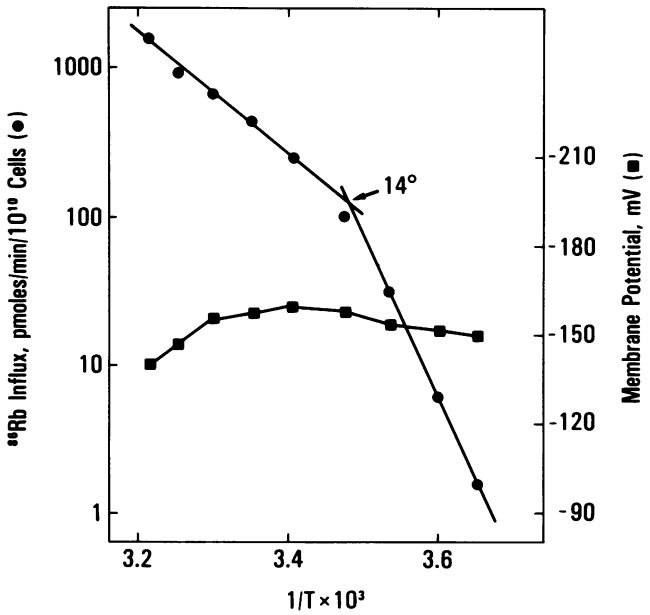

FIG. 2. Dependence of valinomycin-mediated ^{86}Rb influx and membrane potential on the reciprocal of absolute temperature. Measurements were performed as described in Materials and Methods.

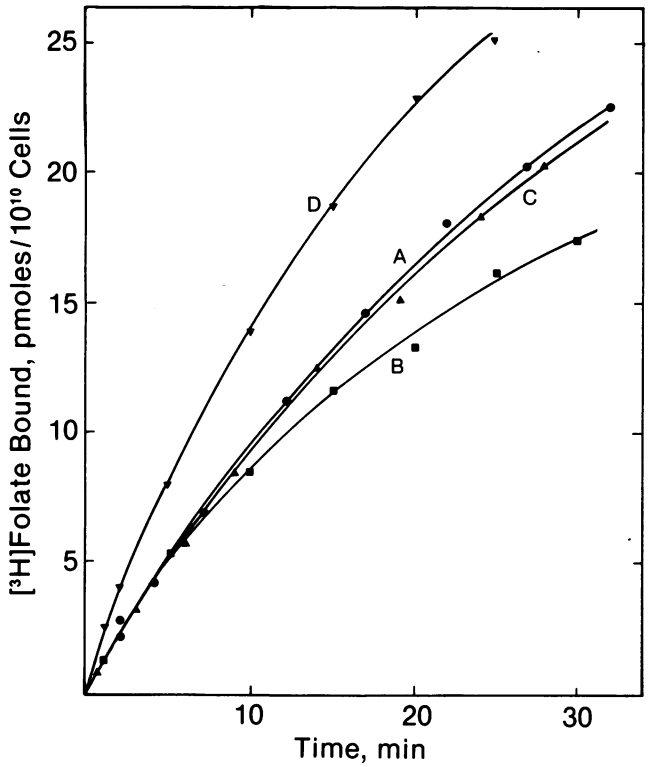

FIG. 3. Rate of [^{3}H]folate binding at 0°C to the transport protein of intact cells. Source of binding protein: A, freshly harvested cells (pH 7.5); B, cells (pH 7.5) preincubated for 60 min at 23°C; C, freshly harvested cells (pH 7.5) preincubated for 2 min at 23°C with 1.0 mM glucose; D, freshly harvested cells (pH 6.0). The [^{3}H]folate concentration was 80 pM; the binding protein concentration was 500 pM. Buffers were HEPES-magnesium (pH 7.5) and MES-magnesium (pH 6.0).

folate approximates the substrate dissociation rate. The dissociation rate constant can then be measured from the time at which half of the bound [^{3}H]folate is displaced by the unlabeled substrate. Freshly harvested cells that had been exposed to [^{3}H]folate for 10 min at 0°C released bound [^{3}H]folate at a relatively slow rate ($t_{1/2}$, 25 min) when a 200-fold excess of the unlabeled folate was added to the medium (Fig. 4). A replot of these data (not shown) as log [^{3}H]folate bound versus time was linear, indicating that the release of bound substrate followed first-order kinetics. The calculated first-order dissociation rate constant (k_{-1}) for this process was 0.028/min. The exchange rate in fresh cells was not affected by the time at which unlabeled folate was added after the initial binding of [^{3}H]folate (over a 1- to 60-min interval) or by preincubation with glucose (1.0 mM) for 2 min at 23°C before the addition (at 0°C) of [^{3}H]folate.

Exchange of bound folate in preincubated cells. Preincubation of the cells for extended times at 23°C before the addition of [^{3}H]folate at 0°C substantially increased the exchange rate (Fig. 4). The rate constant for exchange (k_{-1}) increased from 0.028/min in control cells to values of 0.063, 0.077, and 0.100/min in cells that had been subjected to preincubation intervals of 20, 60, and 120 min, respectively. As was observed in freshly harvested cells, the exchange rate in cells preincubated for 60 min at 23°C did not vary with the time (between 1 and 60 min) at which unlabeled folate was added (data not shown).

Exchange of bound folate in isolated membranes. The folate-binding protein was recovered in high yield in plasma membrane preparations derived from high-pressure homogenization (see Materials and Methods). These preparations, when observed with an electron microscope, consisted almost entirely of open spheres devoid of cytoplasmic constituents. The exchange of bound folate in these membrane preparations proceeded at a faster rate than in intact cells (Fig. 4). k_{-1} for exchange in membranes was 0.15/min, which represented an increase of 5.4-fold relative to freshly harvested cells.

pH dependence. The exchange rate for bound [^{3}H]folate decreased when the pH was reduced from 7.5 to 6.0 (Fig. 5). In freshly harvested cells, k_{-1} at pH 6.0 was 0.014/min, compared with 0.028/min under the same conditions at pH 7.5 (Fig. 4). In addition, preincubation of the cells for 60 min at 23°C increased the exchange rate to a lesser extent (1.9-fold) at pH 6.0 (k_{-1} = 0.026), than at pH 7.5 (2.8-fold). In plasma membranes at pH 6.0, the exchange of bound folate proceeded at a rate (k_{-1} = 0.077/min) which was 5.5-fold higher than in freshly harvested cells under the same conditions (Fig. 5).

Reversibility. The relatively rapid exchange associated with cells that had been preincubated at pH 7.5 for 60 min at 23°C could be reversed by performing a second incubation with added glucose or at pH 6.0 or both, and then shifting the pH back to 7.5 before measuring [^{3}H]folate exchange (Fig. 6). Control cells that had been incubated for 60 min at 23°C before analysis exhibited a k_{-1} of 0.095/min, while a 1.9-fold-slower exchange (k_{-1} = 0.05/min) was observed when these same cells were subjected to a brief (2-min) exposure to glucose (at 23°C) before analysis. The exchange rate decreased to a slightly greater extent (k_{-1} = 0.041/min) when the intermediate incubation was performed in buffer at pH 6.0, while a combination of glucose plus a shift in pH to 6.0 led to a decrease of 7.9-fold (k_{-1} = 0.012).

Effect of preincubation conditions on the dissociation constant (K_D) for folate. The affinity of the binding protein for

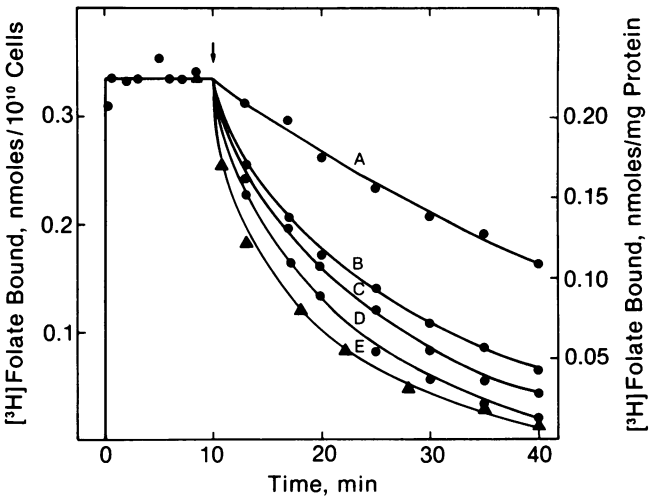

FIG. 4. Effect of preincubation at 23°C on the rate of [^{3}H]folate exchange at 0°C. [^{3}H]folate (0.5 μM) was added at 0°C to cells that had been preincubated for various times at 23°C (A through D) or to isolated plasma membranes (E) and then analyzed at the indicated times for bound [^{3}H]folate. Excess unlabeled folate (100 μM) was added after 10 min to initiate the exchange reaction. Preincubation times for cells (at 23°C) were 0 min (A), 20 min (B), 60 min (C), and 120 min (D).

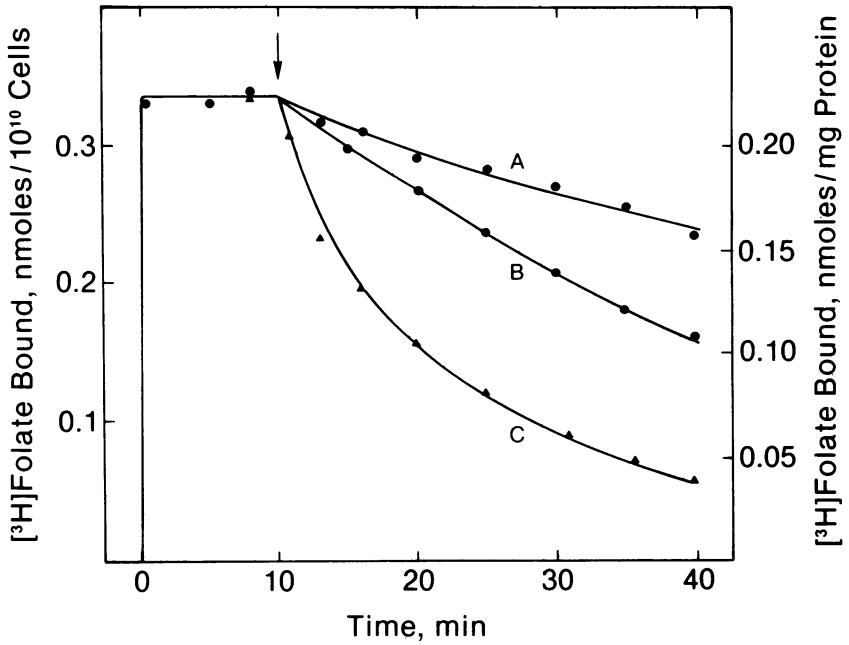

FIG. 5. Exchange rate for bound [³H]folate at pH 6.0. Measurements were performed at 0°C in freshly-harvested cells (A), cells preincubated for 60 min at 23°C (B), and isolated plasma membranes (C). The buffer used was MES-magnesium (pH 6.0).

folate remained relatively unchanged under conditions in which considerable fluctuations were observed in the exchange rate (Table 1). At pH 7.5, the K_D for folate was 0.42 nM in cells that had been preincubated for 60 min at 23°C; slightly lower values were obtained in freshly harvested cells (K_D = 0.26 nM) or in isolated plasma membranes (K_D = 0.35 nM). At pH 6.0, the K_D for folate was approximately 0.1 nM in freshly-harvested cells, preincubated cells, or isolated plasma membranes.

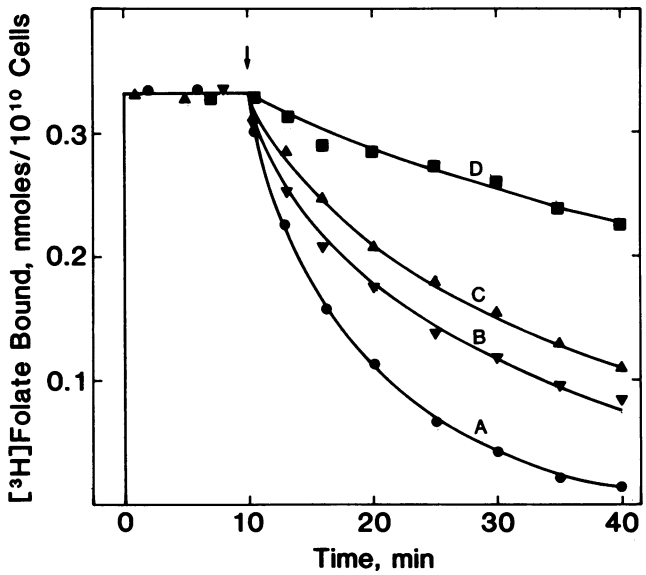

FIG. 6. Variation in exchange rate with preincubation conditions. Washed cells were preincubated for 1 h at 23°C in HEPES-magnesium buffer (pH 7.5) and then analyzed directly at 0°C for [³H]folate exchange (A [control samples]); incubated an additional 2 min at 23°C with added glucose (1.0 mM) before analysis at 0°C (B); centrifuged, incubated in MES-Mg buffer (pH 6.0) for 2 min at 23°C, centrifuged, suspended in pH 7.5 buffer, and analyzed at 0°C for exchange (C); or analyzed as described for curve C, above, except that glucose (1.0 mM) was added during the pH 6.0 preincubation (D). In samples preincubated with glucose, exchange with unlabeled folate was initiated 3 min after the addition of [³H]folate.

DISCUSSION

The ability to readily deplete *L. casei* cells of energy reserves has made it possible to study the binding of folate to its transport protein (at 0°C) without the concurrent accumulation of substrate in the cytoplasm. Under these conditions, essentially all folate taken up by cells is bound to the transport protein (Fig. 1). At higher temperatures, transport activity can be detected, although uptake occurs at less than 10% of the rate of energized cells. The level of binding activity is independent of temperature (Fig. 1) and the phase transition of the membrane (Fig. 2), indicating that even at 0°C, all of the folate-binding sites are directed towards or are readily accessible to the external surface of the cell. Contrasting results have been obtained with L1210 mouse leukemia cells in which (at 0°C) a portion (25%) of the folate transport proteins did not react with an active ester of

TABLE 1. Effect of preincubation time and pH on the K_D for folate in intact cells and in isolated plasma membranes[a]

Source of binding protein	Preincubation time (min) at 23°C	K_D for folate (nM)	
		pH 7.5	pH 6.0
Cells	0	0.26	0.12
	60	0.42	0.10
Membranes	0	0.35	0.13

[a] K_D values were measured at 0°C in buffers of the indicated pH as described in Materials and Methods. Each value is the average of two separate determinations.

methotrexate and, thus, appeared to be oriented towards the cytoplasmic side of the membrane (4).

[^{3}H]folate that is bound to the *L. casei* transport protein exhibits varied patterns of exchange with added unlabeled folate. At pH 7.5 (Fig. 4), the exchange rate is relatively slow in freshly harvested cells, but it increases by 2.8-fold upon preincubation for 60 min at 23°C and by 5.4-fold in plasma membranes. At pH 6.0 (Fig. 5), exchange rates are generally slower than at pH 7.5, although the same pattern of increased exchange is apparent between fresh cells, cells preincubated at 23°C, and isolated membranes. A distinct feature of the exchange process is that the more rapid rate which appears after the preincubation step at pH 7.5 can be reversed (Fig. 6). Thus, by inserting a second incubation with added glucose or at pH 6.0 or both before exchange measurements (at pH 7.5), the exchange rate can be reduced two- to eightfold (Fig. 6).

The proposed explanation for the above results is that the binding of folate causes the transport protein to assume a second form in which its binding site (at 0°C) becomes inaccessible to the added exchange substrate and that the amount of this form is dependent upon both the pH and the energetic state of the cell. A slow exchange would thus be indicative of conditions in which the inaccessible form is relatively abundant. Considerations of protein function suggest that this inaccessible form may be a transport protein with an intracellular orientation. This explanation would be feasible if the binding of folate to externally oriented sites is followed by a rapid translocation of the folate-binding site complex to the inner membrane surface. Exchange of bound folate could then occur only when loaded binding sites return to the cell exterior. The presence of fewer accessible sites would, in turn, cause a reduction in the dissociation rate for bound folate. Since the association rate would also decrease when inaccessible binding sites are present, proportionally similar decreases in k_{+1} and k_{-1} at the steady state could have caused the apparent K_D for folate to remain relatively constant (Table 1), despite substantial changes in the exchange rate (Fig. 4 through 6). In contrast, initial rates of binding would not be expected to vary with preincubation conditions (Fig. 3) since the inaccessible form arises only subsequently to the binding of folate. The decrease in exchange rate that occurs upon preincubation with glucose could be explained if the latter provides an energy source which interacts with a component of the transport system and stabilizes the inward orientation or if an inward orientation of the protein-folate complex is promoted by a glucose-dependent increase in the membrane potential. The latter explanation would be plausible if the binding site-folate-cation complex has a net positive charge. A pH-dependent stabilization of an inward orientation could also contribute to the decrease in exchange rate that occurs in cells preincubated at pH 6.0 (Fig. 6).

The folate transport system of *L. casei* must facilitate a series of steps to achieve active transport. These include the extracellular binding of folate, movement of the substrate across the membrane, substrate release into the cytoplasm, and reorientation of the binding site to the external surface of the membrane. Energy is consumed at some point in this cycle to achieve the large concentration gradient of folate observed in these cells (8). The present results suggest that both substrate binding and internalization occur rapidly at 0°C and therefore cannot be rate limiting to the overall process. It thus appears likely that energy coupling occurs during one of the latter two steps of the transport cycle. In addition, since internalization of the binding site appears to occur rapidly at a temperature (0°C) substantially below the phase transition temperature (14°C) of the membrane (Fig. 2), movement of the binding site across the membrane does not appear to require large conformational changes in the protein.

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