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Binding and Transport of Thiamine by *Lactobacillus casei*

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The relationship between thiamine transport and a membrane-associated thiamine-binding activity has been investigated in *Lactobacillus casei*. Thiamine transport proceeds via a system whose general properties are typical of active uptake processes; entry of the vitamin into the cells requires energy, is temperature dependent, exhibits saturation kinetics, and is inhibited by substrate analogs. A considerable concentration gradient of unchanged thiamine can be achieved by the system, although the vitamin is slowly metabolized to thiamine pyrophosphate. Consistent with these results, *L. casei* also contains a high-affinity, thiamine-binding component which could be measured by incubation of intact cells with labeled substrate at 4°C (conditions under which transport is negligible). Binding was insensitive to iodoacetate, occurred at a level (0.5 nmol per 10^{10} cells) nearly 20-fold higher than could be accounted for by facilitated diffusion, and was found to reside in a component of the cell membrane. Participation of this binder in thiamine transport is supported by the observations that the processes of binding and transport showed similarities in their (i) regulation by the concentration of thiamine in the growth medium, (ii) binding affinities for thiamine, and (iii) susceptibility to inhibition by thiamine analogs.

Cells of *Lactobacillus casei* contain a highly efficient transport system for the accumulation of the vitamin folic acid and various folate compounds (1, 4, 8, 19, 20). In conjunction with this transport capacity, the cells also have the ability to bind folate. The component responsible for this binding could be detected as uptake of the vitamin at 4°C and was shown to reside in the cell membrane (5, 6). When membrane preparations were treated with the nonionic detergent Triton X-100 in the presence of labeled folate, the bound folate was released from the particulate fraction and was recovered stoichiometrically in a water-soluble form (5, 6). The extracted binding component was then purified to homogeneity (6, 7) and shown to be an unusually hydrophobic protein (M_r 25,000) that could bind large amounts of Triton X-100 (8.5 g/g of protein). The participation of this purified binding protein in the folate transport system of intact cells has been established by several criteria (5-8).

In a preliminary communication (G. B. Henderson and E. M. Zevely, Fed. Proc. **35**:1357, 1976), we demonstrated that *L. casei* cells have the ability to transport several other B-vitamins, including thiamine. When examined in more detail, the thiamine transport system of *L. casei* was found to be similar in many respects to the thiamine uptake systems in both *L. fermentii* (14-17) and *Escherichia coli* (9, 10, 18). *L. casei* also contains considerable amounts of a thiamine-binding activity which can be detected by the same procedure (5) employed to measure folate binding by these cells. The general properties of this thiamine binder and its relationship to thiamine transport are described in the present communication.

MATERIALS AND METHODS

Radiolabeled thiamine. [*thiazole*-2-^{14}C]thiamine hydrochloride (14 mCi/mmol) and [^{35}S]thiamine hydrochloride (189 mCi/mmol) were purchased from Amersham/Searle. Radioactivity was measured with the use of a dioxane-based scintillation fluid (4).

Growth of cells. *L. casei* subsp. *rhamnosis* (ATCC 7469) was grown from a 1% inoculum for 24 h at 30°C in the basic medium described by Flynn et al. (2) except that the prescribed amount of thiamine was omitted and the folate concentration was increased to 5 μM. Vitamin-free casein hydrolysate (Nutritional Biochemicals) served as the source of acid-hydrolyzed casein. The yield of cells grown under these conditions was 5 to 7 g/liter.

Thiamine binding. The binding of thiamine by *L. casei* was measured by the same type of procedure as described previously for folate (5). Assay mixtures consisted of 0.8 ml of a washed cell suspension (10^9 cells per ml in 0.05 M potassium phosphate, pH 6.8), 0.1 ml of [^{14}C]thiamine (unless otherwise stated), and the indicated additions in a final volume of 1.0 ml. After incubation for 5 min at 4°C, the cell suspensions were diluted to 8 ml with ice-cold phosphate buffer (see above) and centrifuged (12,000 × *g*) for 5 min at 4°C. The cell pellets were suspended in 0.2 ml of water and transferred to counting vials with two 3-ml portions of scintillation fluid. Control values were ob-

tained from cell samples to which unlabeled thiamine (100 μM) was added 1 min before the labeled vitamin. Results after subtraction of the control are expressed in nanomoles of thiamine bound per 10^{10} cells (ca. 2.2 mg of cells, dry weight).

Thiamine transport. The transport of thiamine (at 37°C) was determined by the same procedure that was used to measure thiamine binding, except that the cells were preincubated with 5 mM glucose for 5 min at 37°C before the addition of labeled thiamine. Thiamine transport (at 37°C) was corrected for the amount of vitamin bound by the cells (at 4°C) under the same conditions. Concentration gradients were based on a cell volume of 2.4×10^{-12} ml; the latter was calculated as described by Winkler and Wilson (21).

Preparation of subcellular fractions. *L. casei* cells (1 g) were suspended in 20 ml of 0.1 M potassium phosphate (pH 7.5) and frozen in a dry ice-acetone bath. After 5 min, the cells were thawed, and [^{14}C]-thiamine was added to a final concentration of 5 μM. The mixture was then subjected to sonic oscillation (Biosonik III) for two 1-min intervals at 4°C, centrifuged for 20 min at 32,000 × *g*, and separated into a supernatant and particulate fraction. After the latter fraction was suspended in 20 ml of buffer, bound thiamine was measured by the same procedure employed for analysis of whole cells (see above). Binding activity in the supernatant fraction was determined by a Sephadex G-25 column chromatography procedure described previously (5). Protein in intact cells (after sonic treatment for 2 min at 23°C) and in the supernatant and particulate fractions was determined by the method of Lowry et al. (11), using bovine serum albumin as the standard.

Thiamine metabolism. *L. casei* cells (3×10^{10}) were suspended in 15 ml of 0.05 M potassium phosphate (pH 6.8) and incubated as described in the legend to Fig. 3. The cells were diluted to 40 ml with ice-cold 0.05 M sodium acetate (pH 4.0), centrifuged for 5 min at 4°C (12,000 × *g*), and resuspended in 2.0 ml of 0.02 M sodium acetate (pH 4.0). Samples were then heated for 10 min at 80°C and centrifuged as above to remove particulate material. After lyophilization, the supernatant fraction was dissolved in 25 μl of water and chromatographed on cellulose sheets (Eastman) in the solvent system described by Kawasaki et al. (9). Sections (1-cm) of the dried plates were cut out, and their radioactivity was determined by liquid scintillation counting.

RESULTS

General characteristics of thiamine uptake by *L. casei*. The time course for the uptake of thiamine at 37°C and in the presence of glucose is shown in Fig. 1. Under these conditions, the initial rate of uptake was rapid (ca. 0.6 nmol/min per 10^{10} cells) and was linear for approximately 1 min. A second phase characterized by a much slower rate of thiamine uptake (ca. 0.01 nmol/min per 10^{10} cells) was observed after 10 min. In various experiments, a total of 1.7 to 2.0 nmol of thiamine was taken up during a 30-min incubation. When the temperature was lowered to 4°C and glucose was omitted, uptake of labeled thiamine was again rapid but reached a plateau within 5 min (Fig. 1). Total uptake of thiamine at 4°C was approximately 0.5 nmol per 10^{10} cells. Thus, by controlling the temperature, the uptake of thiamine (like that of folate [5]) can be separated experimentally into two components: (i) the apparent binding of thiamine by cells (uptake at 4°C) and (ii) the transport of thiamine into cells (uptake at 37°C minus binding at 4°C).

Relationship between thiamine uptake at 4°C by intact cells and thiamine-binding activity in subcellular fractions. Intact cells were disrupted in the presence of [^{14}C]thiamine by sonic oscillation (see Materials and Methods) and were separated into a particulate (membrane) and a soluble (cytoplasmic) fraction by centrifugation. Although thiamine transport was almost completely lost by this treatment, thiamine-binding activity could be recovered in subcellular fractions (Table 1). (The purification and properties of the membrane protein containing this thiamine-binding activity will be presented elsewhere.) Bound thiamine was con-

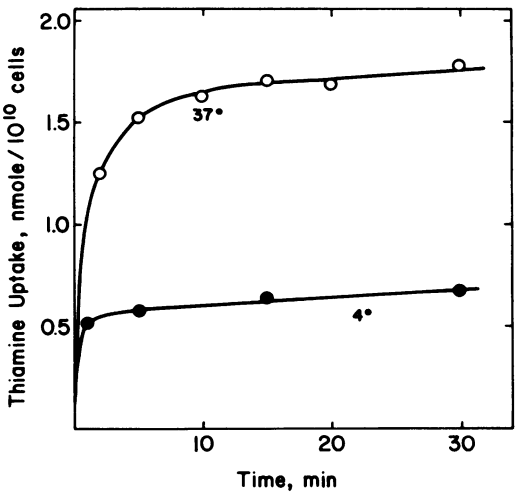

FIG. 1. *Time dependence for the uptake of thiamine (1.0 μM) by L. casei. Uptake at 4°C in the absence of glucose (binding) and at 37°C in the presence of 5 mM glucose (binding plus transport) were determined as described in the text.*

TABLE 1. *Distribution of thiamine-binding activity in subcellular fractions of L. casei*

Cellular prepn	Protein (mg)	Thiamine bound (nmol)	Sp act (nmol/mg of protein)
Intact cells	58.0	17.9	0.30
Cytoplasmic fraction	22.0	1.8	0.09
Membrane fraction	35.0	13.0	0.37

tained predominantly in the insoluble, membrane fraction and in an amount representing 73% of the binding capacity of intact cells; only 10% of the total activity appeared in the cytoplasmic fraction. The specific activity for thiamine bound by the particulate fraction (0.37 nmol/mg of protein) was higher than the corresponding values for cells (0.30) or the soluble fraction (0.09). In an experiment not shown in Table 1, thiamine-binding activity (0.43 nmol/mg of protein) was also recovered in good yield (60%) in a membrane fraction prepared by treatment of intact cells with lysozyme (6).

Energy requirements. Transport (but not binding) of thiamine was highly dependent upon a source of energy (Table 2). When cells were depleted of their energy reserves by prior incubation in phosphate buffer, the 37°C transport process was barely detectable unless glucose (5 mM) was added to the assay mixtures. In addition, treatment of the cells with 5 mM iodoacetate to block glycolysis abolished the transport of thiamine, but did not affect its binding.

Countertransport of thiamine compounds. Efflux of [^{14}C]thiamine compounds from *L. casei* was also determined (Fig. 2). Cells were preloaded by exposure to [^{14}C]thiamine for 20 min at 37°C in the presence of glucose. After removal of free thiamine by centrifugation, the samples were incubated at 37°C in the presence and absence of unlabeled thiamine (100 μM), and the release of the ^{14}C label was monitored. Plateau values were observed in both cases within 30 min; at this time, cells to which no additional substrate was added had lost 45% of their labeled material, but, in cells incubated with unlabeled vitamin, radioactivity was released in somewhat higher amounts (57% after 30 min) and at a more rapid initial rate. Addition of thiamine pyrophosphate (100 μM) to samples already containing unlabeled thiamine (100 μM) did not increase the rate or extent of release of the labeled material.

TABLE 2. *Effect of glucose and iodoacetate on the binding and transport of thiamine by energy-depleted cells of L. casei*[a]

Addition (5 mM)	Thiamine (nmol/10^{10} cells)	
	Bound	Transported
None	0.53	0.13
Glucose[b]	0.55	0.93
Iodoacetate plus glucose[c]	0.55	0.01

[a] Cells were exhausted of their energy reserves by incubation in 0.05 M potassium phosphate buffer (pH 6.8) for 60 min at 23°C. Binding and transport were determined after incubation of the cells with 1.0 μM labeled substrate for 5 min at 4 and 37°C, respectively.

[b] Samples were preincubated with 5 mM glucose for 5 min at 37°C prior to the determination of binding or transport.

[c] Samples were incubated sequentially with 5 mM iodoacetate (5 min, 37°C) and then with 5 mM glucose (5 min, 37°C) prior to the determination of binding and transport.

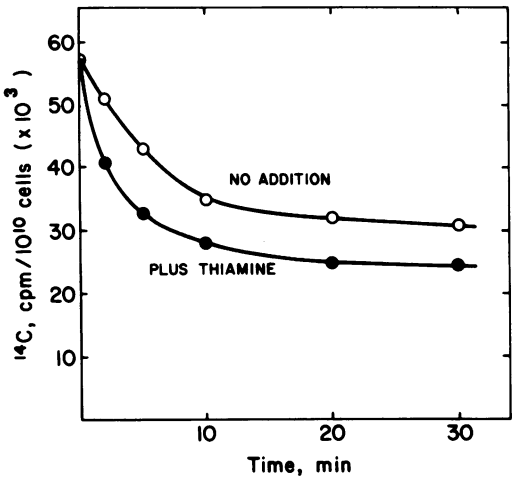

FIG. 2. *Effect of thiamine on the release of [^{14}C]-thiamine compounds from L. casei. Cells (10^{10}) were suspended in 20 ml of 0.5 M potassium phosphate (pH 6.8) containing 5 mM glucose and 1.0 μM [^{14}C]thiamine. After incubation for 20 min at 37°C, the cells were recovered by centrifugation for 5 min at 4°C (12,000 × g) and resuspended at 4°C in 20 ml of phosphate buffer. Cell samples (0.9 ml) and 0.1 ml of either buffer alone or buffer containing 1 mM unlabeled thiamine were combined and incubated again at 37°C. At the indicated times, samples were removed and centrifuged as above, and radioactivity associated with the cell pellets was determined.*

Analysis of bound and transported material. When analyzed by thin-layer chromatography (Fig. 3A), the labeled material bound to cells at 4°C chromatographed with an R_f value (0.62) identical to that of the thiamine standard. Extracts from cells into which labeled vitamin was actively transported in the presence of glucose also contained primarily unchanged thiamine, although small amounts of thiamine pyrophosphate (R_f = 0.14) could be detected (Fig. 3A). In contrast, labeled material associated with cells that had been preloaded with [^{14}C]-thiamine and then exposed to unlabeled vitamin for 30 min at 37°C (see Fig. 2) consisted predominantly of thiamine pyrophosphate (Fig. 3B). Analysis of the material released into the medium during the 30-min efflux period showed that it was mostly unchanged thiamine, although a significant amount (ca. 25%) of an unidentified metabolite (R_f = 0.95) was also present. When preloaded cells were incubated

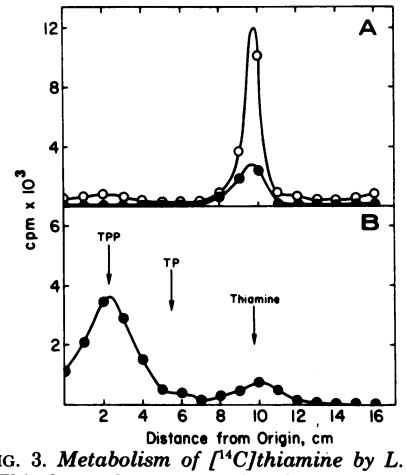

FIG. 3. *Metabolism of [¹⁴C]thiamine by L. casei. (A) Thin-layer chromatography of the thiamine compounds associated with cells that had been incubated with either 1.0 μM [¹⁴C]thiamine for 5 min at 4°C (●) or 1.0 μM [¹⁴C]thiamine and 5 mM glucose for 10 min at 37°C (○). (B) Thin-layer chromatography of the thiamine compounds associated with cells which, subsequent to their accumulation of [¹⁴C]thiamine, were incubated (30 min at 37°C) in the presence of unlabeled thiamine (100 μM). Preloading of the cells with labeled vitamin (see legend to Fig. 2) was achieved by incubation with 1.0 μM [¹⁴C]thiamine and 5 mM glucose for 20 min at 37°C. Arrows show the positions to which standards of thiamine, thiamine phosphate (TP), and thiamine pyrophosphate (TPP) migrate under these conditions (see reference 10).*

for 30 min in the absence of unlabeled thiamine (see Fig. 2), only the unknown compound appeared in the external medium.

Regulation by thiamine in the growth medium. The ability of *L. casei* to bind and transport labeled thiamine was dependent upon the concentration of vitamin added to the growth medium (Fig. 4). (Trace amounts of thiamine may be present in the vitamin-free casein hydrolysate used in the medium.) Both binding and transport were maximal in cells propagated in the presence of very small amounts of added thiamine (less than 10^{-8} M). At higher concentrations (10^{-8} to 10^{-6} M), both activities were repressed coordinately. From the data in Fig. 4, the concentrations for a 50% loss in thiamine binding and transport were calculated to be 17 and 37 nM, respectively. The loss of binding and transport activity was not due to saturation of these processes by added thiamine. Cells that had been exposed to unlabeled vitamin (1 μM) and glucose (5 mM) for 30 min at 37°C and then washed with 100 volumes of vitamin-free buffer retained 60% of their capacity for the uptake (transport plus binding) of [¹⁴C]thiamine. Cells grown in the presence of 1 μM thiamine lost greater than 90% of their ability to take up thiamine (Fig. 4) under these same conditions.

Kinetics of thiamine binding and transport. In *L. casei* cells grown in the absence of thiamine, the binding (5 min, 4°C) and transport (2 min, 37°C) of [¹⁴C]thiamine remained constant when tested over a range (50 to 500 nM) of substrate concentrations. [³⁵S]thiamine was also bound and transported maximally at the lowest substrate concentration (10 nM) permitted by the specific activity of this isotope. It was concconcluded accordingly that the dissociation constant (K_D) for binding and the Michaelis constant for transport (K_m) are both less than 10 nM.

The extremely tight association of thiamine with cells was also evident from the results shown in Table 3. When cells that had been first exposed (at 4°C) to 0.1 μM [¹⁴C]thiamine were treated with unlabeled thiamine at 100 times

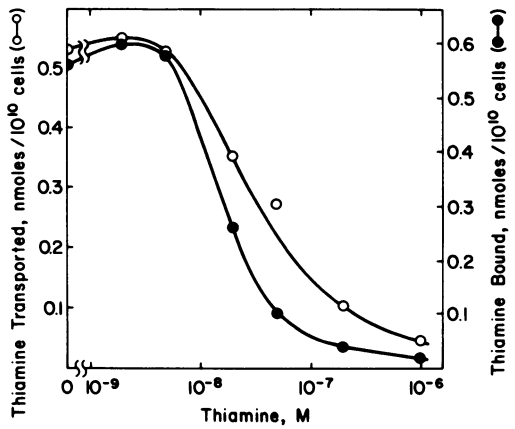

FIG. 4. *Effect of thiamine added to the growth medium upon the binding (●) and transport (○) of thiamine. Assay mixtures contained 1.0 μM [¹⁴C]thiamine and were incubated for 5 min at 4°C or 2 min at 37°C for the measurement of binding and of transport activity, respectively.*

TABLE 3. *Effect of unlabeled thiamine on the binding (at 4°C) of [¹⁴C]thiamine by L. casei*[a]

Addition A (0.1 μM)	Addition B (10 μM)	[¹⁴C]thiamine bound (nmol/10¹⁰ cells)
[¹⁴C]thiamine	None	0.52
[¹⁴C]thiamine	Thiamine	0.45
Thiamine	[¹⁴C]thiamine	0.17

[a] *L. casei* cells (0.8 ml) were combined with addition A (0.1 ml of a 1.0 μM solution), incubated for 5 min at 4°C, and then exposed to addition B (0.1 ml of a 100 μM solution). After incubation for an additional 5 min at 4°C, [¹⁴C]thiamine bound by the cells was determined as described in the text.

this level, the unlabeled material was unable to displace the previously bound vitamin. Similarly, when cells were exposed in the converse experiment to a low concentration (0.1 μM) of unlabeled thiamine, the addition of a large excess (10 μM) of [^{14}C]thiamine did not readily replace the previously bound substrate. In contrast, when these competition experiments were performed at 37°C in cells treated with 10 mM iodoacetate to block transport, a rapid exchange occurred between bound and free thiamine regardless of the order of addition.

Inhibition of [^{14}C]thiamine binding and transport by thiamine compounds. The competition of unlabeled thiamine, thiamine phosphate, and thiamine pyrophosphate with [^{14}C]thiamine for both binding and transport is illustrated in Table 4. To ensure competitive conditions (compare Table 3), the [^{14}C]thiamine and the unlabeled thiamine compounds were mixed before their addition to the cells. The results show that each of these compounds was a good inhibitor of the binding and transport of labeled thiamine and that the sequential addition of phosphate groups onto the vitamin gave rise to analogs with a progressively lower ability to compete with the parent compound. Moreover, as the concentrations of thiamine and its phosphorylated derivatives were varied, the binding and transport of thiamine were each inhibited to the same degree.

TABLE 4. *Inhibition of [^{14}C]thiamine binding and transport by thiamine compounds*[a]

Thiamine compound	Concn (μM)	Inhibition of binding (%)	Inhibition of transport (%)
Thiamine	0.05	28	26
	0.2	56	61
Thiamine phosphate	0.1	14	14
	0.4	38	35
	1.0	60	57
Thiamine pyrophosphate	0.4	10	12
	1.0	30	20
	4.0	40	35

[a] Assay samples were prepared at 4°C by combining 0.1 ml of 1.0 μM [^{14}C]thiamine and 0.1 ml of the indicated unlabeled thiamine compound, followed by the addition of 0.8 ml of cells. The binding and transport of [^{14}C]thiamine were then determined after incubation for 5 min at 4°C or 2 min at 37°C, respectively. Control levels (no addition) were 0.50 nmol bound per 10^{10} cells and 0.49 nmol transported per 10^{10} cells.

DISCUSSION

Thiamine uptake at 37°C by non-proliferating cells of *L. casei* possesses several characteristics common to carrier-mediated active transport systems. The uptake process is dependent upon a source of energy (Table 2), is sensitive to temperature (Fig. 1), displays specificity (Table 4), and follows saturation kinetics. Rapid exchange between intracellular and extracellular thiamine could also be demonstrated (compare Fig. 2 and 3), indicating that the vitamin was not tightly bound by intracellular components and was free to recross the membrane. Intracellular thiamine was, however, slowly converted to thiamine pyrophosphate, which did not exit from the cells. A considerable concentration gradient could also be achieved by the system. When the uptake of thiamine after 10 min at 37°C was corrected for both the small amount of vitamin converted to its phosphorylated forms and the amount of vitamin bound by the cells, the ratio of the internal and external thiamine concentrations was approximately 40. In both *E. coli* (9) and *L. fermentii* (14–16), free thiamine does not accumulate but is converted rapidly to thiamine pyrophosphate upon entry into the cell. However, mutant studies have shown that, at least in *E. coli*, energy can be used to achieve concentration gradients for free thiamine (10).

The energy required for thiamine transport was readily generated by the metabolism of glucose (Table 2). Similarly, transport could be abolished when iodoacetate was used to block glycolysis. Since the cells accumulate free thiamine, the energy requirement appears to be coupled directly to the transport system and not to the subsequent metabolism of this vitamin.

In addition to the active transport process, a considerable portion (ca. 38%) of the total uptake of thiamine by *L. casei* is referable to the binding of the vitamin by the cells. The binding component responsible for this uptake is detected at low temperature (or at 37°C in the absence of energy production), has a high affinity for thiamine (K_D < 10 nM), resides in the cell membrane, and is present in relatively large amounts (0.5 nmol per 10^{10} cells). This latter value exceeds by 20-fold the amount of thiamine which could be expected to be taken up by cells via facilitated (or passive) diffusion. That the energy-independent uptake of thiamine at 4°C is mediated by a species with a very high affinity for the vitamin was further indicated by experiments (Table 3) which showed that at 4°C high concentrations of unlabeled thiamine did not readily displace [^{14}C]thiamine once the latter was taken up by cells. The insensitivity of the binding activity to iodoacetate suggests that re-

active sulfhydryl groups are not present at the thiamine-binding site. The latter result is not characteristic of various amino acid-binding proteins in the cell membrane of *E. coli* which, upon treatment with *p*-chloromercuribenzoate, lose the capacity to bind their respective substrates (3). *E. coli* also contains a periplasmic binding protein which appears to mediate the uptake of thiamine (9, 12, 18). The latter binder also has a high affinity for thiamine ($K_D \simeq 29$ nM) but is present in considerably lower amounts per cell (18) than the *L. casei* thiamine-binding component. *L. fermentii* transports thiamine with a K_m of 480 nM (15) and presumably contains a binding component with a similar K_D value.

L. casei concentrates another vitamin, folic acid, by a system which can also be separated into a transport and binding phase (4, 5, 7). The membrane component responsible for folate binding is present in amounts (0.35 nmol per 10^{10} cells) slightly lower than the thiamine binder and is insensitive to iodoacetate and other sulfhydryl reagents. Studies on regulation (5), cellular location (5, 6), the structural properties of the purified protein (6), and kinetics in wild-type and mutant cells of *L. casei* (7) have provided substantial evidence that the isolated folate binder is a component of the folate transport system. Analysis of the thiamine-binding component of *L. casei* has led to a similar conclusion. Evidence supporting this viewpoint is as follows. (i) The binding of thiamine by cells is mediated by a component of the cell membrane. (ii) Repression of both binding and transport of thiamine by the level of the vitamin in the growth medium occurs in a parallel fashion (Fig. 4). (iii) The K_D for thiamine binding and the K_m for thiamine transport are of the same magnitude (<10 nM). (iv) When various concentrations of thiamine, thiamine phosphate, and thiamine pyrophosphate were tested as inhibitors of [^{14}C]thiamine binding and transport, the relative order of affinities (thiamine > thiamine phosphate > thiamine pyrophosphate) and the apparent values for the affinity constants were the same for each process (Table 4). Although another means to explore this relationship would be to isolate cells defective in thiamine transport, selection of such mutants would be difficult, since a growth requirement for thiamine has not been established in *L. casei* (13).

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