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The Folate and Thiamine Transport Proteins of Lactobacillus Casei

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Two separate binding proteins, one specific for folate and the other for thiamine, have been isolated from membrane fragments of Lactobacillus casei. Purification to homogeneity was achieved by fractionation of the Triton-solubilized proteins with microgranular silica (Quso G-32) and Sephadex G-150. Amino acid analyses revealed that the folate (M_r = 25,000) and thiamine (M_r = 29,000) binders have unusually low polarity constants, 0.32 and 0.26, respectively. Evidence obtained with intact cells has established a direct role for these binding proteins in transport of the corresponding vitamins: A) In each case, the processes of binding and transport showed similarities in substrate affinities and repression by excess vitamin in the growth medium. B) Competition studies employing amethopterin, 5-formyl tetrahydrofolate, and 5-methyl tetrahydrofolate (for folate) and thiamine monophosphate and thiamine pyrophosphate (for thiamine) have shown that the ability of these compounds to inhibit the transport of the corresponding vitamins is paralleled by their ability to inhibit binding. C) Amethopterin-resistant mutants which are defective in folate transport have a comparable defect in ability to bind folate. D) Amethopterin-resistant cells which (compared with the parent cell line) contain folate transport systems with altered affinities for amethopterin also contain binding proteins whose affinities for amethopterin have changed by equivalent amounts. E) Both the transport and binding of folate by one of the mutants were stimulated (approximately 3-fold) in parallel by the addition of mercaptoethanol.

Key words: folate, thiamine, transport, binding proteins, Triton X-100

INTRODUCTION

The active transport of folate (1–4) and thiamine (4, 5) into L. casei proceeds via 2 separate systems readily distinguishable by their substrate specificity. The uptake processes are similar in other respects, however, such as their energy requirements, rates of vitamin uptake, dependence on pH and temperature, and regulation by amount of vitamin in the growth medium. In conjunction with the transport processes, the cells also have the common ability to bind appreciable amounts ($\sim 2 \times 10^4$ molecules/cell) of both folate

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(4, 6, 7) and thiamine (4, 5). The components responsible for this binding are expressed only in cells propagated under conditions of vitamin limitation and have high affinities for their respective substrates. The present report summarizes evidence for a relationship between vitamin-binding activity and vitamin transport. The binding components have been solubilized from membrane preparations of L. casei, purified to homogeneity, and shown to be extremely hydrophobic, water-insoluble proteins. Folate transport mutants have also been isolated and employed in conjunction with the parent cells to demonstrate kinetic similarities between the binding and transport processes.

MATERIALS AND METHODS

Lactobacillus casei var. rhamnosis (ATCC 7469) were grown according to the general procedure described previously (7) in the medium of Flynn et al. (8) containing either 5 nM folate plus 5 μM thiamine (for studies on the folate transport system) or 5 μM folate and no added thiamine (for studies on the thiamine transport system). Folate transport mutants (RX-1 through RX-21) were isolated by selecting for cells with resistance to amethopterin. Details of the procedures employed for this purpose will be described elsewhere. The minimum concentrations of folate (values in parentheses) required to give full growth of the individual mutants were as follows: RX-2 and RX-3 (500 nM); RX-1 (100 nM); RX-4 and RX-5 (10 nM); and RX-6 through RX-21 (5 nM).

Measurement of the binding of folate or thiamine by intact cells was determined in assay mixtures consisting of 0.05 ml of [G-^{3}H] folate (120,000 dpm/nmole) or [thiazole-2-^{14}C] thiamine (35,000 dpm/nmole), 0.05 ml of a desired addition, and 0.9 ml of a washed cell suspension (6×10^{8}/ml in 0.05 M potassium phosphate, pH 6.8). After 5 min at 4°C, the cells were collected on a Millipore filter (0.22 μm) and washed with 2 1-ml portions of ice-cold phosphate buffer. The filters were placed in 5 ml of a dioxane-based scintillation fluid (1), and the radioactivity was determined. Control values (usually 5–10%) were calculated as described previously (7). Vitamin transport was determined by a procedure similar to that described above for binding. In this case, cell suspensions were preincubated with glucose (5 mM) for 5 min at 37°C prior to their addition to the assay mixture. The samples were then incubated for an appropriate interval at 37°C. The amount of folate or thiamine bound by cells (see above) served as the control for the corresponding transport process. A general procedure for measuring vitamin-binding activity in membrane or supernatant fractions derived from intact cells has been described previously (7).

Protein in intact cells (after sonication for 1 min at 23°C) or membrane preparations was determined by the method of Lowry et al. (9). Protein in samples containing Triton was determined by the biuret reaction (10). Bovine serum albumin served as the standard.

RESULTS AND DISCUSSION

I. Characterization of the Binding Proteins

Cellular location. Information on the intracellular location of the folate- and thiamine-binding components of L. casei was obtained by fractionation experiments. Intact cells (having a binding capacity of 0.15 nmole folate or 0.26 nmole thiamine per 20 mg protein) were disrupted either osmotically following treatment with lysozyme or by passage through a homogenizer (7); the supernatant and particulate (membrane)

fractions were separated by centrifugation and analyzed for the presence of the binding components. The crude membrane fractions were found not only to retain a majority (60–80%) of the cellular binding activity but also to bind folate (0.20–0.25 nmole) or thiamine (0.35–0.43 nmole) in higher amounts per mg protein than the intact cells. The supernatant fractions had only a minimal capacity (0.01–0.05 nmole/mg protein) for the binding of either vitamin and contained low amounts (10–30%) of the original binding activity.

Purification and properties. A general procedure was developed for the isolation of both the folate- and thiamine-binding proteins. Crude membranes were prepared by disruption of intact cells (~ 180 g, wet weight) in a Manton-Gaulin homogenizer, exposed to labeled vitamin, and treated with Triton X-100 (5%) to solubilize the binding components. The membrane extracts (containing 50–70% of the binding activity of intact cells) were then fractionated by 2 cycles of adsorption and elution from microgranular silica (Quso G-32), followed by chromatography on Sephadex G-150. Additional details of this procedure are given in Ref. 7, and the results for representative preparations of both binding proteins are given in Table I.

The 2 preparations were similar in a number of respects, including their initial vitamin-binding capacity and the recovery and specific activity of the proteins at each step of the procedure. In fact, since the fractionation properties are virtually identical, separation of the binding proteins can be achieved only by selecting growth conditions under which only 1 of the proteins is synthesized.

The purified proteins contained bound folate or thiamine which could not be removed by dialysis or gel chromatography and did not exchange with vitamin added externally. Thus, it has not been possible to measure the kinetics of vitamin binding by purified preparations. On the other hand, the irreversible nature of this binding provided a convenient means for labeling the proteins during purification. The explanation for these unusual binding characteristics is probably related to the large amounts of Triton (~ 320 moles/mole protein) associated with the purified proteins. Vitamin-binding sites may be inaccessible to the aqueous medium because of the formation of a detergent micelle around the protein-vitamin complex (7).

When analyzed by SDS-gel electrophoresis, the folate- and thiamine-binding proteins appeared homogeneous and had molecular weights of 25,000 and 29,000, respectively. From these values, it could be calculated that the purified proteins prior to electrophoresis contained 0.85 mole of folate and 0.96 mole of thiamine per mole of protein.

TABLE I. Purification of the Binding Proteins

Step	Volume	Protein	Vitamin bound	Yield
	ml	mg	nmoles/mg protein	%
Folate-binding protein				
1. Membrane extract	380	2,040	0.34	–
2. Quso G-32 fractionation	2.1	9.0	20.4	27
3. Sephadex G-150	2.2	4.4	34.0	22
Thiamine-binding protein				
1. Membrane extract	405	2,025	0.45	–
2. Quso G-32 fractionation	2.3	12.4	21.8	30
3. Sephadex G-150	2.0	5.1	33.2	19

The amino acid compositions of the binding proteins are shown in Table II. Examination of the individual amino acid constituents reveals the unique nature of these proteins, but similarities are evident in both their lack of cysteine and their unusually high contents of hydrophobic amino acids, including methionine. The polarity of the folate (0.32) and thiamine (0.26) binders is extremely low even when compared with values for other membrane proteins (13).

II. Involvement of the Binding Proteins in Transport

General considerations. Since binding studies were not feasible with the isolated proteins (see above), the relationship between these binding proteins and their corresponding transport systems was analyzed using intact cells. In these experiments, rates of vitamin transport were compared with amounts of binding material, and Michaelis (K_m) constants for the transport systems were compared with the dissociation (K_D) constants for the corresponding binding proteins. These studies were simplified by the fact that the binding proteins for both folate and thiamine are present in large amounts and each has a high affinity for its substrate. That the isolated binding proteins are the cellular components responsible for the binding (and transport) activity has been supported by considerations of stoichiometry and specificity (cf section I, and Refs. 6 and 7). In the present comparison, cellular regulation, substrate affinities, and transport mutants are examined.

Cellular regulation. The ability of L. casei to transport and bind either folate or thiamine is regulated by the amount of vitamin present during growth. Folate transport is

TABLE II. Amino Acid Composition of the Folate- and Thiamine-Binding Proteins*

	Folate-binding protein	Thiamine-binding protein
	Residues/25,000 g	Residues/29,000 g
Cysteic acid	0.1	0.0
Aspartic acid	14.4	10.3
Methionine sulfone	18.0	9.1
Threonine	15.3	12.0
Serine	14.5	15.6
Glutamic acid	9.3	13.4
Proline	12.3	10.0
Glycine	15.7	28.4
Alanine	22.6	37.3
Valine	13.0	28.2
Isoleucine	18.3	25.4
Leucine	26.6	33.9
Tyrosine	5.0	8.6
Phenylalanine	13.3	18.8
Histidine	2.8	3.9
Lysine	8.3	10.8
Arginine	8.1	5.8
Tryptophan	10.7	7.1

*Protein samples (~ 0.5 mg) were precipitated with ethanol (50%) to remove Triton X-100, hydrolyzed in 6 N HCl for 24, 48, and 72 hr at 105°C, and analyzed for amino acid content as described previously (7). Cysteine and methionine were determined after oxidation with performic acid (11) while tryptophan was calculated by the spectrophotometric method of Goodwin and Morton (12).

maximal in cells grown with 1–10 nM folate, declines progressively at higher concentrations (50% loss at 50 nM), and is totally repressed at 1 μM folate (Fig. 1). Folate-binding capacity showed an identical response (50% loss at 55 nM) to these growth conditions. In a similar fashion, thiamine is transported and bound optimally in cells propagated in medium containing up to 10 nM thiamine (Fig. 2). At higher concentrations, a decline in transport and binding was observed although at slightly different rates; reductions of 50% occurred at 37 nM and 17 nM, respectively, for the 2 processes. An exact coincidence in the latter curves was not obtained, and the reason for this difference is not yet clear. Thiamine added to the growth medium may interfere with these measurements since separate experiments have shown that unlabeled thiamine, once bound to the cells, does not readily exchange at 4°C with the [^{14}C]thiamine of the assay mixture. It is clear, however, that the rates of both folate and thiamine transport are closely related to the amounts of the corresponding binding proteins within the cell.

Binding affinities. Analyses of binding parameters provided an alternative means for comparing the binding and transport processes. For investigation of the folate system, affinity constants were determined for several of the compounds (folate, amethopterin, 5-methyl tetrahydrofolate, and 5-formyl tetrahydrofolate) previously shown to compete with folate for transport (1). The results show that amethopterin was bound with the highest affinity to both the receptor protein and the transport system (Table III). In this respect 5-formyl tetrahydrofolate and folate were intermediate while 5-methyl tetrahydrofolate was bound with the lowest affinity. The actual values for the affinity constants showed considerable variation (16–210 nM), yet for each compound tested, the dissociation constant for the binding protein was 3-fold lower than the Michaelis constant for the transport system (Table III). Thus, a linear relationship could be demonstrated when the affinity constants for transport were plotted against the corresponding values for the binding protein (cf. Fig. 3).

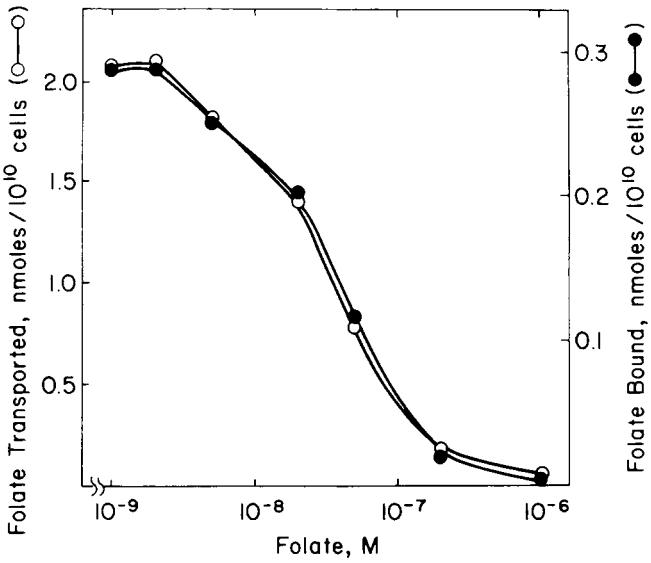

Fig. 1. Effect of folate concentration in the growth medium upon the binding and transport of folate by L. casei. Assay mixtures contained 1.0 μM [^{3}H]folate and were incubated for 10 min at 37°C or 5 min at 4°C for the measurement of transport and binding activity, respectively.

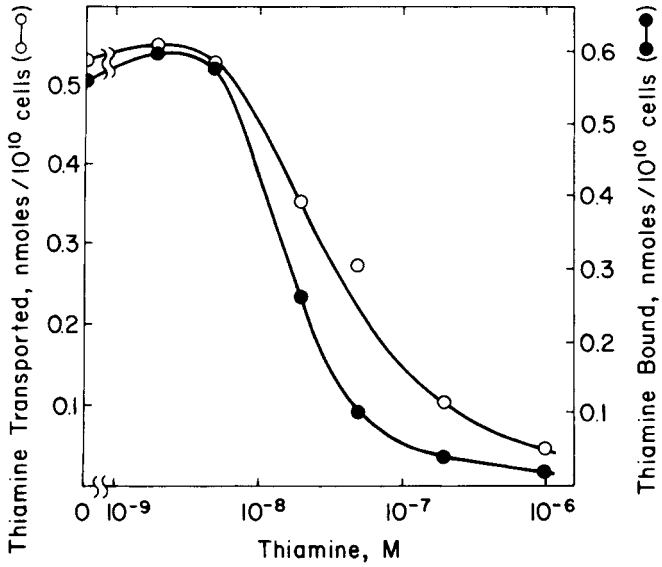

Fig. 2. Effect of thiamine concentration in the growth medium upon the binding and transport of thiamine by L. casei. Assay mixtures contained 1.0 μM [^{14}C] thiamine and were incubated for 2 min at 37°C or 5 min at 4°C for the measurement of transport and binding activity, respectively.

TABLE III. Comparative Affinity of Various Compounds for the Folate-Binding Protein and Folate Transport System of L. casei*

Folate compound	Affinity constant	
	Binding protein	Transport system
	nM	nM
Amethopterin	16	50
5-Formyl tetrahydrofolate	26	60
Folate	36	95
5-Methyl tetrahydrofolate	85	210

*The K_m for [^{3}H] folate transport (1) and the K_D for [^{3}H] folate binding (6) were determined from double-reciprocal plots of folate taken up by cells vs free folate. The corresponding affinity constants for amethopterin, 5-formyl tetrahydrofolate, and 5-methyl tetrahydrofolate were determined as K_i values by the method of Dixon and Webb (14); assay mixtures contained 0.1 μM or 0.4 μM [^{3}H] folate and variable concentrations (0.05–1.0 μM) of the indicated folate derivatives. Samples were incubated for 2 min at 37°C and 5 min at 4°C for measurement of transport and binding, respectively.

A similar relationship could also be established between the transport system and binding protein for thiamine. In this case, percent inhibition, rather than an inhibition constant, was employed for the comparison, since the affinity constants for thiamine of both the transport system ($K_m \cong 10$ nM) and the binding protein ($K_D \cong 10$ nM) were too low to measure conveniently. The competition of unlabeled thiamine, thiamine phosphate, and thiamine pyrophosphate with ^{14}C-labeled thiamine for binding and transport is illustrated in Table IV. The results show that thiamine phosphate and thiamine pyrophosphate are good inhibitors of thiamine binding and transport, and that the sequential addition of

TABLE IV. Inhibition of [^{14}C] Thiamine Binding and Transport by Thiamine Compounds*

Addition	Concentration	[^{14}C] Thiamine bound	Inhibition	[^{14}C] Thiamine transported	Inhibition
	μM	nmoles/10^{10} cells	%	nmoles/10^{10} cells	%
None	–	0.50	–	0.49	–
Thiamine	0.05	0.36	28	0.36	26
	0.2	0.22	56	0.19	61
Thiamine phosphate	0.1	0.43	14	0.42	14
	0.4	0.31	38	0.32	35
	1.0	0.20	60	0.21	57
Thiamine pyrophosphate	0.4	0.45	10	0.43	12
	1.0	0.35	30	0.39	20
	4.0	0.30	40	0.32	35

*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 was then determined following incubation for 5 min at 4°C or 2 min at 37°C, respectively.

phosphate groups onto the vitamin progressively lowers the ability of the analogs to compete with the parent compound. It was also observed that as the concentrations of thiamine and its phosphorylated derivatives were varied, the binding and transport of thiamine were each inhibited to the same degree. Thus, the transport system and binding protein have the same relative affinity for each of these thiamine compounds.

Folate transport mutants. A series (RX-1 through RX-21) of amethopterin-resistant cell lines which contain a defective folate transport system has been isolated. In each case, a reduction in the transport of folate was accompanied by a comparable loss in ability to bind folate. In several of the cell lines, the ability to transport and bind folate was essentially absent, whereas other mutant cells retained up to 50% of the transport and binding activity of the parent cells.

Since the folate transport mutants were selected for resistance to a folate analog (amethopterin), it seemed possible that mutant cells might have arisen which retained an effective means for the transport of folate but not amethopterin. In cells of this type, the binding protein and transport system might be altered in their affinity for folate compounds. To test this possibility, affinity constants for folate and amethopterin were determined in several of the cell lines. One of the mutants selected for this study was RX-13 since it retained the highest capacity to transport folate (~50% of wild-type cells.) In these cells, the K_M for folate transport (110 nM) and the K_D for folate binding (25 nM) were found to be comparable to the corresponding values obtained in the parent cells (cf. Table III), while the affinities of the transport system (K_i = 3,000 nM and the binding protein (K_i = 800 nM) for amethopterin were both 60-fold lower than in the wild-type cells. A similar result was obtained with mutant RX-21. When measured in the presence of mercaptoethanol (see below), the interaction of amethopterin with the transport system (K_i = 650 nM) and the binding protein (K_i = 170 nM) was 10-fold less efficient in this mutant than in the parent cells, while the parameters relative to folate binding were, again, virtually unchanged. When the affinity constants for mutants RX-13 and RX-21 were plotted in Fig. 3, the following relationships were

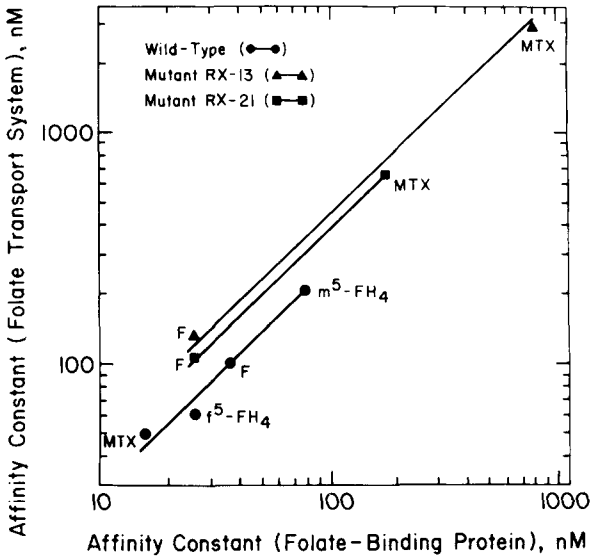

Fig. 3. Relationship between the affinity of various compounds for the folate transport system and the folate-binding protein of wild-type and mutant cells of L. casei. The affinity constants were determined as described in the legend to Table III. Cells of mutant RX-21 were incubated with 25 mM mercaptoethanol for 5 min at 37°C prior to the addition of folate compounds.

apparent: A) The plot of the affinity constants for each mutant was parallel to and nearly coincident with that for the wild-type cells; B) the 3-fold difference between the affinity constants of the binding protein and transport system in the wild-type cells (cf. Table III) was maintained in the mutant cell lines.

The transport system and binding activity of mutant RX-21 were both dependent on added mercaptoethanol. When measured under standard conditions, binding and transport of folate by these cells were only one-third of the levels characteristic of the parent cell line. Mercaptoethanol added to the assay mixtures increased both the binding and transport of folate by 3-fold (Table V). Thus, analyses of the mutant cell lines were able to establish correlations between the transport and binding processes in 3 separate areas, i.e., relative amounts, binding affinities, and responses to an external agent.

III. Concluding Remarks

Evidence has been presented which shows that the membrane-associated folate- and thiamine-binding proteins of L. casei participate in vitamin transport. These conclusions are based primarily upon the cellular location and amino acid compositions of the binding proteins, a parallel relationship between the interaction of specific ligands with the binding proteins and transport system, and, in the case of folate, the properties of transport mutants. The results do not establish whether the binding proteins are merely receptor sites or the actual carriers of the vitamins across the cell membrane, although the extreme hydrophobicities of these proteins argue in favor of the latter possibility. Reconstitution of a vitamin-transporting system by insertion of the purified proteins into liposomes should be able to resolve this question. Other aspects of the transport process, such as the

TABLE V. Effect of Mercaptoethanol on the Binding and Transport of Folate by Mutant RX-21*

Process	No addition	Mercaptoethanol	Stimulation
		25 mM	-fold
Folate binding (nmoles/10^{10} cells)	0.09	0.29	3.2
Folate transport (nmoles/10^{10} cells)	0.8	2.2	2.8

*Cells were preincubated with mercaptoethanol for 5 min at 37°C prior to the addition of labeled folate. The binding and transport of folate was then determined following incubation for 5 min at 4°C or 10 min at 37°C, respectively.

mechanism for energy-coupling, are not yet clear. Evidence has been obtained, however, that folate transport is directly linked to glycolysis and that both oxidoreduction equivalents and ATP may be required as sources of energy (3).

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