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Differential interaction of cations with the thiamine and biotin transport proteins of *Lactobacillus casei*

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Lactobacillus casei **cells contain separate and specific binding proteins which mediate the cellular uptake of thiamine and biotin. In buffered salt solutions, these proteins exhibit a very high affinity for their vitamin substrate. Dissociation constants (K_d values) at pH 7.5 are 0.03 and 0.15 nM for thiamine and biotin, respectively. Optimal binding of biotin requires the presence of cations. This cation dependence is substantial since the K_d for biotin is 60-fold higher in a buffer containing 0.1 mM K-Hepes, compared with a buffer composed of 50 mM K-Hepes and 5 mM $MgCl_2$. Measurements of K_d versus cation concentration showed that Mg^{2+} is 300-fold more effective than K^+ in promoting biotin binding. The extent of cation dependence decreases as the pH is reduced from 7.5 to 5.0, suggesting that protons can partially fulfill the cation requirement. In contrast, binding of thiamine to the thiamine transport protein shows no dependence on the ionic composition of the medium. These results suggest that the transport protein for the anionic vitamin, biotin, contains a binding site for cations. Cotransport of both the vitamin and cation into the cell might then occur during the normal transport cycle, allowing the cellular uptake of the vitamin to occur against the membrane potential. Conversely, the cationic vitamin, thiamine, does not appear to be transported into the cell as a complex with other ions.**

Introduction

The transport of folate compounds in *Lactobacillus casei* proceeds via a single high-affinity transport system [1–3]. The uptake process is active and dependent on an energy source, and folate can be accumulated to intracellular concentrations that are several-thousand fold higher than external levels [4]. The membrane-associated binding protein which mediates folate transport is present in large amounts per cell, exhibits a very high affinity for folate, and has been purified to homogeneity [5–8]. An affinity-labeling agent has also been developed which covalently modifies a lysine residue at the folate-binding site [9]. The transport system is comprised of at least two cellular components, the binding protein and a second shared constituent that is also required for the transport of thiamine and biotin [10].

The folate binding protein assumes a high-affinity state only in the presence of cations [8]. Transfer of the cells from a buffered salt solution to isotonic sucrose causes a 600-fold reduction in binding affinity, while the re-addition of various cations to the medium restores optimum binding. K_d measurements over a range of cation concentrations revealed that a half-maximal restoration of binding occurs with a variety of cations, although divalent cations are 200- to 1000-fold

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

more effective than monovalent cations. These results led to the conclusion that the folate transport protein contains both a folate and cation binding site and that folate may be transported across the cell membrane via a folate/cation symport mechanism. In the present study, the specific binding of thiamine [7,10,11] and biotin [10] to their respective transport proteins has been evaluated. It was observed that binding of the anionic vitamin, biotin, exhibits a comparable dependence on cations as the binding of folate, while the composition of the medium has no effect on the binding of the cationic vitamin, thiamine. These results suggest that folate and biotin, but not thiamine, are translocated across the membrane as a complex with cations.

Material and Methods

Radiolabeled vitamins. [^{3}H]Folate (500 mCi/mmol) and [^{35}S]thiamine (200 mCi/mmol) were obtained from Amersham. The [^{35}S]thiamine was employed directly, while the [^{3}H]folate was diluted to 500000 dpm/nmol with unlabeled vitamin. The purity of the [^{3}H]folate and [^{35}S]thiamine was greater than 95% as judged from the ability of excess amounts of cells containing the respective binding proteins to bind the label. [^{3}H]Biotin (20 Ci/mmol) was obtained from New England Nuclear and was diluted to 1000000 dpm/nmol prior to use. The [^{3}H]biotin had a radiochemical purity of greater than 95% as judged by thin-layer chromatography on cellulose sheets using *n*-butanol/acetic acid/water (4:1:5, v/v) as the solvent.

Growth of cells. Lactobacillus casei subsp. *rhamnosis* (ATCC 7469) cells were grown at 30°C as described previously [12] in the presence of limiting amounts of either folate (2 nM), thiamine (0.5 nM), or biotin (2 nM). Cells were harvested after 16 h.

Binding determinations. Binding measurements were performed with freshly grown cells that had been washed with 100 volumes of 50 mM potassium phosphate buffer (pH 7.5) and incubated for 60 min at 23°C to deplete energy reserves [8]. The cells were then collected by centrifugation at 20000 × *g* (5 min, 4°C), washed, and resuspended in the desired assay buffer to $5 \cdot 10^8$/ml. The binding rate for thiamine was measured in assay mixtures consisting of labeled substrate (0.5 nM), cells ($2 \cdot 10^8$), and buffer in a final volume of 50 ml. After incubation at 4°C for various times, the cells were collected (at 4°C) onto 0.45 μm filters and analyzed directly for bound radioactivity in 10 ml of scintillation fluid (Cytoscint, West Chem Products). Biotin binding was measured similarly, except that the number of cells was increased to 10^9/assay. Controls were prepared identically, except that a 100-fold excess of unlabeled vitamin (which reduced specific binding by greater than 99%) was added prior to the labeled substrate. After correction for the control, which was usually less than 10% of total binding, results were expressed in nanomoles of substrate bound per 10^{10} cells. Cell numbers were determined from the colonies formed after spreading dilutions of cells onto plates containing growth medium and 2% agar and incubating for 48 h at 30°C. 10^{10} cells were found to contain 2.2 mg, dry weight, when a known amount of cells ($5 \cdot 10^{10}$) was pelleted by centrifugation, washed with 100 volumes of water, dried at 100°C for 16 h, and weighed.

K_d values were measured in assay mixtures containing variable amounts of labeled substrate (0.05–50 nM), cells ($(2–10) \cdot 10^8$), and assay buffer in a final volume of 50 ml. After equilibrium binding as obtained (60 to 120 min at 4°C depending upon the substrate employed), the cells were collected onto 0.45 μm filters and analyzed as described above. K_d values were calculated from a double-reciprocal plot of substrate bound versus the free substrate concentration at the steady state.

Results

General characteristics of [^{35}S]thiamine and [^{3}H]biotin binding

The kinetics of substrate binding to the folate transport protein of *L. casei* have been analyzed previously [8]. Binding at 4°C (in energy-depleted cells) is rapid and quantitative at high substrate concentrations, while much longer time intervals (ca. 60 min) are required to achieve steady-state binding near the dissociation constant for folate (ca. 0.5 nM). Similar results were obtained in the present study for the binding of [^{35}S]thiamine [10,11] and [^{3}H]biotin [10] to their respective

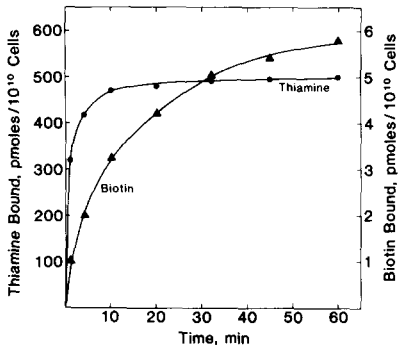

Fig. 1. Time dependence for the binding of [^{35}S]thiamine and [^{3}H]biotin to *L. casei* cells at 4°C. Substrate concentration, 0.5 nM; assay volume, 50 ml. Buffer, 50 mM K-Hepes/5 mM $MgCl_2$ (pH 7.5).

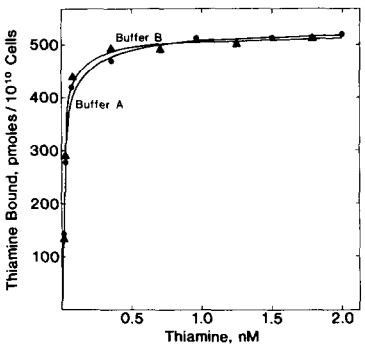

Fig. 2. Effect of buffer composition on the concentration dependence for the binding of [^{35}S]thiamine. Assay volume, 50 ml; incubation time, 60 min at 4°C. Buffer A: 50 mM K-Hepes/5 mM $MgCl_2$ (pH 7.5); buffer B: 0.1 mM K-Hepes (pH 7.5).

transport proteins. Binding was rapid at substrate concentrations above 1 μM, while the rate decreased substantially as the concentration was reduced to the nanomolar range (Fig. 1). Binding at 0.5 nM thiamine reached a plateau after 20 min at 4°C, while biotin binding at the same substrate concentration approached a maximum only after a 60 min interval.

Effect of buffer composition on the K_d for [^{35}S]thiamine and [^{3}H]biotin

Dissociation constants (K_d values) for thiamine and biotin were measured by incubating the cells with varying amounts of labeled substrate and determining steady-state levels of bound and free substrate. Incubation periods of 1 and 2 h at 4°C were required to achieve equilibrium binding for thiamine and biotin, respectively (see Fig. 1), and a large assay volume (50 ml) was employed to permit sufficient amounts of unbound substrate to remain in the medium at the steady state. In a cation-sufficient buffer (50 mM K-Hepes and 5 mM $MgCl_2$, pH 7.5), the K_d for thiamine was 0.03 nM, although an identical K_d value was also obtained in a cation-deficient buffer (0.1 mM K-Hepes, pH 7.5) (Fig. 2). When the same measurements were performed with biotin (Fig. 3), a K_d of 0.15 nM was obtained in the buffer containing excess cations, while a much higher value of 9.0 nM was observed in the cation-deficient buffer. The effect of buffer composition on the K_d values for thiamine and biotin is summarized in Table I.

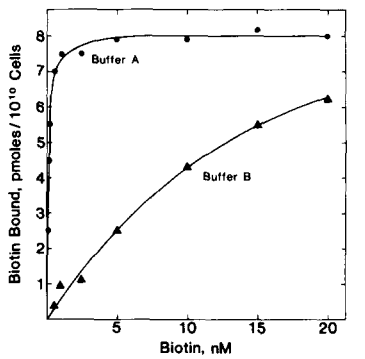

Fig. 3. Effect of buffer composition on the concentration dependence for the binding of [^{3}H]biotin. Assay volume, 50 ml; incubation time, 120 min at 4°C. Buffer A: 50 mM Hepes/5 mM $MgCl_2$ (pH 7.5); buffer B: 0.1 mM K-Hepes (pH 7.5).

TABLE I

EFFECT OF CATIONS AT pH 7.5 ON THE BINDING AFFINITY (K_d) FOR THIAMINE, BIOTIN, AND FOLATE

Buffers employed: limiting cations, 0.1 mM K-Hepes (pH 7.5); excess cations, 50 mM K-Hepes/5 mM $MgCl_2$ (pH 7.5).

Substrate	Level of cations	K_d (nM)
Thiamine	Limiting	0.03
	Excess	0.03
Biotin	Limiting	9.0
	Excess	0.15
Folate	Limiting	300
	Excess	0.5

TABLE II

EFFECT OF CATIONS AT pH 5.0 ON THE BINDING AFFINITY (K_d) FOR THIAMINE, BIOTIN, AND FOLATE

Buffers employed: limiting cations, 0.1 mM potassium acetate (pH 5.0); excess cations, 50 mM potassium acetate/5 mM $MgCl_2$ (pH 5.0).

Substrate	Level of cations	K_d (nM)
Thiamine	Limiting	0.01
	Excess	0.01
Biotin	Limiting	1.0
	Excess	0.1
Folate	Limiting	15
	Excess	0.1

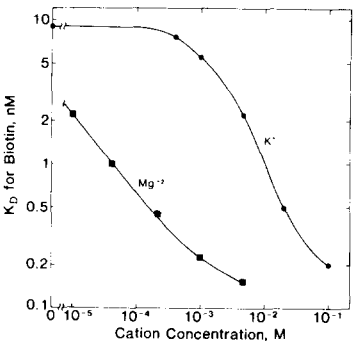

Fig. 4. Enhancement in binding affinity (K_d) for biotin as a function of Mg^{2+} and K^+ concentration. Cations were added as chloride salts. Suspending medium, 0.1 mM K-Hepes (pH 7.5).

Effect of pH on binding

Dissociation constants for thiamine and biotin were also measured at pH 5.0 in buffers that contained a similar composition of excess (50 mM potassium acetate/5 mM $MgCl_2$, pH 5.0) and limiting (0.1 mM potassium acetate, pH 5.0) cations (Table II). The binding of thiamine at pH 5.0 ($K_d = 0.01$ nM) remained independent of the ionic composition of the medium, while optimum binding of biotin still required the presence of added cations. In the latter case, however, the cation dependence was less pronounced at the lower pH. K_d values for biotin measured in the presence or absence of cations differed by 10-fold at pH 5.0, compared with 60-fold at pH 7.5. The dependence of folate binding on buffer composition was also less pronounced (by 4-fold) at pH 5.0 than at pH 7.5 (Table II).

Cation specificity for [^{3}H]biotin binding

K_d values for biotin were measured at various concentrations of K^+ and Mg^{2+} to determine the effectiveness of these cations in promoting the binding of biotin (Fig. 4). The results were quantitated by comparing cation levels required to lower log K_d to one-half the sum of log K_d (maximum) and log K_d (minimum). This mid-point value was calculated (at pH 7.5) to be 1.06 nM from the binding affinity for biotin in dilute buffer alone ($K_d = 9.0$ nM) and dilute buffer plus excess $MgCl_2$ ($K_d = 0.12$ nM). By this method, Mg^{2+} was shown to be much more effective than K^+ in promoting the binding of biotin. A half-maximal increase in affinity was observed at an Mg^{2+} concentration of 37 μM, while a K^+ concentration of 9400 μM was required to give a comparable enhancement in binding.

Discussion

Lactobacillus casei cells possess separate transport systems for the cellular uptake of folate, thiamine, and biotin [1,10,12]. These systems each contain a specific binding protein but appear also to share a second component which has been proposed to act as an energy-coupling factor [10]. Evidence for this shared component was obtained from a kinetic analysis of the simultaneous transport of three vitamins. Each vitamin was found to inhibit non-competitively the transport of the other vitamins and the inhibition reached a maximum level whose magnitude was directly proportional to the amount of binding protein for each vitamin. An additional relationship between these transport systems is apparent from the observation that the purified binding proteins for two of the vitamins (folate and thiamine) are structurally very similar [6,7].

The transport proteins for folate, thiamine, and biotin can be induced by growth of the cells in the presence of limiting amounts of their respective vitamin substrates [1,10]. The binding proteins each

have a high substrate binding affinity (K_d range, 0.01 to 0.5 nM), but differ in their total amount per cell. The relative abundance is thiamine, folate, biotin, with a molar ratio of 60 : 40 : 1. The thiamine and folate binding proteins comprise approximately 0.3 and 0.2% of total cellular protein, respectively, and thus represent relatively abundant cellular constituents. Each of the binding proteins has the additional property that binding can be efficiently and conveniently measured without the concurrent transport of the respective substrates into the cell. The ability to separate binding from transport is possible because of a combination of factors. In each case, the substrate binding affinity is very high, which enables binding measurements to be performed under conditions in which the specific association of substrate with the cells is substantially higher than the nonspecific component. A high binding affinity also limits the accumulation of the substrate within the cells caused by the release of carrier-bound substrate at the inner membrane surface. A second major factor is the ability to block the energy-dependent transport of the vitamin substrates into the cell. This is achieved by lowering the temperature to 4°C and by using cells depleted of energy reserves [4,8].

Optimal binding to the folate transport protein requires the presence of cations [8]. The binding affinity for folate is reduced by 600-fold unpon transfer of the cells from a buffered saline medium to isotonic sucrose or water, and this reduction is reversible since cations added back to cells in sucrose regain the ability to bind folate with a high affinity. The proposed explanation for these findings is that the transport protein contains both a folate and cation binding site and that the binding of cations is required to convert the folate binding site to a high-affinity state. A possible role for cations in this process could be to maintain electroneutrality during the movement of folate across the membrane. Since folate is a divalent anion, it must enter the cell against a considerable membrane potential [4]. The latter represents a force which could potentially prevent the movement of the folate dianion across the cell membrane. A method for neutralizing this outward force could be to mediate an electroneutral uptake of folate along with a cation or cations of equal net charge. Mechanistically, this could be achieved if the transport protein contains a binding site for both folate and divalent cation and if both sites function in a concerted fashion. The bound substrates would then be free to move to the inner membrane surface, prior to being released from the binding protein via a subsequent energy-coupling step [4]. Accumulated folate would then undergo reduction (to tetrahydrofolate) followed by polyglutamylation [13], while cations could be used for metabolic processes or extruded from the cell via separate transport routes.

Measurements in the present study demonstrate that substrate binding to the biotin transport protein of *L. casei* is also dependent upon cations (cf. Figs. 3 and 4). The affinity of the binding protein for biotin is reduced 60-fold when the cells are transferred from a cation replete medium to one deficient in cations, and divalent cations are much more effective than monovalent cations in returning the binding protein to a high-affinity state. The cation dependence of binding decreases with pH (Table II), indicating that protons can partially fulfill the cation requirement. These results are very similar to those obtained for substrate binding to the folate transport protein [8], except that the cation dependence for the latter substrate is substantially higher (Table I). It thus appears that both the biotin and folate transport proteins of *L. casei* contain a relatively similar cation binding site and that the function of this site is to mediate the translocation of a cation (or cations) in concert with the vitamin substrate. The net charge of the transported complexes has not been determined, although the specificity of binding indicates that divalent cations and biotin would combine to form a complex with a +1 charge which could then be drawn into the cell by the membrane potential. A complex between a divalent cation and the folate dianion would be electroneutral under the same conditions, although protonation of one of the carboxyl groups of folate could result in a positively charged complex. Monovalent cations could bind to the folate transport protein in pairs to form a complex with the same net charge as with divalent cations, or a single monovalent cation and a proton could combine to give an electroneutral complex.

Substrate binding to the thiamine transport of

L. casei does not require cations to exhibit an optimum binding affinity (Fig. 2). The K_d for thiamine was the same when measurements were performed in buffer containing either excess or limiting amounts of cations, and the lack of a cation dependence was observed both at pH 7.5 and at pH 5.0 (cf. Tables I and II). This finding is consistent with the prediction that a cation binding site on the thiamine transport protein would not be necessary for thiamine transport. Thiamine already has a net +1 charge and therefore upon binding to the transport protein could move directly to the inner membrane surface. The inward movement of thiamine would be energetically favored under these conditions and the cell would not have to contend with the uptake of potentially unwanted cations.

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