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Osmotic and Chill Activation of Glycine Betaine Porter II in *Listeria monocytogenes* Membrane Vesicles

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***Listeria monocytogenes* is a foodborne pathogen known for its tolerance to conditions of osmotic and chill stress. Accumulation of glycine betaine has been found to be important in the organism's tolerance to both of these stresses. A procedure was developed for the purification of membranes from *L. monocytogenes* cells in which the putative ATP-driven glycine betaine permease glycine betaine porter II (Gbu) is functional. As is the case for the *L. monocytogenes* sodium-driven glycine betaine uptake system (glycine betaine porter I), uptake in this vesicle system was dependent on energization by ascorbate-phenazine methosulfate. Vesicles lacking the *gbu* gene product had no uptake activity. Transport by this porter did not require sodium ion and could be driven only weakly by artificial gradients. Uptake rates could be manipulated under conditions not affecting secondary transport but known to affect ATPase activity. The system was shown to be both osmotically activated and cryoactivated. Under conditions of osmotic activation, the system exhibited Arrhenius-type behavior although the uptake rates were profoundly affected by the physical state of the membrane, with breaks in Arrhenius curves at approximately 10 and 18°C. In the absence of osmotic activation, the permease could be activated by decreasing temperature within the range of 15 to 4°C. Kinetic analyses of the permease at 30°C revealed K_m values for glycine betaine of 1.2 and 2.9 μM with V_{max} values of 2,200 and 3,700 pmol/min · mg of protein under conditions of optimal osmotic activation as mediated by KCl and sucrose, respectively.**

Listeria monocytogenes is a foodborne pathogen responsible for a variety of infective syndromes in humans (7, 24). The organism is considered to be saprophytic and is known for its ability to survive and grow under adverse conditions (31, 32). It is especially well known for its ability to grow under conditions of high osmolarity and desiccation and at low temperatures (3, 6, 30). One mechanism of adaptation to osmotic stress by eubacteria is accumulation of compatible solutes, organic osmolytes that have comparatively minimal deleterious effects on cell physiology at high concentrations (2, 5, 9, 20). The compatible solute glycine betaine (*N*,*N*,*N*-trimethylglycine) has been shown to be accumulated to high levels in several species of bacteria (11, 25, 26), including *L. monocytogenes* (16, 23), and has been shown to confer a substantial degree of both osmotolerance and cryotolerance upon *L. monocytogenes* (16).

In eubacteria, glycine betaine is taken up through both primary and secondary transport systems. We have previously described a sodium-driven, osmotically activated glycine betaine uptake system in *L. monocytogenes* (glycine betaine porter I) that is functional in membrane vesicles (10). Ko and Smith have cloned glycine betaine porter II, sequenced the *gbu* locus, and determined that it belongs to the family of ATP-binding cassette (ABC) transporters (17). We now report that glycine betaine porter II is also functional in membrane vesicles and can be activated either by an osmotic gradient or by low temperature.

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MATERIALS AND METHODS

Materials. ^{14}C-labeled glycine betaine was synthesized from [^{14}C]choline HCl (NEN, Boston, Mass.) as described by Landfald and Strøm (19). Egg white lysozyme, mutanolysin, and DNase were obtained from Sigma Chemical Co. (St. Louis, Mo.). Other materials were of reagent grade and were obtained from commercial sources. The bicinchoninic acid reagent (Pierce Chemical, Rockford, Ill.) was used to determine protein concentrations (28). Mixed-ester filters (Millipore GSWP; 0.22-μm pore size, 25-mm diameter) were used in uptake assays, and liquid scintillation counting was performed with Scintiverse I fluid (Fisher Scientific, Pittsburgh, Pa.). The osmotic strength of assay mixtures was adjusted with appropriate concentrations of sucrose and KCl using values given previously (33). Culture densities were measured with a Klett-Summerson colorimeter with a no. 45 (green) filter.

Growth conditions and preparation of membrane vesicles. *L. monocytogenes* DP-L1044 (*hly*::Tn*917*-LTV3) (29) was grown aerobically at 30°C in a 5-liter batch culture of tryptose broth (Difco Laboratories, Detroit, Mich.), supplemented with 281 g of sucrose per liter, to a mid-exponential-phase culture density of 80 to 85 Klett units. Cells were harvested (13,200 × *g*, 30 min at 4°C) and washed twice in 100 mM potassium phosphate buffer (pH 7.0).

Vesicles were prepared using a modification of procedures for gram-negative bacteria described by other groups (13, 27). The washed cell pellet was resuspended to a volume of 100 ml in 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM $MgSO_4$, 25% (wt/vol) sucrose, 1.4×10^6 U of egg white lysozyme, and 1.0×10^4 U of mutanolysin and incubated under gentle shaking at 30°C. Protoplast formation, which was monitored by phase-contrast microscopy, was allowed to proceed to about 95% completion (50 to 55 min). Protoplasts were then concentrated by centrifugation (27,000 × *g*, 15 min at 4°C) and resuspended on ice by means of a Potter-Elvehjem homogenizer to a volume of 3 ml in 50 mM potassium phosphate (pH 7.0) containing 20 mM $MgSO_4$, 25% sucrose, and 16 mg of DNase. The mixture was allowed to incubate on ice for 10 min, after which it was injected by means of a syringe (19-gauge needle) into 1.5 liters of rapidly stirring 50 mM potassium phosphate (pH 7.0) at 37°C. The solution was gently stirred at 37°C for 15 min, after which potassium-EDTA was added to a final concentration of 10 mM; after a further 15 min of stirring, $MgSO_4$ was added to a final concentration of 15 mM. Cell debris and remaining whole cells were removed by centrifugation (3,300 × *g*, 15 min, 4°C). Purified membranes were collected by centrifugation (21,500 × *g*, 60 min, 4°C) and suspended by means of a Potter-Elvehjem glass homogenizer in 50 mM potassium phosphate (pH 7.0) containing 10 mM $MgSO_4$. Aliquots of 300 μl containing membrane protein levels of 6 to 8 mg/ml were then frozen and stored in liquid nitrogen.

For experiments involving preloading of the vesicles with ATP or *ortho*-vanadate, either 5 mM ATP or 1 mM sodium *ortho*-vanadate was included in the buffer during lysis.

Transport assays. Membrane vesicles were thawed rapidly at 45°C, held on ice, and used within 120 min of thawing. Incubation mixtures for transport studies contained 50 mM potassium phosphate, 10 mM $MgSO_4$, 200 μM phenazine methosulfate (PMS), 20 mM potassium ascorbate, [^{14}C]glycine betaine, and solutes at concentrations yielding the osmotic pressures indicated in the figure legends. Unless stated otherwise, assays were conducted at 30°C. These conditions were used in all of the experiments except those employing artificial gradients for energization of the transporter. Vesicles were preincubated for 10 min in buffer containing the appropriate solutes, after which ascorbate and PMS were added in that order. The reaction was initiated by the addition of [^{14}C]glycine betaine (0.05 μCi per assay). For each time point, a 50-μl aliquot containing approximately 100 μg of protein was withdrawn. The reaction was terminated by diluting the aliquot into 2 ml of an ice-cold solution containing 100 mM LiCl and KCl of the same osmotic strength as that used in the uptake mixture. The samples were filtered and then washed with 2 ml of the same LiCl-KCl solution; radioactivity was determined by liquid scintillation counting. Transport rates obtained from duplicate experiments using a single preparation of vesicles differed by <10%.

Measurement of ATP production. Vesicles were incubated under a steady flow of water-saturated oxygen gas at 30°C in potassium phosphate buffer containing $MgSO_4$ and sucrose for 2 min before the reaction was initiated by addition of PMS. Final concentrations were 3.5 mg of protein per ml, 610 mM sucrose, 20 mM potassium ascorbate, 20 μM PMS, 4.8 mM magnesium sulfate, and 25 mM potassium phosphate, pH 7. Except for the omission of glycine betaine, the conditions were the same as those used to measure osmotically activated transport (e.g., see Fig. 1, inset). ATP present in the solution was determined as described by Prossnitz et al. (27). Aliquots (25 μl) were withdrawn at various time intervals, the reaction was quenched, and the vesicles were lysed by addition of 650 μl of cold 9% perchloric acid. After incubation for 10 min in an ice bath, the solution was neutralized by the addition of 188 μl of cold 4 M potassium hydroxide, followed immediately by 188 μl of 2 M potassium bicarbonate. After an additional 10 min, the samples were centrifuged at 11,500 × *g* for 10 min. Aliquots (100 μl) of the supernatant solution were assayed for ATP using 50 μl of luciferin-luciferase reagent (Sigma) and a TD 20/20 luminometer (Turner Designs, Sunnyvale, Calif.). The reagent, which contained glycine buffer, luciferin, and luciferase, was used at a concentration of 8 mg/ml. Experiments were run in duplicate.

Artificial gradient-driven transport. Concentrated membrane vesicles (20 mg of protein/ml) were incubated on ice for 60 min in 20 mM potassium phosphate, pH 6.0, containing 100 mM potassium acetate and 10 mM $MgSO_4$ in either the presence or the absence of valinomycin (2 nmol/mg of protein). The equilibrated vesicles were then collected by centrifugation (48,200 × *g*, 20 min, 4°C) and suspended (with or without 1 μM valinomycin) in 20 mM potassium phosphate (pH 6.0) containing 100 mM potassium acetate, 10 mM $MgSO_4$, and 350 mM sucrose. The vesicles were then recentrifuged and resuspended in the same buffer to a concentration of approximately 30 mg of protein/ml.

Valinomycin-treated vesicles (6-μl aliquots) were then diluted 100-fold with buffers at 30°C as indicated. All diluting buffers contained 20 μM [^{14}C]glycine betaine (0.2 μCi), 500 mM sucrose, and 10 mM $MgSO_4$, pH 6. The valinomycin-treated vesicles were used to establish driving forces consisting of a membrane potential ($\Delta\Psi$), pH gradient (ΔpH), and sodium gradient ($\Delta\mu_{Na^+}/F$) created by dilution into 120 mM sodium phosphate; a membrane potential and sodium gradient created by dilution into 100 mM sodium acetate-20 mM sodium phosphate; a membrane potential and pH gradient (ΔP) created by dilution into 120 mM Tris-phosphate; or a membrane potential alone created by dilution into 100 mM Tris-acetate–20 mM Tris-phosphate.

Vesicles not treated with valinomycin were used to establish driving forces consisting of a pH gradient and sodium gradient created by dilution into 120 mM sodium phosphate; a pH gradient created by dilution into 120 mM potassium phosphate; or a sodium gradient alone created by dilution into 100 mM sodium acetate–20 mM sodium phosphate. To determine uptake rates, aliquots (80 μl) of the reaction mixture were withdrawn, immediately placed on prewashed mixed-ester filters, and washed and processed as described for the ascorbate-PMS-energized system.

Membrane phase transition temperature. Membrane phase transition temperatures were measured by Fourier transform infrared (FTIR) spectroscopy (4). Membranes were collected by centrifugation (30 min, 48,000 × *g*, 4°C) and resuspended to a level of approximately 30 mg of protein per ml in 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM $MgSO_4$ and with solutes at concentrations indicated in the figure legends. A 30-μl aliquot of the membrane suspension was then placed between glass plates and then inserted into a temperature controller. The CH_2 symmetric stretching frequency (within the wave number range of 2,850 to 2,854 cm^{-1}) at different temperatures was then measured using a Perkin-Elmer 1750 Fourier-Transform Infrared Spectrometer.

RESULTS

Vesicle preparation methodology. Glycine betaine transport activity could be demonstrated upon energization by the ascorbate-PMS electron donor system in vesicles derived from cells grown in tryptose broth supplemented with 281 g of sucrose per liter and prepared as described in Materials and Methods. Activity also was apparent in vesicles prepared using conventional methods for mesophilic gram-positive bacteria (e.g., see references 10 and 22) or methods originally used for gram-negative bacteria (13, 27) and modified (EDTA treatment omitted, lysin treatment optimized) for *L. monocytogenes*. Transport activity was, however, extremely variable in preparations using the method for *L. monocytogenes* described previously (10); uptake rates ranged from 20 to 70% of those consistently obtainable using the procedure described in this work. Furthermore, membrane yields were also approximately fourfold greater using the methodology described in the present work. In the absence of large amounts of sodium ion in the assay mixtures, transport activity could not be demonstrated in vesicles prepared from cells grown under conditions of osmotic stress mediated by either NaCl or KCl instead of sucrose.

Energetics of transport. The use of ionophores can result in inhibition of ABC-type transporters, either by direct inhibition of the permease or by indirect inhibition due to effects on internal pH. We therefore chose instead to investigate the porter's energetics through intravesicular incorporation of *ortho*-vanadate and ATP and through the use of artificial chemical gradients to selectively generate components of the proton motive and sodium motive forces. Vesicles prepared by lysis of protoplasts in hypotonic potassium phosphate buffer containing 1 mM sodium *ortho*-vanadate had uptake rates approximately one-fifth of those prepared in the absence of *ortho*-vanadate (Fig. 1), while lysis in the same buffer containing 5 mM ATP enhanced uptake rates by approximately one-third. Uptake was dependent on addition of ascorbate-PMS.

Artificial gradient-driven transport of glycine betaine in the presence of high concentrations of Na^+ has been previously demonstrated for glycine betaine porter I (10). Under conditions of energization provided by acetate and valinomycin-mediated potassium diffusion potentials (Fig. 1, inset), uptake was rapid in the presence of both sodium and a proton motive force, ΔP (closed circles), or sodium and a charge gradient, $\Delta\Psi$ (closed squares). Uptake was apparent but low in the presence of ΔP or $\Delta\Psi$ alone. The results show that the vesicles are electrochemically robust because glycine betaine can be taken up by glycine betaine porter I, a sodium-driven porter having low affinity for sodium ion. However, glycine betaine transport by Na^+-independent glycine betaine porter II is slow under conditions that would directly energize a porter that uses H^+ as the coupling ion. ΔP or $\Delta\Psi$ alone is able to support only a low level of activity, suggesting that energization occurred through an indirect mechanism.

ATP production by vesicles. ATP levels in PMS-ascorbate-energized vesicles, vesicles containing 2 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and unenergized controls were determined under osmotic stress provided by sucrose (data not shown). ATP was found to be present at a level of 0.4 nmol/mg of protein in unenergized controls, CCCP-treated vesicles, and energized vesicles at time zero. Upon addition of ascorbate-PMS, the ATP level increased to 1.4 nmol/mg of protein after 5 min; CCCP-treated vesicles did not show ATP synthesis, nor did unenergized vesicles. These data are similar to those obtained with *Escherichia coli* by Prossnitz et al. (27), who termed the basal level of ATP in unenergized vesicles "not accessible for energizing transport," suggesting it to be bound or sequestered. Our result demonstrates that there is sufficient ADP and phosphate in the vesicles for the production of ATP and that the machinery for doing so operates under the conditions under which transport is observed.

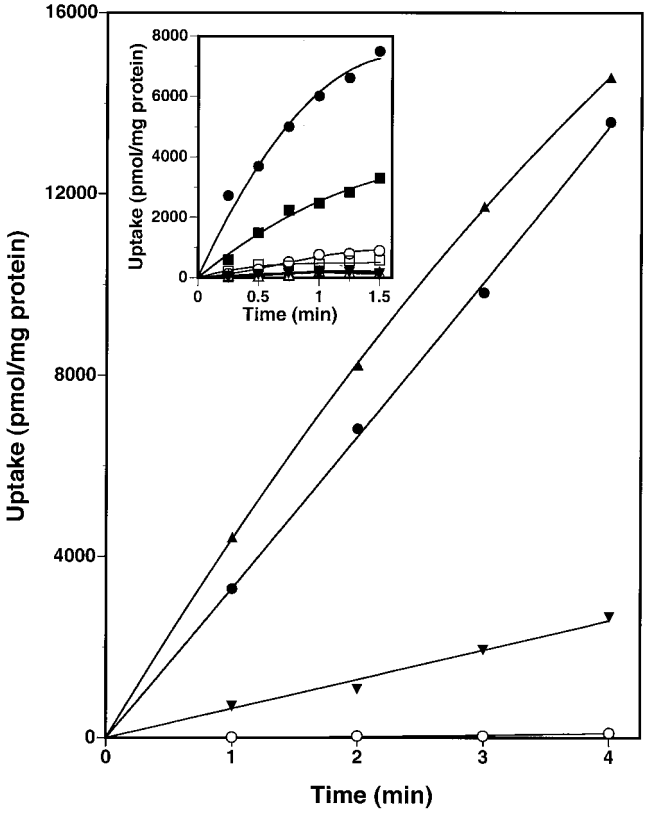

FIG. 1. Energetics of transport: enhancement and inhibition of ATP-dependent glycine betaine transport activity in membrane vesicles of *L. monocytogenes*. ATP and sodium *ortho*-vanadate were loaded into vesicles during the membrane purification procedure by addition of the compounds to the lysis buffer. The vesicles were stressed with 600 mOsm (506 mM) sucrose at pH 7 and energized with ascorbate-PMS as described in Materials and Methods. Symbols: ●, no additions; ○, no additions, unenergized (PMS omitted); ▲, 5 mM ATP; ▼, 1 mM sodium *ortho*-vanadate. (Inset) Artificial gradient-driven uptake of 20 mM [^{14}C]glycine betaine into membrane vesicles of *L. monocytogenes*. Vesicles were loaded and gradients were imposed at pH 6.0 and 30°C as described in Materials and Methods. Symbols: ●, $\Delta\mu_{Na}^{+}/F$ and ΔP; ■, $\Delta\mu_{Na}^{+}/F$ and $\Delta\Psi$; ▼, $\Delta\mu_{Na}^{+}/F$ and ΔpH; ▲, $\Delta\mu_{Na}^{+}/F$; ○, ΔP; □, $\Delta\Psi$; ▽, ΔpH; △, control (osmotic gradient alone).

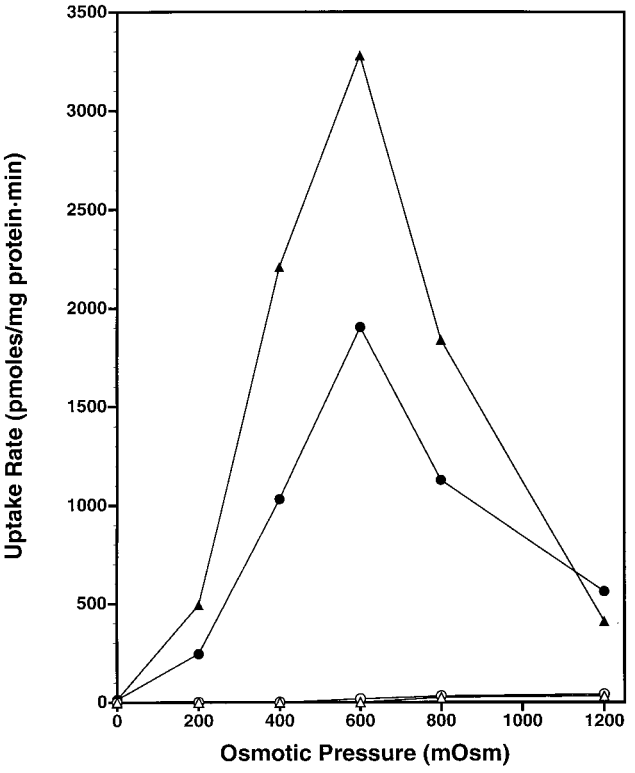

FIG. 2. Osmotic activation of glycine betaine uptake in membrane vesicles of *L. monocytogenes*. Systems were supplemented with either KCl or sucrose to provide osmotic pressures at the levels indicated. Vesicles were energized with ascorbate-PMS at pH 7 as described in Materials and Methods. Strain DP-L1044 (*gbu*$^{+}$) was tested with KCl (●) or sucrose (▲). Strain LTG59 (*gbu*) was also tested with KCl (○) or sucrose (△).

Osmotic activation of the permease. Glycine betaine porter II, like glycine betaine porter I, is osmotically activated. Glycine betaine was taken up by glycine betaine porter II at a high rate under hypertonic conditions (Fig. 2). Uptake was completely dependent on energization by the exogenous energy source ascorbate and PMS. Uptake under energized conditions was very low (<2 pmol/min · mg of protein) at 30°C in the absence of hypertonic stress but could be activated by over 1,000-fold in the presence of a hypertonic solute gradient. The system showed maximal activation at an osmotic pressure of 600 mOsm for either potassium chloride or sucrose (Fig. 2). The dependence of transport on osmotic strength was similar for KCl and sucrose, and activity decreased rapidly above the osmotic optimum. Activity was approximately 50% higher in the presence of a sucrose gradient than in the presence of a KCl gradient at 30°C, where sucrose-mediated activation was optimal. In vesicles derived from strain LTG59, in which the *gbu* genes are disrupted by a transposon insertion, uptake was not observed below an osmotic pressure of 600 mOsm mediated by either sucrose or KCl; at 600 mOsm, activity of the LTG59-derived vesicles was less than 2% of that observed at maximal activation of vesicles derived from strain DP-L1044, which bears the same transposon at an unrelated site.

A kinetic analysis of glycine betaine uptake was carried out under conditions of optimal osmotic activation by sucrose and KCl at 30°C. The initial rate of glycine betaine uptake within the concentration range of 1.0 to 20.0 μM yielded Michaelis constants (Table 1) for glycine betaine of 2.9 and 1.2 μM with activation by sucrose and KCl, respectively. Values of V_{max} under these conditions were predicted to be 3,700 and 2,200 pmol/mg of protein · min, respectively.

Temperature influence on osmotic activation. The effect of temperature under conditions of optimal osmotic activation

TABLE 1. Kinetics of osmotic-gradient- and cold-activated glycine betaine transport[a]

Conditions[b]	K_m (μM GB)	V_{max} (pmol of GB/min · mg of protein)
30°C + 600 mOsm sucrose	2.9 ± 0.1	3,730 ± 60
30°C + 600 mOsm KCl	1.2 ± 0.1	2,200 ± 200
4°C, no additions	15.7 ± 0.3	28 ± 2.4

[a] Abbreviations: GB, glycine betaine; V_{max}, maximum rate of transport. Reported errors are standard errors of at least two experiments, each run in duplicate. Data were analyzed using the program Enzyme Kinetics, version 1.1 (Trinity software); values were calculated using fourth-power weighting.

[b] All assay mixtures contained 50 mM potassium phosphate (pH 7.0), 10 mM $MgSO_4$, 20 mM potassium ascorbate, and 200 μM PMS.

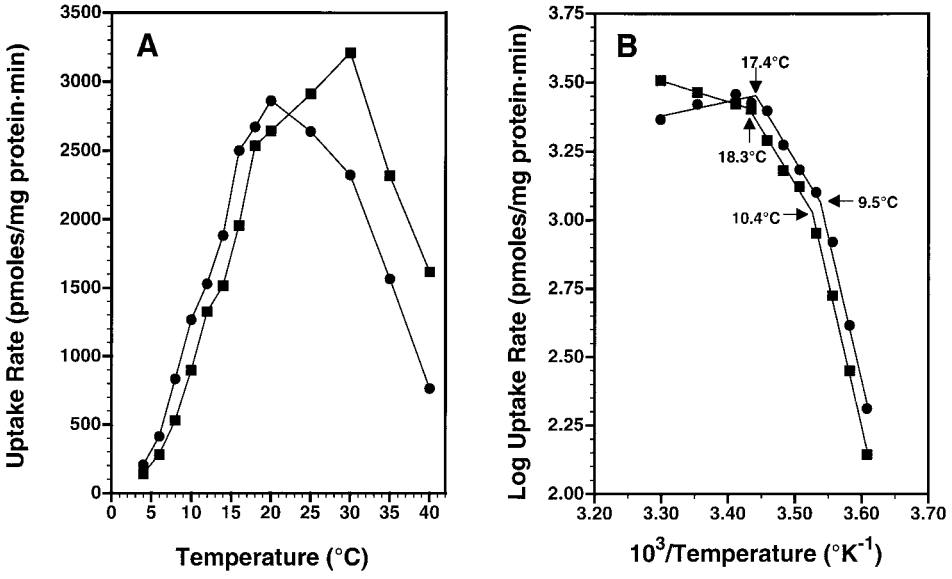

FIG. 3. Effect of temperature on glycine betaine uptake under conditions of optimal osmotic activation. (A) Effect of temperature on uptake in the presence of 600 mOsm KCl (●) or sucrose (■). Vesicles were energized with ascorbate-PMS at pH 7 as described in Materials and Methods. (B) Arrhenius plot of data from panel A within the temperature range of 4 to 30°C. Symbols: ●, 600 mOsm KCl; ■, 600 mOsm sucrose. Arrows indicate temperatures corresponding to discontinuities in lines.

was examined. Interestingly, activity was optimal at 30°C (the temperature at which the culture was grown) under hypertonic conditions mediated by sucrose, while activation under hypertonic conditions mediated by KCl was optimal at 20°C (Fig. 3A). Because of the difference in temperature optima, KCl-elicited activities were higher than those of sucrose at temperatures below 20°C; the opposite effect was true at temperatures above 20°C. A sharp decrease in glycine betaine uptake rates in the presence of either sucrose or KCl was observed with increasing temperature above 30°C.

When the temperature dependence data (Fig. 3A) are presented as Arrhenius plots (Fig. 3B), data derived from either the sucrose or the KCl curves exhibit three distinct phases. Sharp breaks occur at about 10 and 18°C. Activation energies (E_a) for the permease are not solute dependent below 20°C, as slopes are parallel, and both the KCl- and sucrose-activated systems have E_a of about 48 kcal/mol below and E_a of about 18 kcal/mol above the 10°C breaks (Table 2). Between 18°C and its optimum temperature of 30°C, under conditions of osmotic activation by sucrose, the system continues to show temperature activation consistent with the Arrhenius equation whereas activity in the KCl-mediated system shows decreasing activity. The fact that the temperature dependence of transport below 18°C is independent of the nature of the solute suggests that the membrane plays an important role in determining the rate of transport.

TABLE 2. Effect of transport-activating conditions on energies of activation for the rate of transport of glycine betaine in *L. monocytogenes* membrane vesicles[a]

Condition	Temp range (°C)	Slope[b]	E_a (kcal/mol)[c]
600 mOsm KCl	18–30	+520	
	12–18	−3,990	18.3
	4–10	−10,500	48.1
600 mOsm sucrose	18–30	−750	3.4
	12–18	−3,960	18.1
	4–10	−10,610	48.6
Low temp[d]	4–15	+6,020	−115

[a] All systems were energized by addition of ascorbate-PMS.
[b] Values were obtained from data in Fig. 3 and 5.
[c] $E_a = -2.303R \times$ (slope of Arrhenius plot).
[d] In the absence of osmotic stress.

Measurement of membrane order. Membrane phase transitions can be determined by changes in the vibrational frequency of the CH_2 bands in membrane phospholipids. Analysis of the CH_2 symmetric stretch band in FTIR spectra revealed a broad membrane lipid phase transition (Fig. 4A). The lower boundary of the phase transition is 0°C, and the upper boundary is 29°C. The addition of sucrose or KCl to 600 mOsm had no apparent effect on the phase transition temperature. When lipid phase transition data obtained in the presence of either 600 mOsm sucrose or 600 mOsm KCl are analyzed by means of Arrhenius curves depicting the degree of membrane ordering (log percent maximum wave number versus inverse absolute temperature) (Fig. 4B), a very strong correlation is revealed. Two distinct breaks are evident in the plot, suggesting a triphasic phase transition in the membrane. In the presence of either solute, breaks are observed at about 10 and 18°C. There is thus a close correlation between the phase of the *L. monocytogenes* membrane and permease activity in the presence of either sucrose or KCl at 600 mOsm. Results summarizing predicted phase transitions as measured by solute gradient-mediated enzymatic activity and FTIR spectroscopy are presented in Table 3. These results show the dependence of porter activity on membrane order.

Temperature influence in the absence of osmotic activation. In whole cells of *L. monocytogenes*, transport of glycine betaine has been shown to be stimulated by low temperature (16) in the absence of osmotic stress. In membrane vesicles energized by ascorbate-PMS, glycine betaine transport was clearly stimulated by decreasing temperature within the range of 15 to 4°C (Fig. 5A). Transport could be stimulated approximately seven-

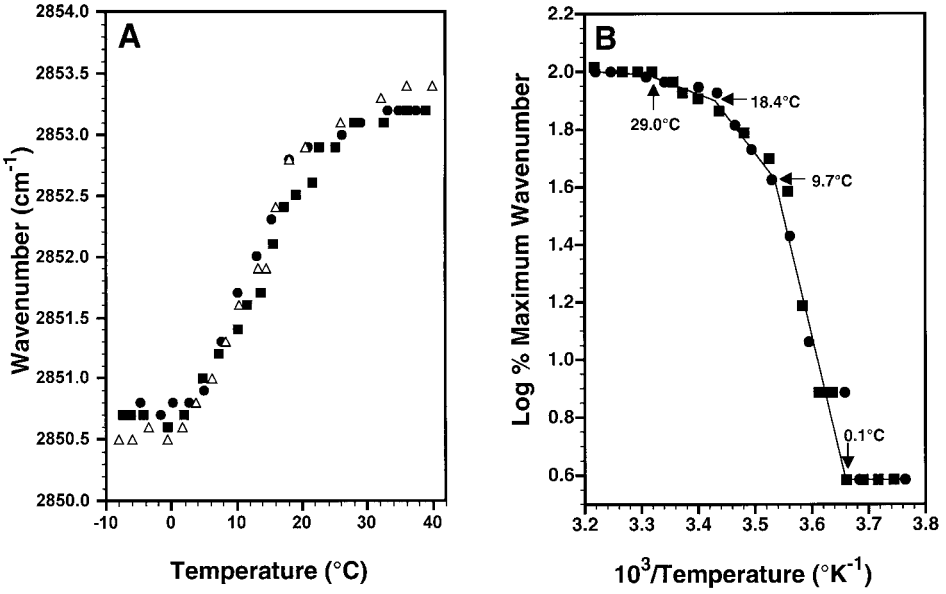

FIG. 4. Effect of temperature on *L. monocytogenes* membrane order. Vibrational frequencies of CH_2 symmetric stretch bands in membrane vesicles of *L. monocytogenes* as measured by FTIR spectroscopy are shown. (A) Effect of temperature on CH_2 symmetric stretching frequency. A higher wave number signifies a more disordered state. Membranes were tested in 600 mOsm KCl (■), in 600 mOsm sucrose (●), or without added solutes (△). (B) Arrhenius plot of data in panel A. The log percentage of the maximum wave number versus the inverse of the absolute temperature in systems containing KCl and sucrose is shown. Membranes were tested in 600 mOsm KCl (■) or 600 mOsm sucrose (●). Temperatures at discontinuities in the lines are noted; arrows at 29.0 and 0.1°C mark the ends of the phase transitions.

fold within this range but was very low or undetectable above 10°C and fell to approximately 50% of the maximal value at 1°C. Uptake rates in the presence of 20 mM ascorbate and 200 μM PMS reached approximately 28 pmol/mg of protein · min. As in the case for osmotic activation, this effect is energy dependent, as transport at low temperature could not be observed in the absence of the electron donor system (open circles), nor was this activity apparent in energized vesicles prepared from strain LTG59, which lacks *gbu* (closed squares). When these data are presented as Arrhenius plots (Fig. 5B), a positive slope is obtained within the temperature range of 4 to 15°C, resulting in a calculated apparent negative activation energy of −27.5 kcal/mol. A kinetic analysis of chill-activated glycine betaine uptake conducted within the range of 1.0 to 30.0 μM glycine betaine yielded a Michaelis constant (Table 1) for glycine betaine of 16 μM and a V_{max} value of 28.1 pmol/mg of protein · min.

TABLE 3. Predicted values of phase transitions in *L. monocytogenes* membrane vesicles as measured by solute gradient-mediated enzymatic activity and FTIR spectroscopy[a]

Condition[b]	$T_{m(FTIR)}$ (°C)	$T_{m(enz)}$ (°C)
600 mOsm KCl	9.7	9.5
	18.4	17.4
600 mOsm sucrose	9.7	10.4
	18.4	18.3

[a] Abbreviations: $T_{m(FTIR)}$, phase transition temperature as predicted by log percent maximum wave number as measured by FTIR spectroscopy; $T_{m(enz)}$, phase transition temperature as predicted by breaks in Arrhenius activation plots of enzymatic activity. Values were obtained by extrapolation of the curves in Fig. 3B and 4B.

[b] All systems were energized by addition of ascorbate-PMS.

DISCUSSION

Transport activity by the putative *L. monocytogenes* ATP-driven glycine betaine porter encoded by *gbu* (glycine betaine porter II) can be effectively recovered and studied as an iso-

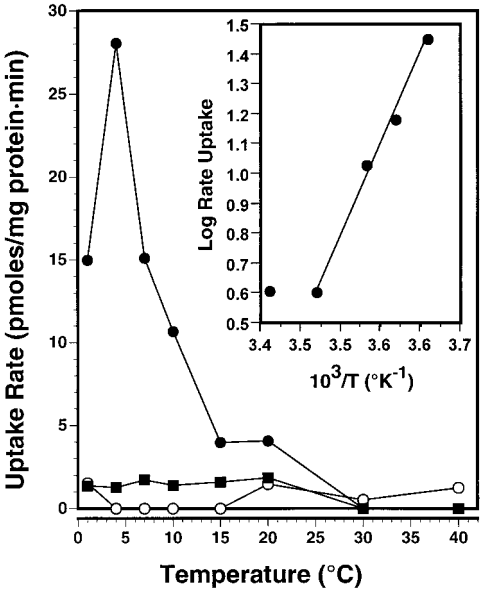

FIG. 5. Chill activation of glycine betaine transport. Vesicles were assayed without osmotic stress at pH 7.0 as described in Materials and Methods. Vesicles prepared from strain DP-L1044 (*gbu*$^+$) were tested with (●) or without (○) energization by addition of PMS; vesicles prepared from strain LTG59 (*gbu*) were energized by addition of 200 μM PMS (■). The inset shows an Arrhenius plot of the transport rate in energized vesicles without osmotic stress between 4 and 20°C.

lated system in membrane vesicles derived from cells grown in tryptose broth supplemented with sucrose. This Na^+-independent activity likely is the result of retention by vesicles of a cytoplasmic constituent (presumably ADP), allowing in situ generation of ATP and the concomitant transport of substrates. Such a system has previously been demonstrated for the histidine permease of *Salmonella enterica* serovar Typhimurium(27) using vesicles prepared essentially as described for this system. Ko and Smith have sequenced the *gbu* open reading frame (17), and the deduced amino acid sequences were found to have high homology to components of the OpuA glycine betaine permease of *Bacillus subtilis* (14, 15). In whole-cell studies in our laboratory, we have shown this porter to be osmotically activated and responsible for cold-activated glycine betaine transport.

Initial experiments utilizing ionophores provided little information on the nature of system energetics (Fig. 1). Experiments employing artificial gradients were used to determine whether betaine transport by this porter could be driven directly by transmembrane gradients. Only very low levels of transport were observed (Fig. 1, inset, open circles and squares). To assess the significance of these low levels of transport, transport by a genuine $\Delta\Psi$-driven porter, glycine betaine porter I (10), was measured as a positive control. This activity required Na^+ (the coupling ion for this symporter) in the assay mixture in addition to the other components of energization. Substantial transport (Fig. 1, inset, closed circles and squares) was catalyzed by porter I under these conditions, which is consistent with the data reported in reference 10. This result strongly suggests that the potentials we provided would be adequate to drive porter II if it were directly powered by $\Delta\Psi$ or ΔP. Our conclusion was that porter II cannot be driven directly by gradients. It would be expected that ΔP should drive ATP formation, which should, in turn, drive porter II, as is the case for energization by ascorbate-PMS. However, it might also be expected that ascorbate-PMS is capable of generating a larger pool of ATP and is therefore much more efficient at driving porter II, albeit indirectly, than are artificial gradients.

If transport by the permease is ATP dependent, manipulation of conditions not affecting components of ΔP but affecting either the intravesicular ATP pool or the permease directly should influence transport rates. Preliminary evidence that a ΔP drives transport indirectly was shown by inhibition of the F_0F_1 ATPase, as glycine betaine uptake rates decreased approximately 50% in the presence of 100 μM *N*,*N*′-dicyclohexylcarbodiimide (data not shown). The results illustrated in Fig. 1 show a clear sensitivity to 1 mM sodium *ortho*-vanadate and clear stimulation by 5 mM ATP (provided during vesicle formation). In addition, direct measurement of ATP production corresponded well with the observation of transport after energization with ascorbate-PMS. The biochemical evidence presented in this study is therefore in complete agreement with the genetic evidence available (17). If glycine betaine is not taken up via an ATP-dependent transporter, the system should remain insensitive to *ortho*-vanadate, a potent inhibitor of both P-type ATPases and binding protein-dependent transporters (8), and incorporation of ATP into the intravesicular space also should have no significant effect on the glycine betaine uptake rate.

At 30°C, glycine betaine is taken up at a high rate only in the presence of a hypertonic solute gradient (Fig. 2). Uptake in the absence of added sodium ion was completely energy dependent, as uptake was not observed when PMS was omitted from assay mixtures (Fig. 1). Interestingly, no downhill efflux of glycine betaine along its concentration gradient was observed, indicating that this carrier, unlike glycine betaine porter I, is not capable of mediating influx in the absence of an energy source. Such a lack of influx is consistent with evidence provided by several groups that hydrolysis of ATP is a required step in substrate transport by ABC-type porters (12).

Uptake was also dependent on the presence of the *gbu* gene product, since in the presence of solute gradients of either sucrose or KCl, uptake was practically nil in fully energized membranes lacking Gbu. This experiment shows that although glycine betaine porter I is clearly present and active in these vesicles, porter II-dependent transport can be studied in isolation under conditions of optimal osmotic activation if Na^+ is withheld. While sucrose appears to be a better osmotic activator of the permease than does KCl, this effect is dependent on assay temperature. The identity of the stressing solute also influences the affinity for glycine betaine (Table 1).

In the presence of a 600 mOsm hypertonic solute gradient, several kinetic parameters of glycine betaine transport are influenced both by the temperature and by the stressing solute (Fig. 3A). For example, the temperature optimum for activation by sucrose occurred at 30°C but maximal activation produced by KCl occurred at 20°C. Arrhenius plots of the data (Fig. 3B) show discontinuities (corresponding to changes in E_a) at about 18°C for both the sucrose and KCl systems. These discontinuities correlate very well with discontinuities in membrane order observed by FTIR spectroscopy at about 18°C in systems containing either sucrose or KCl at 600 mosM. A second region correlating breaks in enzymatic activity and membrane order is apparent at 10°C.

The temperature dependence data suggest that transport is influenced by both the physical state of the membrane and the nature of the stressing solute between 4 and 18°C. The slopes of the activity curves are parallel, and the calculated activation energies are indistinguishable within this range. Above 18°C, the state of the membrane is no longer the dominant influence on the activation of glycine betaine porter II and the species of the stressing solute has a strong influence on reaction velocity, possibly by direct action on the permease. Responses of permeases to changes in lipid fluidity are quite diverse and do not necessarily always correlate positively (1). Clearly, the activity of glycine betaine porter II is not influenced by lipid fluidity within the temperature range of 20 to 30°C.

In the absence of a solute gradient, permease activity is maximal at 4°C and is barely measurable above 15°C (Fig. 5). Elevated transport activity appears to correlate with the discontinuity in membrane phase order observed at 10°C, and activity exhibits a linear positive Arrhenius slope within the decreasing temperature range of 15 to 4°C (Fig. 5, inset). An apparent negative activation energy is typically observed in enzyme-catalyzed reactions at temperatures sufficiently high to denature the enzyme. While, in this case, the permease is not denatured between 4 and 15°C, it is certainly reasonable to ascribe the decrease in activity with increasing temperature to the reverse of an activation process. The mechanism of activation remains obscure, but activation could result from a temperature-dependent change in the lipid composition of the fluid portion of the membrane. As higher-melting-point lipids crystallize out into the gel phase at lower temperatures, the fluid portion of the membrane would become enriched in the lower-melting-point lipid components which might solvate the permease differently than would the complete mixture of lipids in the membrane. The concentration of a specific annular lipid might also be increased to a critical level by this mechanism. A change in the topology of the membrane at lower temperatures is also a possible mechanism of activation. The suggestion that one or more of the protein components of the permease might spontaneously undergo a conformational change seems un-

likely in view of the correspondence between activity and lipid order (Fig. 3B and 4B). Likewise, involvement of additional soluble components seems unlikely.

The chill activation of glycine betaine transport exhibits a maximum at 4°C, rather than continuing to increase, despite the fact that the membrane phase transition reaches completion at about 0°C (by extrapolation of the data in Fig. 4A). Under hyperosmotic conditions, the permease follows the Arrhenius equation, with an activation energy of ~48 kcal/mol over this temperature range when maximally activated by either KCl or sucrose (Table 2 and Fig. 3B). Therefore, the enzyme is expected to catalyze the reaction faster at 4°C than at 1°C, providing that it is in an active state. The decreasing transport rate between 4 and 1°C could simply correspond to normal Arrhenius behavior rather than a decrease in the population of active permease molecules. However, it is also possible that the transport rate decrease in this temperature range results from a decrease in activation. For instance, the system may require a certain concentration of a specific annular lipid, the concentration of which in the liquid-crystalline state may be optimal at 4°C. Alternatively, the effect may be due to the collapse in the energetics of the system (decreased F_0F_1 ATPase function).

Muench et al. (21) have proposed a general model that could produce an apparent negative activation energy in microbiological systems. This model ascribes the effect to a three-state (two-step) process consisting of a membrane lipid phase transition from the liquid-crystal state to the gel state, followed by an irreversible metabolic reaction (involving a membrane-bound enzyme) which is the consequence of that phase transition. The ABC transporters catalyze essentially irreversible vectorial reactions (18) and have not been found to be capable of mediating both uptake and efflux of substrates (12). Unidirectional catalysis by a traffic ATPase would satisfy the second condition for the phenomenon of non-Arrhenius activation.

Activation of glycine betaine porter II is dependent on the presence of a solute gradient or the presence of low temperature, two seemingly different modes of activation. Our results further suggest that the physical state of the membrane determines permease activity. At 4°C, glycine betaine porter II activity is only approximately fivefold higher in the presence of 600 mOsm sucrose than in its absence, suggesting that forces mediated by the two seemingly different activating phenomena acting on the porter are comparable in magnitude. It therefore seems possible, or perhaps even likely, that the porter is acted on by the same or a similar mechanism, a mechanism likely determined by specific interactions with membrane components. We are currently investigating mechanisms by which the physical state of the membrane alters glycine betaine porter II function.

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