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Regulation of the Glucose:H^+ Symporter by Metabolite-Activated ATP-Dependent Phosphorylation of HPr in *Lactobacillus brevis*

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Lactobacillus brevis **takes up glucose and the nonmetabolizable glucose analog 2-deoxyglucose (2DG), as well as lactose and the nonmetabolizable lactose analoge thiomethyl β-galactoside (TMG), via proton symport. Our earlier studies showed that TMG, previously accumulated in *L. brevis* cells via the lactose:H^+ symporter, rapidly effluxes from *L. brevis* cells or vesicles upon addition of glucose and that glucose inhibits further accumulation of TMG. This regulation was shown to be mediated by a metabolite-activated protein kinase that phosphorylates serine 46 in the HPr protein. We have now analyzed the regulation of 2DG uptake and efflux and compared it with that of TMG. Uptake of 2DG was dependent on an energy source, effectively provided by intravesicular ATP or by extravesicular arginine which provides ATP via an ATP-generating system involving the arginine deiminase pathway. 2DG uptake into these vesicles was not inhibited, and preaccumulated 2DG did not efflux from them upon electroporation of fructose 1,6-diphosphate or gluconate 6-phosphate into the vesicles. Intravesicular but not extravesicular wild-type or H15A mutant HPr of *Bacillus subtilis* promoted inhibition (53 and 46%, respectively) of the permease in the presence of these metabolites. Counterflow experiments indicated that inhibition of 2DG uptake is due to the partial uncoupling of proton symport from sugar transport. Intravesicular S46A mutant HPr could not promote regulation of glucose permease activity when electroporated into the vesicles with or without the phosphorylated metabolites, but the S46D mutant protein promoted regulation, even in the absence of a metabolite. The V_{max} but not the K_m values for both TMG and 2DG uptake were affected. Uptake of the natural, metabolizable substrates of the lactose, glucose, mannose, and ribose permeases was inhibited by wild-type HPr in the presence of fructose 1,6-diphosphate or by S46D mutant HPr. These results establish that HPr serine phosphorylation by the ATP-dependent, metabolite-activated HPr kinase regulates glucose and lactose permease activities in *L. brevis* and suggest that other permeases may also be subject to this mode of regulation.**

Many but not all low-GC gram-positive bacteria possess the phosphoenolpyruvate:sugar phosphotransferase system (PTS) that catalyzes the concomitant uptake and phosphorylation of its sugar substrates. The PTS-catalyzed process requires the sequential phosphorylation of four proteins or protein domains, enzyme I, HPr, IIA^{sugar}, and IIB^{sugar}, before sugar phosphorylation and concomitant transport can occur (8, 20). The PTS proteins function in numerous biochemical and genetic regulatory capacities (17–19, 21). In early studies, it was shown that addition of a rapidly metabolizable sugar such as glucose to streptococci, lactococci, or lactobacilli resulted in inhibition of the uptake of other sugars (inducer exclusion) as well as rapid efflux of preaccumulated sugars or sugar metabolites (inducer expulsion). For example, lactose and its nonmetabolizable analog thiomethyl β-galactoside (TMG) were taken up via the PTS in streptococci and lactococci but via H^+ symport in lactobacilli, and uptake in all three organisms was inhibited by glucose (10, 11, 16, 23).

These in vivo studies led to the search for and discovery of an ATP-dependent, metabolite-activated HPr kinase and an HPr(serine)phosphate [HPr(Ser-P)] phosphatase that reversibly phosphorylate and dephosphorylate HPr (1). The kinase was shown to phosphorylate serine 46 in HPr and to be present in a wide variety of low-GC gram-positive bacteria, including some that apparently lack a complete PTS (2, 16; reviewed in reference 13). *Lactobacillus brevis* is one of the bacteria that possesses HPr and the kinase/phosphatase system that reversibly phosphorylates serine 46 in this protein. Although these bacteria were previously thought to lack enzyme I and the various sugar-specific enzyme II complexes (12), recent studies have shown that at least enzyme I, and possibly a fructose-enzyme II complex, is present after anaerobic growth in the presence of fructose (3a). We have recently shown that one function of HPr and its reversible ATP-dependent phosphorylation in *L. brevis* is the regulation of lactose:proton symport, as demonstrated by using vesicles derived from this organism (25).

In this study, we further used *L. brevis* vesicles to test the postulate that HPr plays a direct role in the regulation of the glucose as well as the lactose permease of *L. brevis*. We show that both of these transport systems, and probably others, are subject to inhibition by HPr(Ser-P) by a qualitatively similar process.

MATERIALS AND METHODS

Organisms, growth, and vesicle preparations. Growth conditions and the bacterial strain used, *L. brevis* ATCC 367, were described previously (16). Cells were grown for 18 h at 30°C in the presence of 25 mM galactose, harvested, washed, and used directly for transport experiments or for preparation of membrane vesicles as described by Kaback for *Escherichia coli* (3), with the modifications described previously (25). These vesicles were shown to possess only 12% of the cellular β-galactosidase-specific activity (7) and about 5% of the total cellular HPr, but they retained substantial activities of the membrane-associated HPr kinase and HPr(Ser-P) phosphatase (25).

Uptake measurements. Cells or vesicles were suspended in

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50 mM Tris-maleate buffer (pH 7.0) at a cell density of 1.5 to 2.0 mg (dry weight)/ml or a vesicle density of 2 mg of protein per ml. Various additions as specified in Results or as described below were made to a 100-μl cell or vesicle suspension incubated at 30°C. [^{14}C]TMG (New England Nuclear Corp., Boston, Mass.) was added to a final concentration of 60 μM (58 mCi/mmol) at zero time except where otherwise noted. [^{14}C]2-deoxyglucose ([^{14}C]2DG) was similarly added to a final concentration of 2 mM (20 μCi/μmol) at zero time, except where otherwise noted. The energy source (arginine, 20 mM) was usually added at $t = -1$ min, while glucose or other potential external inhibitors were added at $t = -5$ min. Samples of 10 to 20 μl were removed at appropriate intervals, filtered on 25-mm-diameter membrane filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.), and washed with cold 50 mM Tris-maleate buffer (pH 7.0). Washed filters with cells or vesicles were then transferred to vials containing 5 ml of scintillation fluid for determination of radioactivity. All data reported are the means of three or four experiments. Estimation of the ratio of vesicular [^{14}C]2DG-phosphate to free [^{14}C]2DG was conducted by ion exchange (4).

Preparation of [^{14}C]2DG-loaded vesicles for efflux studies. The preloading medium (100 μl) contained 50 mM Tris-maleate buffer (pH 7.0), 2 mM [^{14}C]2DG (20 μCi/μmol), and 200 μg of *L. brevis* vesicle protein. After an incubation period of 10 min at 30°C, after which time the maximal accumulation level of [^{14}C]2DG in *L. brevis* vesicles had been achieved, the suspension was chilled rapidly to 0°C. When appropriate, vesicles were then subjected to electroporation with designated compounds present as specified below. The vesicles were collected by centrifugation (12,000 × *g*, 1 min), the supernatant fluid was removed by aspiration, and excess liquid was removed with cotton buds. The [^{14}C]2DG-loaded vesicle pellet was resuspended in 100 μl of ice-cold 50 mM Tris-maleate buffer (pH 7.0) by rapid mixing with a Vortex stirrer; 100 μl of this suspension (equivalent to 0.2 mg of vesicle protein) was used for 2DG efflux studies. Aliquots of 10 to 20 μl were periodically removed for determination of intravesicular [^{14}C]2DG as indicated above.

Counterflow studies with *L. brevis* vesicles. Counterflow studies were conducted essentially as described by Romano et al. (16) except that vesicles rather than intact cells were used and the substrate was 2DG. Briefly, two sets of *L. brevis* vesicle preparations (10 mg of vesicle protein per ml) were electroporated with HPr with or without 20 mM fructose-1,6-diphosphate (FDP) and incubated in the presence of 20 mM L-arginine alone for 10 min at 30°C. Nonradioactive 2DG (5 mM, final concentration) was then added to preload the vesicles. After a 10-min incubation period at 30°C, all vesicle suspensions were diluted 20-fold with 50 mM Tris-maleate (pH 7.0) containing 20 mM L-arginine essentially as described previously (25) except that 2 mM [^{14}C]2DG (specific radioactivity of 1.4 μCi/μmol) was added instead of [^{14}C]TMG. Sampling was taken immediately after dilution and at suitable time intervals thereafter. The same procedure was used to examine the effect of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 50 μM) except that HPr and FDP were lacking, and CCCP was added shortly before addition of the [^{14}C]2DG.

Electroporation of HPr and metabolites into vesicles. *Bacillus subtilis* HPr (usually at 100 μM) or one of the mutant proteins, H15A HPr, S46A HPr, or S46D HPr (also at 100 μM) (14), was added to a Gene Pulser cuvette (Bio-Rad, Richmond, Calif.) containing 50 to 100 μl of *L. brevis* vesicles. The mixture was then electroporated twice at 0°C and 700 V for 1.5 ms at a resistance of 200 Ω and a capacitance of 25 μF. It was left on ice for at least 10 but not more than 30 min before the electroporated vesicles were used for 2DG uptake or efflux studies as described above.

Materials and protein assays. Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., and were of the highest purity available. HPr and the various site-specific mutants of this protein were overexpressed and purified to homogeneity, as estimated by the presence of a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and native polyacrylamide gel electrophoresis (5) as described previously (14, 15). Protein concentrations and the protein contents of vesicles prepared from *L. brevis* cells were determined as described by Lowry et al. (6).

RESULTS

Inducer exclusion and expulsion in intact *L. brevis* cells. Figures 1A and C show the effects of glucose on the arginine-energized uptake of both [^{14}C]TMG and [^{14}C]2DG in intact cells of *L. brevis* (16). With arginine as an energy source, TMG and 2DG were rapidly accumulated against substantial concentration gradients; when glucose was present during the uptake experiment, accumulation was blocked (Fig. 1A and C).

When TMG or 2DG was preaccumulated in the cytoplasm with arginine as the energy source and glucose was subsequently added, rapid efflux was observed (Fig. 1B and D). The rate of glucose-induced efflux of 2DG was comparable to that of TMG efflux even though the concentration of 2DG used in this experiment was higher than that of TMG. The results are consistent with the conclusion that glucose metabolites regulate both the glucose and lactose permeases by similar mechanisms.

Regulation of 2DG accumulation in *L. brevis* membrane vesicles. Vesicles prepared from *L. brevis* cells as described in Materials and Methods accumulated 2DG in the presence of arginine to an extent approaching that observed with intact cells (Table 1; Fig. 2A). By using an ion-exchange resin (4), the accumulated radioactivity was shown to be free 2DG to an extent greater than 90%. Less than 10% was 2DG-6-phosphate (data not shown). As shown in Table 1, 2DG accumulation was dependent on intravesicular ATP or extravesicular arginine which provides ATP via the arginine deiminase system. Electroporation either of a metabolite (FDP or gluconate-6-phosphate [gluconate-6-P]) or of HPr into the vesicles did not result in inhibition of 2DG uptake. However, when one of these two metabolites as well as wild-type HPr or the H15A mutant protein was electroporated into the vesicles, appreciable inhibition was observed (Table 1). While FDP was more effective than gluconate-6-P (which was more effective than 2-phosphoglycerate [glycerate-2-P]; see below), wild-type and H15A HPr were about equally effective in promoting metabolite-activated inhibition of 2DG uptake (Table 1). Electroporation of S46A HPr into the vesicles was not effective in promoting 2DG uptake inhibition by intravesicular FDP, but S46D HPr inhibited even in the absence of an intravesicular metabolite (Table 1). These results are qualitatively similar to those reported previously for the lactose:H$^+$ permease of *L. brevis* with TMG as the substrate (25).

Figure 2 presents time courses for 2DG uptake into and efflux from vesicles which had been electroporated in the presence or absence of wild-type *B. subtilis* HPr and/or FDP. In the absence of electroporated HPr, FDP neither inhibited uptake nor stimulated efflux appreciably (Fig. 2A and B). However, with wild-type HPr present in the vesicles, intravesicular FDP strongly inhibited 2DG uptake while stimulating efflux of preaccumulated 2DG (Fig. 2C and D).

Dependency of regulation on serine 46 in HPr. Figure 3

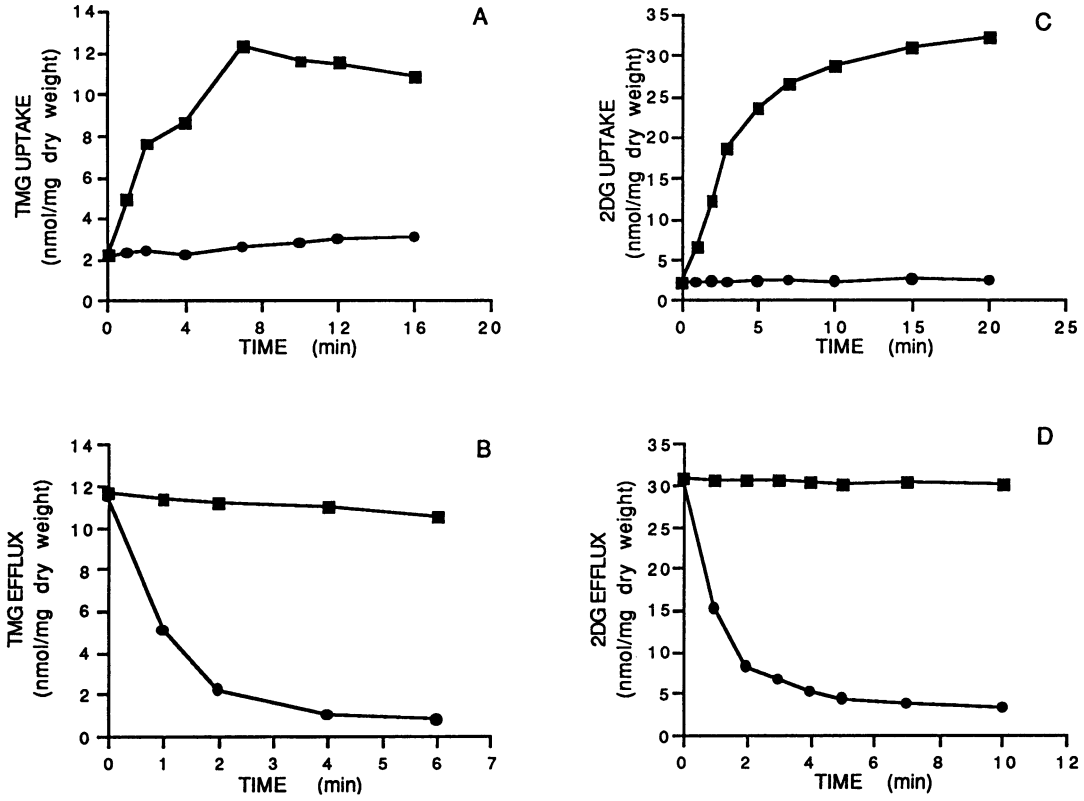

FIG. 1. Time courses for the uptake (A and C) and efflux (B and D) of [^{14}C]TMG (A and B) or [^{14}C]2DG (C and D), using intact galactose-grown *L. brevis* cells. The procedure for measuring ^{14}C-sugar (0.06 mM, 58 μCi/μmol for TMG; 2 mM, 20 μCi/μmol for 2DG) accumulation was as described in Materials and Methods, with 20 mM arginine (added 1 min before the ^{14}C-sugar) providing energy. For the uptake experiments (A and C), glucose was lacking (■) or was added to a final concentration of 20 mM (●) 5 min before the addition of the ^{14}C-sugar. For the efflux experiments (B and D), glucose was lacking (■) or was added at t = 0 to a final concentration of 20 mM (●) following a 10-min preincubation of the cells with the radioactive substrate.

portrays the results of experiments in which intravesicular wild-type *B. subtilis* HPr was replaced with mutant forms of HPr in which the seryl residue target of ATP-dependent kinase-catalyzed phosphorylation was replaced with a neutral alanyl residue (S46A; Fig. 3A and B) or with the negatively charged aspartyl residue (S46D; Fig. 3C and D). In the former case, 2DG uptake occurred to the extent comparable to that observed with the wild-type HPr, and an insignificant degree of inhibition was observed when FDP was electroporated into the vesicles (Fig. 3A). FDP could not promote 2DG expulsion in these vesicles (Fig. 3B). By contrast, when S46D HPr was used, uptake of 2DG was greatly reduced regardless of the presence of FDP (Fig. 2C). Moreover, 2DG-preloaded vesicles containing S46D HPr rapidly lost most of the radioactive sugar, even in the absence of FDP (Fig. 3D). These results are qualitatively similar to those obtained with TMG (25) and are consistent with the observation that the S46D mutation renders HPr conformationally similar to seryl-phosphorylated HPr, as has been revealed by multidimensional nuclear magnetic resonance studies (24). S46D (but not S46A) HPr also behaves like seryl-phosphorylated HPr in in vitro PTS enzyme assays (14, 15).

Metabolites that influence intravesicular 2DG accumulation. Table 2 provides information regarding the metabolites that promote inhibition of 2DG accumulation into vesicles derived from galactose-grown *L. brevis* cells, and the results are compared with those observed when TMG accumulation was

TABLE 1. Regulation of 2DG uptake into *L. brevis* vesicles by HPr, FDP, and gluconate-6-P[a]

Conditions	2-DG (nmol/mg of protein) at 10 min
L. brevis vesicles	24.6 ± 2.2
L. brevis vesicles (no arginine)	4.3 ± 0.3
L. brevis vesicles (no arginine, e/5 mM ATP)	22.1 ± 3.2
Vesicles + 20 mM FDP	24.2 ± 3.4
Vesicles (e/20 mM FDP)	22.6 ± 4.5
Vesicles (e/100 μM HPr)	24.5 ± 2.1
Vesicles (e/100 μM HPr, 20 mM FDP)	11.7 ± 1.8
Vesicles (e/100 μM H15A HPr)	24.1 ± 2.5
Vesicles (e/100 μM H15A, 20 mM FDP)	11.5 ± 2.7
Vesicles + 20 mM gluconate-6-P	24.5 ± 2.3
Vesicles (e/20 mM gluconate-6-P)	24.2 ± 2.5
Vesicles (e/100 μM HPr, 20 mM gluconate-6-P)	13.3 ± 1.1
Vesicles (e/100 μM H15A HPr, 20 mM gluconate-6-P)	13.1 ± 1.0
Vesicles (e/100 μM S46D HPr)	13.5 ± 3.6
Vesicles (e/100 μM S46A HPr)	24.5 ± 2.9
Vesicles (e/100 μM S46A HPr, 20 mM FDP)	24.2 ± 2.8

[a] Values of three experiments were averaged and are reported ± standard deviations. Arginine (20 mM) was added to the vesicle preparation at −1 min unless otherwise indicated; 20 mM FDP or gluconate-6-P and/or 100 μM HPr or one of its derivatives was electroporated into the vesicles (as indicated by e/) or merely added to the extravesicular fluid at −30 s; 2 mM [^{14}C]2DG (specific activity, 20 mCi/mmol) was added to the vesicle suspension at zero time, and samples were removed at t = 10 min for 2DG uptake measurement (see Materials and Methods).

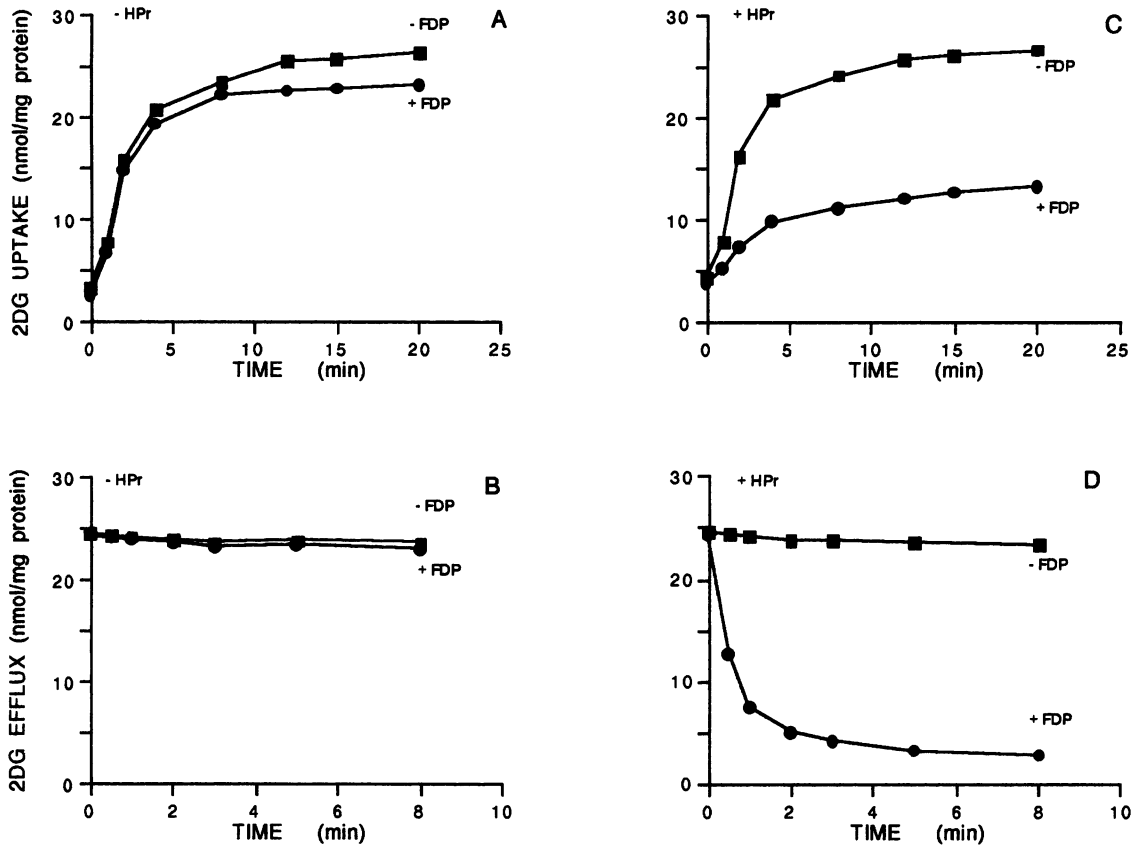

FIG. 2. Time courses for the uptake (A and C) or efflux (B and D) of [^{14}C]2DG, using membrane vesicles of galactose-grown *L. brevis* cells with L-arginine as an energy source. FDP was either present at a concentration of 20 mM (●) or absent (■) during electroporation of the vesicles as described in Materials and Methods. In panels A and B, osmotically shocked vesicles were subjected to electroporation without HPr. In panels C and D, HPr (100 μM) was included during electroporation. The experimental protocol is described in Materials and Methods.

studied. The HPr(Ser) kinase is known to be activated by three metabolites in vitro, FDP, gluconate-6-P, and glycerate-2-P (9). Inhibition of TMG and 2DG accumulation was observed when any one of these three compounds was electroporated into the vesicles. Inhibition of 2DG accumulation was substantially less than the inhibition of TMG accumulation, possibly in part because of the higher sugar concentration used in the 2DG studies (see Fig. 5). 2DG uptake was consistently inhibited most strongly with FDP, less so with gluconate-6-P, and least with glycerate-2-P. Other metabolites, including glucose-6-P, fructose-6-P, and mannose-6-P, were much less effective in inhibiting uptake of radioactive TMG or 2DG. Interestingly, when 25 mM Mg^{2+} was electroporated into the vesicles, about 30% inhibition was observed with respect to both TMG and 2DG uptakes (data not shown). This effect may be due to activation of the HPr(Ser) kinase, which is dependent on Mg^{2+} for activity in vitro (9).

Regulation of the uptake of metabolizable sugars. The studies reported above and previously (25) used nonmetabolizable sugar analogs in order to facilitate interpretation of the results. Table 3 summarizes results obtained under selective conditions when the uptake of several metabolizable as well as nonmetabolizable sugars was studied. It can be seen that intravesicular FDP plus wild-type HPr, or S46D HPr alone, inhibited uptake of both [^{14}C]glucose and [^{14}C]mannose, and inhibition was comparable to that observed for [^{14}C]2DG. Lactose uptake was more strongly inhibited under the same conditions, in agreement with the fact that TMG uptake is more strongly inhibited than is 2DG uptake (Table 3). Finally, examination of [^{14}C]ribose uptake, which occurs in *L. brevis* via an independent, ribose-inducible transport system (unpublished observations), was also inhibited (Table 3). The results suggest that metabolite-inhibited uptake of sugars via at least three permeases, and perhaps many more, is apparently mediated by seryl phosphorylation of HPr.

Evidence that HPr(Ser-P) promotes 2DG:2DG counterflow. The results summarized in Figure 4A provide evidence consistent with the conclusion that HPr(Ser-P) in vesicles results in the partial uncoupling of 2DG transport from H^+ transport. In the experiments shown, vesicles were preloaded with nonradioactive 2DG and then diluted 20-fold into buffer containing [^{14}C]2DG with or without intravesicular FDP. In both experiments, HPr had been electroporated into the vesicles. In both cases, [^{14}C]2DG was rapidly accumulated, but only in the presence of intravesicular HPr and FDP was uptake followed by appreciable efflux. CCCP similarly allowed rapid uptake followed by efflux. In this case, the final basal level of 2DG remaining in the cells at equilibrium (t = >8 min) was lower than that observed in the presence of HPr and FDP (Fig. 4A). The initial rate of [^{14}C]2DG uptake was shown to be independent of intravesicular FDP (Fig. 4B). The behavior observed in the presence of either HPr and FDP or CCCP is characteristic of counterflow and suggests that the permease normally catalyzes obligatory 2DG:H^+ symport, but that HPr(Ser-P) uncouples sugar uptake from H^+ transport. Since appreciable (although slight) accumulation of 2DG, above the level observed

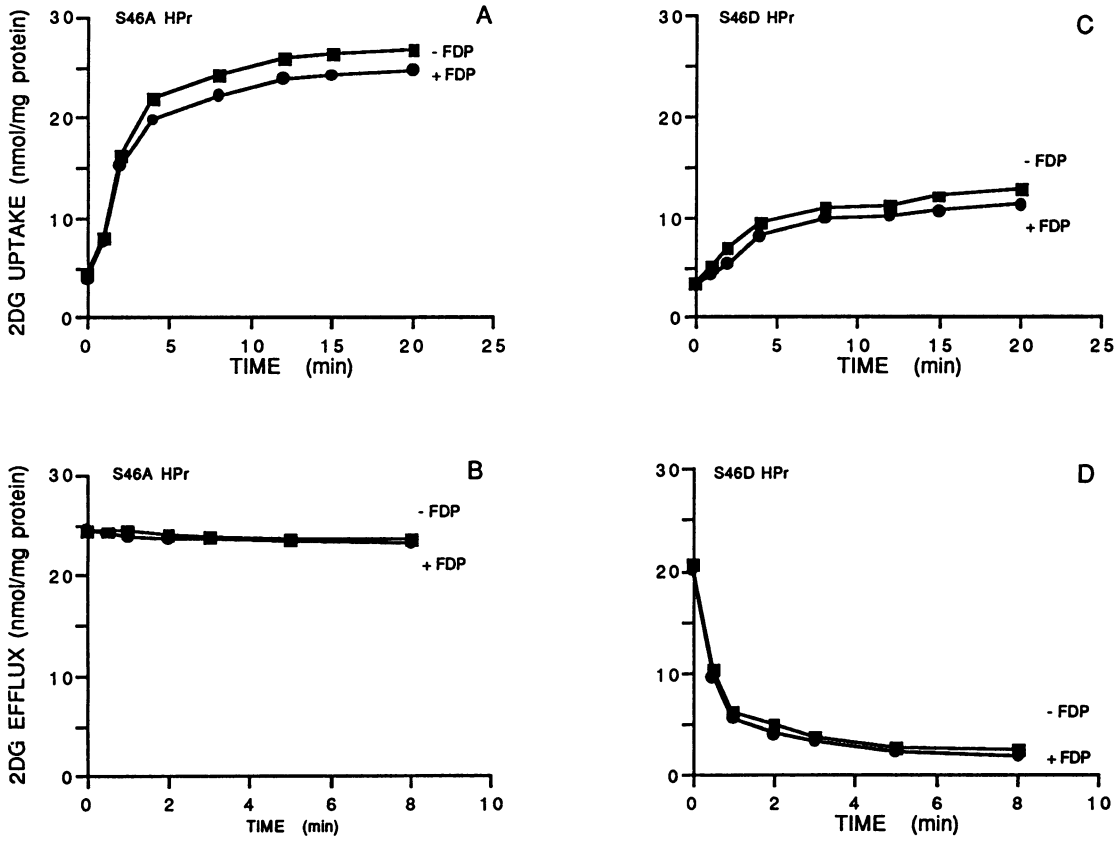

FIG. 3. Effects of intravesicular S46A (A and B) and S46D (C and D) mutant HPr proteins (each at 100 μM during electroporation) on 2DG uptake into (A and C) or efflux from (B and D) *L. brevis* vesicles with (●) or without (■) 20 mM FDP. The experimental protocol was as described in Materials and Methods.

in the presence of excess CCCP, was observed in the presence of HPr and FDP, 2DG and H^+ transport may not be fully uncoupled (see reference 25 for comparison with results obtained for the lactose:H^+ symporter).

The conclusion that 2DG transport is not fully uncoupled from H^+ symport by HPr(Ser-P) under the conditions used in Fig. 4 was supported by studies in which the accumulation level was examined as a function of the external [^{14}C]2DG concentration, in the presence of either excess HPr and FDP or excess CCCP (Fig. 5). Regardless of the sugar concentration used, accumulation of the radioactive sugar was greater in the presence of HPr and FDP than in the presence of CCCP. As expected, the [^{14}C]2DG accumulation ratio observed when the experiment was conducted without versus with either HPr and FDP or CCCP was greatest when the external sugar concentrations were low.

Kinetic analysis of the inhibition of TMG and 2DG uptake. The cell and vesicle systems were used to measure the kinetic parameters for TMG or 2DG uptake under conditions of glucose-promoted or FDP-promoted inhibition, respectively (Fig. 6). In intact *L. brevis* cells, glucose (Fig. 6A) depressed the maximal velocity (V_{max}) of TMG uptake without altering the K_m. When HPr (50 μM) was electroporated into vesicles derived from these same cells and these vesicles were used for TMG uptake measurements with and without glucose, the double-reciprocal plot revealed that glucose again decreased only the V_{max} value without altering the K_m (Fig. 6B). Finally, when 2DG uptake was examined in vesicles containing HPr plus FDP, the intravesicular metabolite also depressed the

TABLE 2. Effects of metabolites on TMG and 2DG uptake into *L. brevis* vesicle[a]

Conditions	Uptake (nmol/mg of protein)	
	TMG	2DG
Control (vesicles)	10.2 ± 1.4	35.0 ± 3.4
Glucose	1.7 ± 0.1	3.1 ± 1.1
Glucose-6-P	8.7 ± 1.0	34.2 ± 5.2
Frucose-6-P	10.5 ± 1.3	33.8 ± 3.7
FDP	1.1 ± 0.3	14.4 ± 2.0
Mannose-6-P	9.5 ± 1.4	32.4 ± 4.2
Ribose-5-P	9.6 ± 1.1	34.2 ± 2.7
Ribulose-5-P	10.2 ± 1.3	34.8 ± 3.2
Gluconate-6-P	8.7 ± 1.0	15.6 ± 3.6
Glycerate-2-P	2.3 ± 0.5	17.2 ± 4.1
Fructose-1-P	10.5 ± 1.3	35.1 ± 2.3
Glucose-1-P	10.3 ± 1.1	34.8 ± 4.2
Glucose-1,6-diP	9.7 ± 2.1	34.7 ± 3.2
Phosphoenolpyruvate	11.1 ± 1.4	34.9 ± 3.7
Pyruvate	10.6 ± 1.2	35.2 ± 1.2
Glycerate-3-P	9.1 ± 2.1	34.3 ± 1.9
Lactate	11.2 ± 1.0	34.4 ± 3.2
Gluconate	10.3 ± 1.4	34.9 ± 4.5

[a] Values of three experiments were averaged and are reported ± standard deviations. Arginine (20 mM) was added at −1 min, and 100 μM HPr and/or 10 mM Mg ATP were electroporated into the vesicles together with the sugar or sugar metabolites indicated; 60 μM [^{14}C]TMG (final concentration; specific activity, 58 mCi/mmol) or 2 mM [^{14}C]2DG (final concentration; specific activity, 20 mCi/mmol) was added to the system at zero time, and samples were removed at t = 10 min for sugar uptake measurement (see Materials and Methods).

TABLE 3. Regulation of sugar uptake into *L. brevis* vesicles[a]

Conditions	^{14}C-sugar uptake (nmol/mg of protein/min)
[^{14}C]2DG	
L. brevis vesicles alone	7.7 ± 0.3
Vesicles (e/100 μM HPr)	7.6 ± 0.1
Vesicle (e/100 μM HPr, 20 mM FDP)	3.8 ± 0.1
Vesicles (e/100 μM S46D)	3.6 ± 0.1
[^{14}C]glucose	
L. brevis vesicles alone	6.1 ± 0.2
Vesicles (e/100 μM HPr)	6.3 ± 0.1
Vesicle (e/100 μM HPr, 20 mM FDP)	3.7 ± 0.2
Vesicles (e/100 μM S46D)	3.8 ± 0.2
[^{14}C]mannose	
L. brevis vesicles alone	1.8 ± 0.3
Vesicles (e/100 μM HPr)	1.9 ± 0.5
Vesicle (e/100 μM HPr, 20 mM FDP)	1.0 ± 0.1
Vesicles (e/100 μM S46D)	0.9 ± 0.2
[^{14}C]TMG	
L. brevis vesicles alone	11.7 ± 1.1
Vesicles (e/100 μM HPr)	10.5 ± 1.4
Vesicle (e/100 μM HPr, 20 mM FDP)	1.1 ± 0.3
Vesicles (e/100 μM S46D)	1.9 ± 0.5
[^{14}C]lactose	
L. brevis vesicles alone	7.8 ± 0.3
Vesicles (e/100 μM HPr)	7.5 ± 0.2
Vesicle (e/100 μM HPr, 20 mM FDP)	2.7 ± 0.6
Vesicles (e/100 μM S46D)	2.6 ± 0.1
[^{14}C]ribose	
L. brevis vesicles alone	2.1 ± 0.6
Vesicles (e/100 μM HPr)	1.9 ± 0.2
Vesicle (e/100 μM HPr, 20 mM FDP)	1.1 ± 0.5
Vesicles (e/100 μM S46D)	1.1 ± 0.3

[a] Conditions were essentially as specified in the footnote to Table 1 except that uptake was for a 1-min time interval at 30°C and that 0.1 mM [^{14}C]glucose (specific activity, 50 mCi/mmol), 1 mM [^{14}C]lactose (specific activity, 0.75 mCi/mmol), 2 mM [^{14}C]2DG (specific activity, 20 mCi/mmol), 60 μM [^{14}C]TMG (specific activity, 58 mCi/mmol), 1 mM [^{14}C]mannose (specific activity, 10 mCi/mmol), or 0.5 mM [^{14}C]ribose (specific activity, 1.2 mCi/mmol) was used as the substrate.

V_{max} value (Fig. 6C). It is relevant that inhibition of carbohydrate uptake via the IIA^{Glc}-mediated regulatory mechanism controlling permease activities in enteric bacteria also depresses the V_{max} value without affecting the K_m (18, 22).

DISCUSSION

In earlier studies, we provided direct evidence that serine phosphorylation of HPr by an ATP-dependent, metabolite-activated protein kinase regulates TMG uptake and efflux via the PTS in *Lactococcus lactis* as well as via the lactose:H$^+$ symporter in *L. brevis* (25, 26). In both cases, inhibition of sugar uptake may be equated to the important physiological phenomenon of inducer exclusion, while stimulation of efflux may be equated to the equally important phenomenon of inducer expulsion (see the introduction). However, the applicability of this regulatory mechanism to other transport systems and its physiological relevance prior to the present report were not known.

In this study, we used vesicles of *L. brevis* to allow examination of the role of HPr serine phosphorylation in the regulation of 2DG:H$^+$ symport, catalyzed by the glucose permease of this organism. In doing so, we have followed up earlier work of Romano et al. (16) showing that in vivo, the glucose permease of this organism probably functions by sugar:H$^+$ symport and that glucokinase activity observed with 2DG as the substrate is negligible following growth of these cells in medium containing galactose as the carbon source. It was not known, however, if this transport system exhibits the phenomenon of metabolite-promoted inducer exclusion or expulsion.

The experiments reported here provide strong evidence for the supposition that metabolite-activated HPr kinase-catalyzed phosphorylation of serine 46 in HPr of *L. brevis* regulates the activity of the glucose:H$^+$ symporter by a mechanism analogous to that previously established for the lactose permease of this organism. The evidence is as follows. (i) Uptake of 2DG is dependent on an energy source (arginine as an ATP-generating system or intravesicular ATP) but not on the small

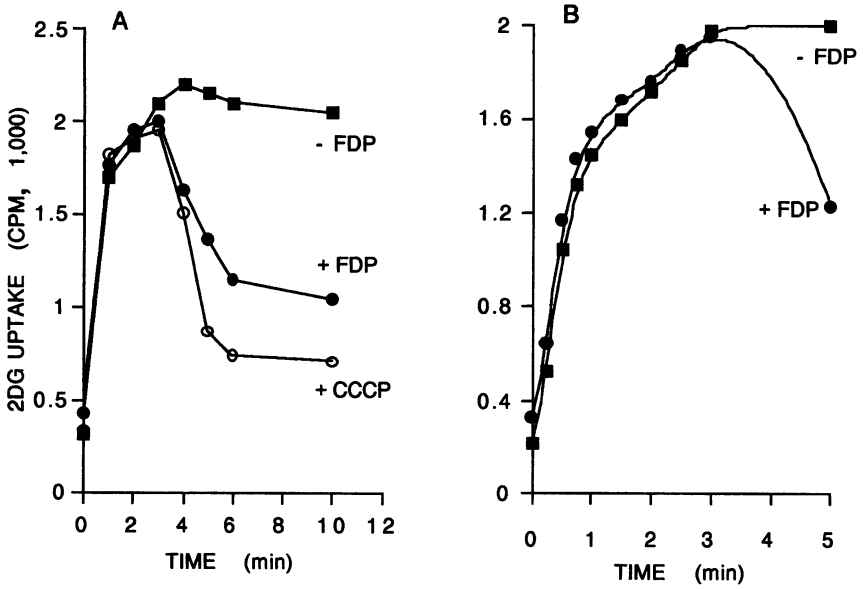

FIG. 4. 2DG counterflow in the presence of intravesicular HPr with or without FDP. (A) The experiments were conducted with vesicles preloaded with a high concentration of nonradioactive 2DG in the presence of 100 μM HPr and in the absence (■) or presence (●) of 20 mM FDP. In the control experiment (○), 50 μM CCCP replaced HPr and FDP. (B) Initial rates of 2DG uptake were measured under the same conditions as in panel A. The experimental protocol was as described in Materials and Methods.

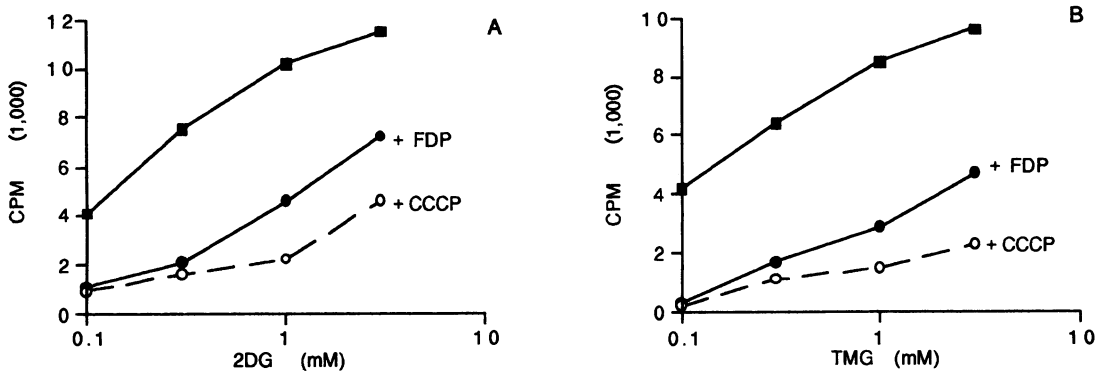

FIG. 5. Comparison of 2DG (A) and TMG (B) accumulation in vesicles incubated with HPr alone (■), HPr plus FDP (●), or HPr plus CCCP (○). Concentrations of these agents were as follows: HPr, 100 μM; FDP, 20 mM; and CCCP, 50 μM. Uptake was measured as a function of radioactive sugar concentration after a 10-min incubation period with the radioactive substrate as outlined in Materials and Methods.

phosphocarrier protein of the PTS, HPr or a metabolite of glucose (e.g., FDP or gluconate-6-P). (ii) Vesicles that have been subjected to osmotic shock, and are largely depleted of HPr, are insensitive to inhibition of 2DG uptake and stimulation of 2DG efflux by glucose metabolites. (iii) Electroporation of wild-type HPr into the vesicles (but not extravesicular HPr) restores sensitivity of the glucose permease to metabolite-promoted regulation. (iv) Intravesicular metabolites of glucose such as FDP, gluconate-6-P, and glycerate-2-P, but not various other metabolites, promote inhibition of 2DG uptake and/or

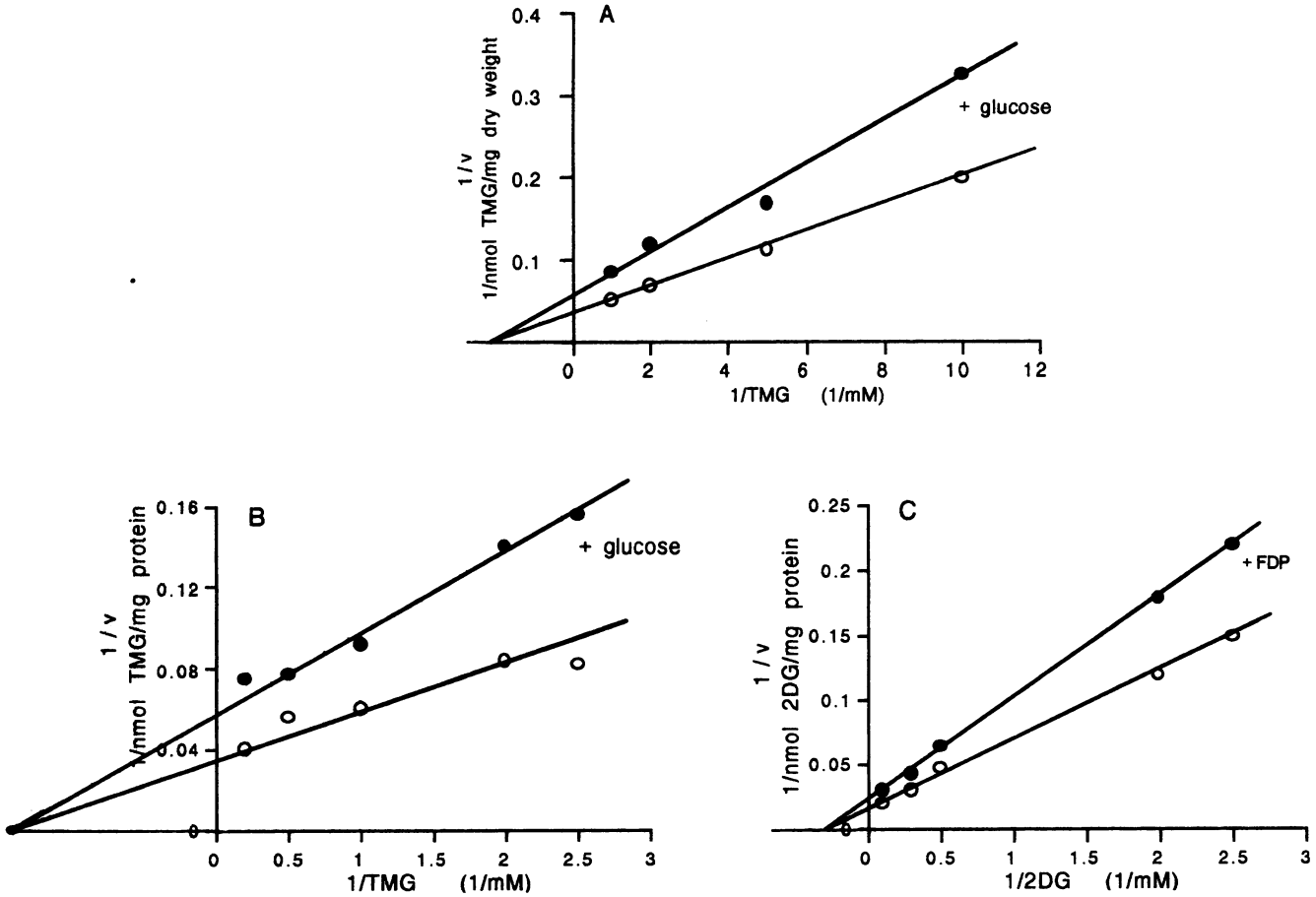

FIG. 6. Kinetic analysis of the glucose-promoted inhibition of TMG uptake into *L. brevis* cells (A) and membrane vesicles (B) and of the FDP-promoted inhibition of 2DG uptake into *L. brevis* vesicles (C). The double-reciprocal plots (Lineweaver-Burk plots) show results obtained when uptake was conducted with cells with (●) or without (○) 2 mM glucose (A) or with vesicles containing wild-type *B. subtilis* HPr (50 μM during electroporation) with (●) or without (○) 2 mM glucose (B) or 5 mM FDP during electroporation (C). Uptake was measured after 10 min.

stimulation of 2DG release. This specificity corresponds to the activation specificity of many gram-positive bacterial HPr kinases, including that from *L. brevis* (9, 12, 13). (v) Replacement of serine 46 with alanine (S46A HPr), a neutral, nonphosphorylatable residue, renders the protein completely inactive as a regulatory molecule. (vi) Replacement of serine 46 with aspartate (S46D HPr), a permanently negatively charged residue resembling phosphorylated serine with regard to its charge, gives rise to a constitutively regulated permease, in agreement with conformational and functional properties of the mutant protein as described previously (14, 15, 24). (vii) Replacement of the active site histidine by alanine (H15A) in HPr has no effect on regulation of the glucose permease, showing that phosphorylation of this residue does not play a role in regulation. (viii) Regulation may convert the vesicular transporter from a strict 2DG:H$^+$ symporter to a partially uncoupled symporter, capable of catalyzing counterflow at an increased rate (16). (ix) Finally, regulation depresses the maximal velocity of 2DG uptake as well as the maximal extent of 2DG accumulation without altering the K_m when 2DG uptake (rather than exchange or counterflow) is studied. The same is true of the lactose:H$^+$ symporter of this organism.

These results, taken together, argue strongly for a direct allosteric mechanism whereby HPr(Ser-P) binds to both the lactose:H$^+$ symporter (25) and the glucose:H$^+$ symporter of *L. brevis*. It is possible that this mechanism is also applicable to other carbohydrate permeases such as the ribose permease of *L. brevis* (Table 3). It is worthy of note that a comparable mechanism occurs in *E. coli*, except that the inhibitory species is the free form of the glucose-specific enzyme IIA of the PTS (18, 22). In this case, the enzyme I-catalyzed phosphorylation of enzyme IIA^{Glc} on histidine prevents binding to the target permeases and consequent inhibition of inducer uptake (21).

Several observations reported in this report are likely to prove to be of physiological importance. First, uptake of metabolizable as well as nonmetabolizable sugars is apparently inhibited by seryl phosphorylation of HPr. Second, relative rates of uptake for different sugars may be differentially regulated by partially uncoupling one system from proton symport more than another system, as suggested by the results presented in Fig. 4 and 5. Third, the fact that glucose uptake is inhibited suggests that the function of HPr(Ser) phosphorylation is not merely to create a hierarchy of preferred sugars with glucose at the top. Metabolite activation of the kinase may serve more generally as a feedback mechanism to control the entry of carbohydrates into the cell, even for the dominant glucose permease. Finally, the fact that ribose uptake into vesicles is also inhibited by HPr(Ser-P) clearly suggests that the mechanism is applicable to permeases other than the only two well-characterized permeases in *L. brevis*, those specific for glucose and lactose (16). Possibly permeases specific for carbon sources other than sugars (i.e., polyols, fatty acids, amino acids, etc.) will also be regulated by this mechanism. Further studies will be required to define the range of permeases that have evolved sensitivity to HPr(Ser-P) inhibition.

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