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Involvement of the Central Loop of the Lactose Permease of *Escherichia coli* in Its Allosteric Regulation by the Glucose-Specific Enzyme IIA of the Phosphoenolpyruvate-Dependent Phosphotransferase System

CHRISTIAN HOISCHEN, JONATHAN LEVIN, SUPOJ PITAKNARONGPHORN, JONATHAN REIZER, AND MILTON H. SAIER, JR.*

Department of Biology, University of California at San Diego, La Jolla, California 92093-0116

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Allosteric regulation of several sugar transport systems such as those specific for lactose, maltose and melibiose in *Escherichia coli* (inducer exclusion) is mediated by the glucose-specific enzyme IIA (IIA^{Glc}) of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). Deletion mutations in the cytoplasmic N and C termini of the lactose permease protein, LacY, and replacement of all cysteine residues in LacY with other residues did not prevent IIA^{Glc}-mediated inhibition of lactose uptake, but several point and insertional mutations in the central cytoplasmic loop of this permease abolished transport regulation and IIA^{Glc} binding. The results substantiate the conclusion that regulation of the lactose permease in *E. coli* by the PTS is mediated by a primary interaction of IIA^{Glc} with the central cytoplasmic loop of the permease.

In *Escherichia coli*, the phosphoenolpyruvate:sugar phosphotransferase system (PTS) regulates the uptake of various non-PTS sugars such as lactose, maltose, melibiose, galactose, raffinose, and glycerol (inhibition of non-PTS inducer uptake by PTS sugars; inducer exclusion) as well as the synthesis of cyclic AMP (catabolite repression) (19, 22, 24, 28, 32). It has been shown that the lactose permease (LacY) is allosterically regulated by the PTS employing a mechanism that involves the direct binding of the glucose-specific enzyme IIA (IIA^{Glc}) to the permease (14–16).

Nelson et al. (14) and Overath et al. (17) described a lactose permease triple mutant defective in its N terminus (carrying a T-to-I mutation at position 7 [T7I], M11I, and G24R) and a partial revertant (T7I and M11I), which were reported to lack IIA^{Glc} binding. These results suggested that the N-terminal 11 residues of LacY might be involved in IIA^{Glc} binding. Wilson et al. (36) sequenced two mutations in the lactose permease that abolished inhibition of lactose uptake by IIA^{Glc}. Both mutants showed single amino acid substitutions in that part of the protein which, according to the topology of LacY described by Calamia and Manoil (3), must be in the central cytoplasmic loop of the permease (S209I and A198V). These observations provided evidence that the central loop of LacY may be involved in IIA^{Glc} binding. However, direct binding experiments were not performed.

The experiments presented here provide both in vivo and in vitro evidence that the binding site in the lactose permease for IIA^{Glc} is localized primarily in its central cytoplasmic loop. Insertional and point mutations in the central loop are shown to abolish regulation and binding, while deletion mutations in the C terminus or the N terminus of the protein have little effect.

IIA^{Glc} binding and lactose uptake experiments were executed with strain T184 [*lacI*$^+$*O*$^+$ *ZYA*$^+$ *rpsL met thr recA hsdM hsdR*/F′ *lacI*^{q}*O*$^+$*Z*^{U118}(*Y*$^+$*A*$^+$)] (31) harboring various plasmids. In the absence of a plasmid, strain T184 lacks β-galactosidase, lactose transport activity, and LacY immuno-cross-reactivity (76). Plasmids pGM21 [*lac*Δ(*I*)*O*$^+$*P*$^+$Δ(*Z*)*Y*$^+$Δ(*A*)Tet^r] (31) and pT7-5 [Amp^r, T7 ϕ10 promoter J] (30) were both used for overproduction of the wild-type lactose permease and its various mutated forms (1, 12, 13). These plasmids are described in Table 1. Strain JLV86 (*nagE crr*) (35) was used for IIA^{Glc} complementation assays.

Transport assays were performed with cells harboring the plasmids described in Table 1. Cells were grown at 37°C to an A_{600} of 0.6 to 0.8 (3 to 4 h) in Luria-Bertani medium, and at various times, synthesis of the lactose permease was induced for 120 min by the addition of isopropyl β-thiogalactoside (IPTG) to a final concentration of 1 mM. These cells express fairly high levels of lactose permease activity in the absence of IPTG because of the presence of multiple copies of the plasmid-expressed *lacY* gene. The glucose PTS was induced by addition of glucose to a final concentration of 0.4% 90 min before the cells were harvested by centrifugation (6,000 × *g* for 10 min at 4°C). After three washes in 100 mM potassium phosphate (pH 7.5) containing 10 mM $MgCl_2$ (KM buffer), cells were resuspended in a minimal volume of the same buffer and stored on ice. Transport assays were performed within 3 h of harvesting.

Transport was initiated by addition of [1-^{14}C]lactose (0.4 mM final concentration; approximately 850 cpm nmol^{-1}) to cells incubated for 10 min (37°C) in KM buffer (final volume, 1 ml; A_{600} = 5 optical density units) and was assayed at 37°C in induced and uninduced cells by modification of a rapid filtration procedure described previously (13, 29). Aliquots (100 μl each) were removed at various intervals and filtered rapidly through nitrocellulose filters (Millipore HA; pore size of 0.45 μm). The filters were immediately washed with two 15-ml aliquots of KM buffer and dried with a heat lamp. Radioactivity on the dry filters was measured by liquid scintillation counting. Experiments were performed simultaneously with and without methyl α-glucoside (αMG) (0.1%) preincubated for 10 min at 37°C. This nonmetabolizable glucose analog causes

* Corresponding author. Phone: (619) 534-4084. Fax: (619) 534-7108. Electronic mail address: msaier@ucsd.edu.

TABLE 1. Plasmids used in this study

Plasmid	Parental plasmid	*lacY* gene or phenotype	Reference
pACYC184			4
pGM21	pACYC	Wild type	31
pGM21/P192A	pGM21	P192A	5
pT7-5			30
pT7-5/lacY	pT7-5	Wild type	
pT7-5/2His	pT7-5	Insertion of two His after S-194	12
pT7-5/6His	pT7-5	Insertion of six His after S-194	12
pT7-5/HisP	pT7-5	Insertion of HisP gene after S-194 (about 800 bp)	7c
pT7-5/KpOAD	pT7-5	Insertion of the biotinylation domain of oxalacetate decarboxylase from *K. pneumoniae* after S-194 (about 300 bp)	7c
pT7-5/S401t	pT7-5	Stop codon for S-401	13
pT7-5/Δ7	pT7-5	Deletion of N-terminal amino acids 2 through 7	2
pT7-5/Δ8	pT7-5	Deletion of N-terminal amino acids 2 through 8	2
pT7-5/C-less	pT7-5	C-less: C117S, C148S, C154V, C176S, C234S, C333S, C353S, C355S	33, 34

dephosphorylation of IIA^{Glc} and consequent binding of IIA^{Glc} to the cytoplasmic surface of the lactose permease in intact cells. Binding and allosteric regulation of the permease can be monitored by measurement of the inhibition of lactose uptake (15, 16, 29).

The overproduction of the wild-type lactose permease or one of its mutant derivatives was carried out essentially as described above with prior induction of *lacY* expression with IPTG, except that synthesis of the glucose enzyme II complex was not induced. Cells were harvested by centrifugation (6,000 × *g* for 10 min at 4°C), washed three times in 20 mM Tris-HCl buffer (pH 7.2) containing 5 mM $MgCl_2$, 3 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (TM buffer) and either stored at −70°C until used or used directly. Intact cells were checked for lactose uptake activity prior to freezing or preparation of membranes. Cells resuspended in a minimal volume of TM buffer were lysed by two passages through an Aminco French pressure cell at 10,000 lb/in^2. To remove cell debris and unbroken cells, the resulting suspensions were centrifuged for 10 min at 10,000 × *g*. Membranes were prepared by centrifugation for 90 min at 100,000 × *g*, rinsed three times in 30 mM Tris-HCl (pH 7.5) containing 3 mM DTT and 0.1 mM PMSF, resuspended in the same buffer, and used for binding assays (15, 16).

Binding experiments (120-μl final volume) were performed at room temperature in 30 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 3 mM DTT, and 0.1 mM PMSF. A 1-mg aliquot of membrane protein, 15 μg of IIA^{Glc} (purified from *Bacillus subtilis* as described and characterized previously with respect to regulation in *E. coli* [21]), and 10 mM melibiose as the substrate of the lactose permease were added as indicated for the figures and tables. These procedures were performed essentially as outlined by Osumi and Saier (16). After a 10-min incubation period at room temperature, the membranes were pelleted by centrifugation at 90,000 × *g* (20 min) in a Beckman Airfuge ultracentrifuge. After the supernatants were discarded, the membranes were rinsed three times with 140 μl of ice cold 20 mM Tris-HCl (pH 7.5) containing 3 mM DTT and 0.1 mM PMSF. Subsequently the membranes were resuspended in 100 μl of 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl, 3 mM DTT, and 0.1 mM PMSF and centrifuged for 20 min at 90,000 × *g*. The supernatants were assayed for the phosphoryl transfer activity of IIA^{Glc}. The amount of IIA^{Glc} specifically bound to the membranes was determined by PTS complementation assays by using a *crr* mutant (strain JLV86). As a control for the determination of nonspecific IIA^{Glc} binding, strain T184 harboring plasmid pACYC184 or pT7-5 (lacking the *lacY* gene) was used.

PTS assays were performed essentially as described previously (23, 25). Assay mixtures (100-μl final volume) contained 50 mM potassium phosphate buffer (pH 7.4), 2.5 mM $MgCl_2$, 25 mM KF, 2.5 mM DTT, 10 μM [^{14}C]glucose (5 μCi μmol^{-1}), and 5 mM phosphoenolpyruvate. IIA^{Glc} activity was determined by complementation assays in the presence of crude extracts of the *crr* mutant strain JLV86 (38 μg of protein per assay). In each binding or control experiment, the amount of membrane-bound IIA^{Glc} was determined by employing calibration curves obtained with standard IIA^{Glc} solutions. Each sample was assayed in duplicate, and the two values obtained (the variation was usually within 20%) were averaged. Reactions were initiated by addition of IIA^{Glc} and were terminated after 20 min (37°C) by rapid dilution in 1 ml of ice-cold water. [^{14}C]glucose-phosphate was separated from the free sugar by ion-exchange chromatography (Bio-Rad AG1-X2 anion-exchange resin [analytical grade, 50/100 mesh, chloride form]), and the amount was determined by liquid scintillation counting as described previously (9).

Protein was measured by the method of Bradford (2). [U-^{14}C]glucose was from ICN Radiochemicals (Irvine, Calif.). [1-^{14}C]lactose was from Amersham, Arlington Heights, Ill. Sugars and other chemicals, obtained from Sigma Chemical Co. (St. Louis, Mo.) or Boehringer (Mannheim, Germany), were of analytical grade or the highest purity available.

Representative time courses for [^{14}C]lactose uptake in IPTG-induced and uninduced cells are shown in Fig. 1A and B, respectively. Induction with IPTG enhanced the rate and extent of [^{14}C]lactose uptake in these *lacZ* deletion-containing cells approximately threefold. In cells bearing the plasmid-encoded wild-type *lacY* gene, 2-fold inhibition by αMG was observed under inducing conditions but 4.5-fold inhibition was observed under noninducing conditions. Diminished inhibition under conditions of induction is due to the desensitization phenomenon described previously (26). The *lacY* mutant 2His (Table 1) exhibited normal rates of lactose uptake (12) but was insensitive to inhibition by αMG regardless of whether the cells were induced (Fig. 1). These results establish that a small insertion in the central loop of the lactose permease between residues S-194 and A-195 abolishes transport regulation.

Table 2 summarizes corresponding data for a number of different *lacY* mutants as listed in Table 1. As can be seen, the N-terminal-deletion mutants Δ7 and Δ8 exhibited reduced lactose uptake activities but normal sensitivities to PTS-mediated

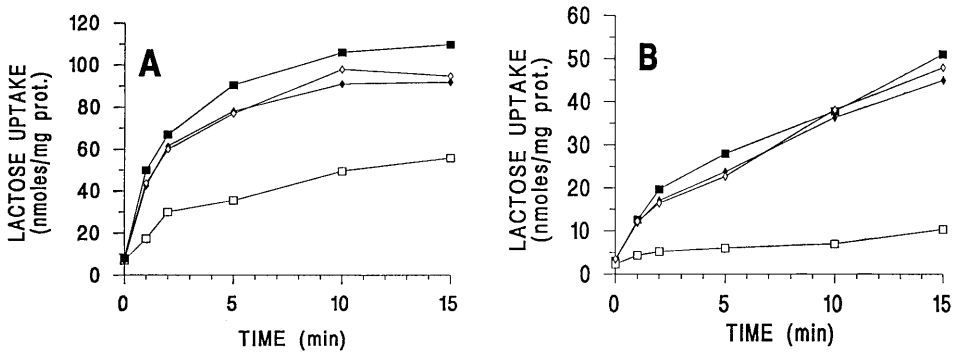

FIG. 1. Regulation of lactose uptake by the PTS in LacY-induced cells (A) and in uninduced cells (B). Uptake rates are compared for the wild-type permease (squares) and a mutant with a 2H insertional mutation of the central loop (diamonds) (Table 1). Uptake in the presence of αMG (0.1%) is indicated by open symbols, while that in the absence of the glucose analog is indicated by closed symbols.

inhibition. The cysteine-less (C-less) mutant exhibited somewhat reduced transport activity and somewhat reduced sensitivity to PTS-mediated inhibition (38% compared with 54% for induced cells; 43% compared with 77% for uninduced cells). The central loop mutation P192A resulted in almost complete abolition of transport regulation without substantially reducing lactose uptake. Various insertional mutations between residues S-194 and A-195 all abolished PTS-mediated regulation while reducing lactose uptake to various degrees (Table 2). Finally, the S401t mutation, which caused early C-terminal termination (at position 401), was without effect on lactose uptake but reduced PTS-mediated regulation significantly.

Table 3 presents representative data on the binding of IIA^{Glc} to the lactose permease obtained by using the assay of Osumi and Saier (15, 16). IIA^{Glc} binding to membranes expressing high LacY levels was greatly stimulated by the presence of a lactose permease substrate such as melibiose, as reported previously (15, 16). By contrast, each of the central loop insertional mutations essentially abolished IIA^{Glc} binding (compare values obtained for these mutants with those obtained for the strain which lacks the lactose permease altogether). It is therefore probable that loss of PTS-mediated transport regulation in these mutants (Table 2) resulted from decreased affinity of the mutant permease proteins for the IIA^{Glc} protein (Table 3).

The results presented above show that deletion of N-terminal residues 2 through 8 in LacY had no effect on the potency of IIA^{Glc} regulation. This fact was not expected since Nelson et al. (14) and Overath et al. (17) have reported that an N-terminal triple mutant (T7I, M11I, and G24R) as well as an R24G revertant with mutations only at positions 7 and 11 abolished IIA^{Glc} binding. It is possible that the mutationally altered N-terminal peptide can interact with and block IIA^{Glc} binding even though the N-terminal deletion is without effect.

Deletion of the C-terminal 17 residues, which include almost all of the hydrophilic tail of LacY following the putative transmembrane spanner 12 (Fig. 2), as a result of the S401t mutation, diminished, but did not abolish, IIA^{Glc}-mediated inhibition of lactose permease activity. In this regard it is of interest that site-specific mutations in the melibiose permease of *E. coli* (MelB) that abolished IIA^{Glc}-mediated inhibition of this permease were localized in the C-terminal tail (10). In view of this fact, it is worth noting that LacY and MelB are not demonstrably homologous (11, 18, 20). Furthermore, the consensus sequence for the LacY, MalK, and RafB proteins could not be found in MelB (32) and is present in GlpK only to a limited extent (unpublished observations). It is therefore possible that the melibiose permease (and glycerol kinase) binds IIA^{Glc} in a fashion quite different from that observed for the other mentioned proteins which do exhibit this consensus sequence. The

TABLE 2. Allosteric inhibition of the activities of the wild type and mutant derivatives of the lactose permease by IIA^{Glc} in vivo

Cell phenotype[a]	Lactose uptake in uninduced cells[b]		Inhibition (%)	Lactose uptake in induced cells[b]		Inhibition (%)
	−αMG	+αMG (0.1%)		−αMG	+αMG (0.1%)	
Wild type	20	4.5	77	67	31	54
$LacY^-$	1.1	1.0	9	1.2	1.2	0
Δ7	—[c]	—	—	24	11	54
Δ8	—	—	—	9	4.5	50
C-less	16	9	43	42	26	38
P192A	21	18	13	51	43	16
2H	17	16	6	61	60	2
6H	9	9	0	41	40	2
KpOAD	20	19	5	56	57	0
$HisP^+$	5.4	5	7	49	51	0
S401t	27	17	37	61	38	38

[a] Cells harbored the plasmid-encoded wild-type lactose permease, one of its mutant forms, or the plasmid without a *lacY* gene insert ($LacY^-$). Mutations are defined in the text, Table 1, and the legend to Fig. 2.
[b] Nanomoles per milligram of protein after 2 min of uptake.
[c] —, not determined.

TABLE 3. Binding of IIA^{Glc} to the wild type and representative mutant derivatives of the lactose permease in the presence and absence of substrate

LacY form[a]	Binding of IIA^{Glc} (μg/mg of membrane protein)[b]	
	+Melibiose (10 mM)	−Melibiose
Wild type	2.3 (0.15)	1.1 (0.1)
$LacY^-$	0.9 (0.2)	0.9 (0.15)
2His	1.0 (0.2)	1.0 (0.15)
6His	1.3 (0.15)	1.0 (0.2)
KpOAD	1.0 (0.15)	0.9 (0.25)
HisP	0.9 (0.2)	0.8 (0.1)

[a] The mutation resulting from the form of the *lacY* gene present on the plasmid is indicated as in Table 2.
[b] Values are the averages of three or four experiments performed with different membrane preparations. Standard deviations are in parentheses.

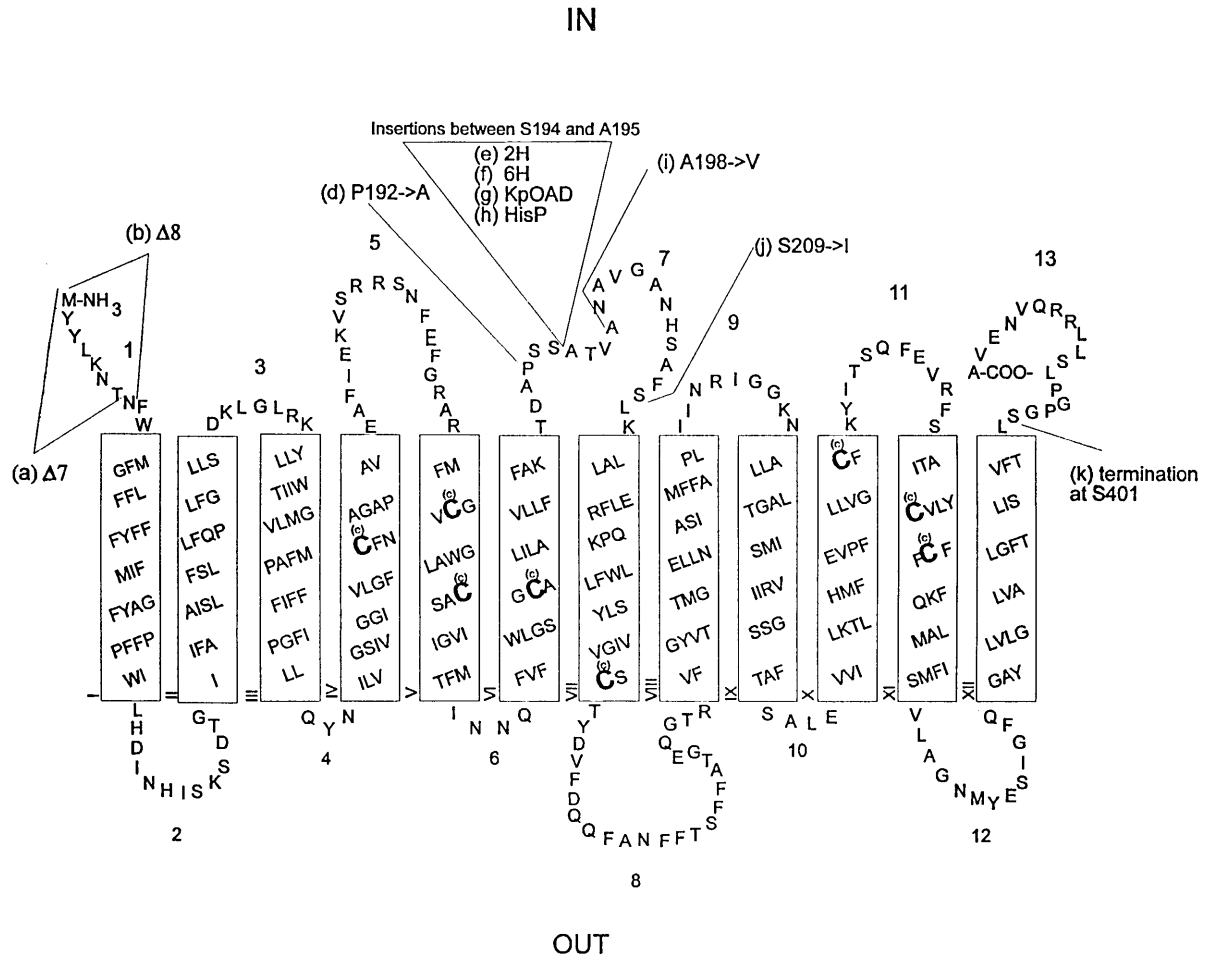

FIG. 2. Secondary structural model of the lactose permease (7). Mutations examined in this study and in other related studies which favor the central loop as the site of interaction with IIA^{Glc} are indicated by lowercase letters (Table 1). Δ7 and Δ8, deletion of N-terminal amino acids 2 through 7 and 2 through 8; **C**, replacement of all cysteines; 2H and 6H, insertion of two and 6 histidines between residue S-194 and A-195; KpOAD, insertion of the *Klebsiella pneumoniae* oxaloacetate decarboxylase (~300 bp) between S-194 and A-195; HisP, insertion of HisP (~800 bp) between S-194 and A-195.

fact that the C-terminal deletion mutation S401t in LacY reduces regulation of LacY by the PTS suggests a potential, secondary role for this region. It is possible that the melibiose and lactose permeases both use their central loops and their C-terminal hydrophilic tails to bind IIA^{Glc} but that the former region of LacY and the latter region of MelB are of greater importance for binding.

A series of insertional mutations of various sizes at a single position (between residues S-194 and A-195) in the central loop of LacY abolished both IIA^{Glc}-mediated regulation of lactose uptake in vivo and IIA^{Glc} binding to LacY in vitro (Tables 2 and 3). Even the insertion of just two histidyl residues (the smallest of the insertions studied) abolished this allosteric interaction. Moreover, the nearby point mutation P192A similarly abolished LacY transport regulation by the PTS. These results clearly point to the central loop of LacY as the primary binding site for IIA^{Glc}. They are in full agreement with the results and conclusions of Wilson et al. (36).

Our results also show that the C-less mutant of LacY, in which all cysteyl residues are replaced with serine (and in one case with valine) (33, 34), retains partial PTS-mediated regulation. This observation shows that these cysteyl residues do not play an important or specific role in IIA^{Glc} binding or in mediation of the functionally significant conformational changes that presumably accompany binding of IIA^{Glc} to LacY. The transmembrane signaling noted previously (15, 16, 27) is evidently not strongly dependent on the acid-base or oxidation-reduction properties of cysteine. It is worth noting, however, that the C-less LacY protein was somewhat less sensitive to IIA^{Glc}-mediated regulation than the wild-type protein (Table 2).

In addition to published reports (6, 8, 36), the work reported here supports the notion of a bona fide consensus sequence for IIA^{Glc} binding (32). Screening of the protein sequence database by using this sequence revealed two potentially IIA^{Glc}-regulated proteins, the raffinose permease (RafB) and arabinose isomerase (AraA), both of *E. coli*. Examination of these two proteins for allosteric regulation by IIA^{Glc} in in vivo and in vitro experiments, respectively, revealed inhibition of the activities of both proteins (references 32 and 7a, respectively). These results substantiate the utility of the consensus sequence for the identification of novel transport proteins and enzymes which may be subject to PTS-mediated regulation.

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