

Quantification of the Regulation of Glycerol and Maltose Metabolism by IIA^{Glc} of the Phosphoenolpyruvate-Dependent Glucose Phosphotransferase System in *Salmonella typhimurium*

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The amount of IIA^{Glc}, one of the proteins of the phosphoenolpyruvate:glucose phosphotransferase system (PTS), was modulated over a broad range with the help of inducible expression plasmids in *Salmonella typhimurium*. The in vivo effects of different levels of IIA^{Glc} on glycerol and maltose metabolism were studied. The inhibition of glycerol uptake, by the addition of a PTS sugar, was sigmoidally related to the amount of IIA^{Glc}. For complete inhibition of glycerol uptake, a minimal ratio of about 3.6 mol of IIA^{Glc} to 1 mol of glycerol kinase (tetramer) was required. Varying the level of IIA^{Glc} (from 0 to 1,000% of the wild-type level) did not affect the growth rate on glycerol, the rate of glycerol uptake, or the synthesis of glycerol kinase. In contrast, the growth rate on maltose, the rate of maltose uptake, and the synthesis of the maltose-binding protein increased two- to fivefold with increasing levels of IIA^{Glc}. In the presence of cyclic AMP, the maximal levels were obtained at all IIA^{Glc} concentrations. The synthesis of the MalK protein, the target of IIA^{Glc}, was not affected by varying the levels of IIA^{Glc}. The inhibition of maltose uptake was sigmoidally related to the amount of IIA^{Glc}. For complete inhibition of maltose uptake by a PTS sugar, a ratio of about 18 mol of IIA^{Glc} to 1 mol of MalK protein (taken as a dimer) was required.

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) in bacteria is responsible for the uptake and concomitant phosphorylation of PTS carbohydrates (e.g., glucose, mannitol, and mannose) (for reviews, see references 20 and 35). The phosphoryl group originates from PEP and is transferred via the general cytoplasmic PTS proteins, enzyme I and HPr, to the carbohydrate-specific membrane-bound enzyme II. Enzyme II consists of three domains, A, B, and C. In some cases, the IIA domain exists as a cytoplasmic protein (e.g., IIA^{Glc} of enteric bacteria, formerly called enzyme III^{Glc} [41]). The enzymes II catalyze the uptake and concomitant phosphorylation of the PTS carbohydrates (20, 35) (see Fig. 1).

Apart from its role in the uptake and phosphorylation of PTS carbohydrates, the PTS in enteric bacteria also regulates the transport and metabolism of a number of non-PTS carbohydrates. IIA^{Glc} plays a central role in this process, and its phosphorylation state is crucial in PTS-mediated regulation of metabolism (for reviews, see references 20, 35, and 38). The unphosphorylated form of IIA^{Glc} inhibits the uptake and metabolism of several non-PTS carbohydrates (lactose, melibiose, maltose, and glycerol) by interacting with the uptake systems for these carbohydrates, a process called inducer exclusion (6, 7, 21, 27–29, 33, 34, 42). As a consequence of the binding of IIA^{Glc}, the target proteins (e.g., lactose permease, melibiose permease, MalK protein [part of the maltose transport system], and glycerol kinase) cannot function any more in the uptake or metabolism of their substrates, which are also the inducers of their respective operons. Upon the addition of a PTS carbohydrate, the PTS proteins, including IIA^{Glc}, will be dephosphorylated and inducer exclusion can occur. In addition

to the phosphorylation state of IIA^{Glc}, the number of target molecules is important for inducer exclusion to occur (IIA^{Glc} itself is expressed at a constant level [46]). In this context, it is important to note that the interaction between unphosphorylated IIA^{Glc} and its target proteins takes place only in the presence of their substrates (27, 29, 34, 40).

A second mode of regulation by the PTS is at the level of gene expression. Phosphorylated IIA^{Glc} is thought to activate adenylate cyclase and thus to influence the cyclic AMP (cAMP) level and consequently the transcription of a large number of genes, including genes encoding catabolic enzymes (for reviews, see references 2 and 30). The addition of a PTS carbohydrate will result in dephosphorylation of phospho-IIA^{Glc} and in a lowering of the intracellular cAMP level. A model of the functioning of the (glucose) PTS in carbohydrate uptake and regulation of metabolism is summarized in Fig. 1 (for reviews, see references 20, 35, and 38).

Although the model described above is generally accepted, it is mainly based on qualitative experiments. Our aim is to quantify the model with respect to uptake of PTS carbohydrates and PTS-mediated regulation of metabolism of non-PTS carbohydrates in order to be able to predict more exactly the behavior of cells under a certain set of environmental conditions.

In order to quantify the role of IIA^{Glc} in PTS-mediated regulation of metabolism, it is necessary to be able to modulate the amount of IIA^{Glc} in vivo. In this report, we describe the construction of plasmids for the controlled synthesis of IIA^{Glc} and their use in studying the in vivo effects of different levels of IIA^{Glc} on the regulation of glycerol and maltose metabolism in *Salmonella typhimurium* by PTS carbohydrates.

MATERIALS AND METHODS

Bacterial strains. The following *S. typhimurium* strains have been used: wild-type strain SB3507 (*trpB223*), SB1690 (*ptsI34*)

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subcloned by ligating the 1.6-kbp *EcoRI* fragment from pT-SHIC9 with plasmid pJFHE118 (5.3 kbp) (12) digested with *EcoRI*. The ligation mixture was first transformed into *E. coli* JA221. Transformants were selected on LB agar plates supplemented with ampicillin (50 µg/ml). Plasmid DNA was isolated from a JA221 transformant and retransformed into *E. coli* LM1 (*crr ptsM*). Transformants that were able to ferment glucose on eosine methylene blue plates containing glucose (1%), ampicillin (50 µg/ml), and IPTG (100 µM) were selected. This procedure resulted in plasmid pBCP206 (6.9 kbp) which contains the *crr* gene under control of the *tac* promoter (Fig. 2B). The 1.6-kbp *EcoRI* fragment does not contain the promoter of the *crr* gene, as described by De Reuse and Danchin (8). Plasmid pSU18, provided by F. de la Cruz (Universidad de Cantabria, Santander, Spain), was used to construct an intermediate-copy-number vector (p15A replicon, about 15 copies per chromosome). Plasmid pSU18 is similar to plasmid pSU2718 (19), except that the *EcoRI* site, located in the *cat* gene, and the *AccI* site, located near the p15A replicon in pSU2718, are not present in pSU18 (7a). The origin of replication and half of the *bla* gene of pBCP206 were removed by digesting the plasmid with *PvuI* and *NruI*. The ends of the resulting 4.1-kbp *PvuI-NruI* fragment were made blunt, using T4 DNA polymerase, and the fragment was subsequently ligated to pSU18 (2.3 kbp) digested with *SmaI*. Selection of transformants was done as described for plasmid pBCP206, with the exception that chloramphenicol (34 µg/ml) was used. The resulting plasmid is called pBCP208 (6.4 kbp) (Fig. 2C).

Preparation of cell extracts, enzyme assays, and transport studies. Cells were ruptured by passage through an Aminco French pressure cell at 1,100 kg/cm². Crude cell extracts, prepared as described previously (32), were centrifuged at 230,000 × *g* (2 h, 4°C). Enzyme IICB^{Glc} activity was determined in the membrane fraction as PEP-dependent phosphorylation of methyl α-glucopyranoside (αMG), as described by Roseman et al. (37), with the high-speed supernatant from ZSC112 (*ptsG ptsM*) as a source of the soluble PTS proteins. Enzyme I activity was determined in the high-speed supernatant as described by Roseman et al. (37), with the high-speed supernatant and the membrane fraction from SB1690 (*ptsI*) as sources of HPr and IIA^{Glc} and of enzyme IICB^{Glc}, respectively. The relative HPr activity was determined in the high-speed supernatant by using the high-speed supernatant and the membrane fraction from SB2226 (*ptsH*) as sources of enzymes I and IIA^{Glc} and enzyme IICB^{Glc}, respectively. The relative IIA^{Glc} activity was determined in a similar way, with LM1 (*crr ptsM*) as a source of enzyme I, HPr, and enzyme IICB^{Glc}. The high-speed supernatants and membrane fractions from SB2226 and LM1, used in the *in vitro* PTS assays, all originated from a single batch. When the HPr or IIA^{Glc} concentration in the supernatant was too high, samples were diluted until HPr and IIA^{Glc} activities were in the linear range of the assay system (37). Transport of labelled sugars (final concentration of 0.5 mM [U-¹⁴C]glycerol or [U-¹⁴C]maltose) at room temperature was done as described by Postma (32).

Source of purified proteins and antibodies. Purified IIA^{Glc} and maltose-binding protein (MBP) and antisera raised against these proteins were available from earlier studies (25, 46). Purified MalK protein and an antiserum raised against the MalK protein were kindly provided by E. Schneider (University of Osnabrück, Osnabrück, Germany) (52). Pure *E. coli* glycerol kinase (EC 2.7.1.30) was prepared by loading 3 mg of commercially available glycerol kinase (83% pure; Fluka Chemie AG, Buchs, Switzerland) on a sodium dodecyl sulfate (SDS)-15% polyacrylamide gel. After electrophoresis, the gel was stained for 10 min in a 0.05% bromophenol blue solution

and briefly destained in demineralized water. The 55-kDa band (glycerol kinase monomer) was cut out of the gel and potted in a 25-ml potter tube with 10 ml of demineralized water. The resulting slurry was incubated overnight at 4°C and potted again. The acrylamide particles were spun down, and the supernatant was lyophilized. The protein was dissolved in 1.5 ml of a 0.9% NaCl solution. As judged from SDS-polyacrylamide gel electrophoresis and rocket immunoelectrophoresis, this procedure resulted in a completely pure preparation of glycerol kinase, with a recovery of 90%. An antiserum against glycerol kinase was obtained by injecting a rabbit with portions of 100 µg of the pure glycerol kinase, as described previously for IIA^{Glc} (46).

Immunological methods. IIA^{Glc}, glycerol kinase, and MBP were quantified by rocket immunoelectrophoresis performed as described previously (1, 46). To determine the amount of glycerol kinase, cell extracts were prepared in a 50 mM bicine buffer (pH 8.5) supplemented with 10 mM MgCl₂, 1 mM glycerol, and 2.5 mM D,L-dithiothreitol. Rocket immunoelectrophoresis of glycerol kinase was performed with 5% (vol/vol) antiserum. IIA^{Glc} and MBP were determined by using 1% (vol/vol) antiserum. In all cases the purified proteins were used as standards. The MalK protein was quantified by means of an enzyme-linked immunosorbent assay (ELISA) in cell extracts prepared in a 50 mM Tris-HCl buffer (pH 7.5) supplemented with 50 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 20% (vol/vol) glycerol. Wells of a microtiter plate (Maxisorp-F96; Nunc, Roskilde, Denmark) were coated with 100 µl of several serial dilutions of MalK-containing cell extracts by incubation for 1 h at 37°C. After the wells were rinsed two times with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ [pH 7.2]), 200 µl of a 20-mg/ml bovine serum albumin solution (in PBS) was added to each well, and the plates were subsequently incubated for 1 h at 37°C. The wells were rinsed two times with PBS supplemented with 0.1% [wt/vol] Tween 20 (PBST), and 150 µl of an appropriate dilution (in PBST) of an anti-MalK antiserum was added to each well. After incubation for 1 h at 37°C, the wells were washed three times with PBST, and 200 µl of a 1:1,000 dilution (in PBST) of goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Bio-Rad, Richmond, Calif.) was added to each well. After an incubation of 1 h at 37°C, the wells were rinsed four times with PBST. Subsequently, 200 µl of a color reagent (100 mM sodium/potassium phosphate buffer [pH 6] supplemented with 2 mg of *o*-phenylenediamine per ml and 0.015% H₂O₂) was added to each well, and the plates were incubated for 5 to 10 min at room temperature in the dark. The reaction was stopped by adding 50 µl of 2 N H₂SO₄ to each well, and A₄₉₂ was measured with a Titertec Multiscan Plus (EFLABoy, Helsinki, Finland). Purified MalK protein was used as a standard.

Protein and dry weight determinations. The amount of protein was determined as described by Peterson (31), with bovine serum albumin as a standard. Dry weights were determined as described previously (13).

Chemicals. [U-¹⁴C]αMG (5.68 GBq/mmol) and [U-¹⁴C]maltose (20 GBq/mmol) were obtained from Amersham International (Amersham, England); [U-¹⁴C]glycerol (5.7 GBq/mmol) was obtained from NEN Research Products, Du Pont (Boston, Mass.); PEP was obtained from Sigma Chemical Co. (St. Louis, Mo.); IPTG, restriction enzymes, and other DNA modification enzymes were obtained from Pharmacia (Uppsala, Sweden); and cAMP was obtained from Serva (Heidelberg, Germany).

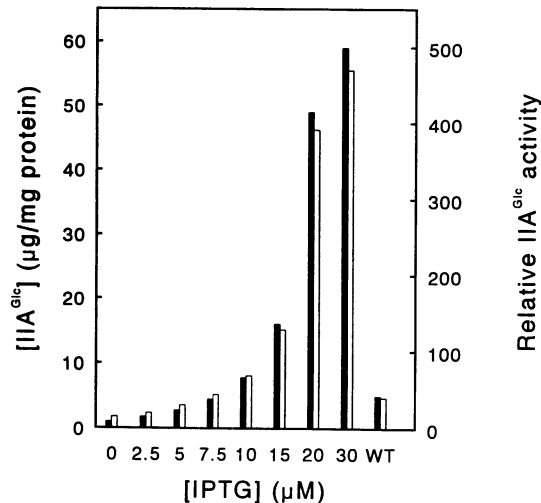


FIG. 3. Synthesis and relative activity of IIA^{Glc} as a function of the IPTG concentration by using plasmid pBCP206. PP2005/pBCP206 was grown as described in Materials and Methods with the indicated concentrations of IPTG. The relative activity of IIA^{Glc} (open bars) was measured by the PEP-dependent phosphorylation of 10 mM [U-¹⁴C]αMG (specific activity, 200 dpm/nmol); a value of 40 corresponds to an activity of 40 nmol/min/mg of protein as determined in the wild-type strain under experimental conditions (see Materials and Methods). The amount of IIA^{Glc} (closed bars) was determined by rocket immunoelectrophoresis as described in Materials and Methods. The wild-type (WT) levels and relative activity of IIA^{Glc}, determined in SB3507/pJFHE118, are indicated.

RESULTS

Controlled expression of IIA^{Glc}. As mentioned in the Introduction, the quantification of the role of IIA^{Glc} in inducer exclusion and stimulation of adenylate cyclase requires the modulated expression of IIA^{Glc}. We have constructed an expression plasmid that allows the controlled synthesis of IIA^{Glc} and studied the in vivo effects of different levels of IIA^{Glc} on glycerol and maltose metabolism in an *S. typhimurium* *crr* strain completely lacking chromosomally encoded IIA^{Glc}.

In plasmid pBCP206, the promoter of the *crr* gene was replaced by the IPTG-inducible *tac* promoter. Expression of IIA^{Glc} from this plasmid was tested in *S. typhimurium* PP2005 (*crr::Tn10*) grown in the presence of various concentrations of IPTG. The amount of IIA^{Glc}, as measured with an antiserum against IIA^{Glc}, could be varied from about 20% (no IPTG present) to more than 1,000% (30 μM IPTG present) of the wild-type level, with increasing concentrations of IPTG in the culture medium (Fig. 3). The relative activity of IIA^{Glc}, determined as PEP-dependent phosphorylation of αMG, increased from about 35% of the wild-type level with no IPTG present to more than 1,000% of the wild-type level in the presence of 30 μM IPTG (Fig. 3). The synthesis of IIA^{Glc} in the absence of IPTG is most likely due to readthrough of the *tac* promoter. The discrepancy between the amount and the activity of IIA^{Glc} expressed from pBCP206, noticeable only at very low IPTG concentrations, is due to membrane-bound IIA^{Glc}-like activity still present in *S. typhimurium* *crr::Tn10* strains that completely lack cytoplasmic IIA^{Glc} (25, 51).

Since the amount of IIA^{Glc} expressed from pBCP206 in the absence of IPTG is still considerable, another plasmid, pBCP208 (Fig. 2), which is derived from the intermediate-copy-number vector pSU18 (19), was constructed. By using

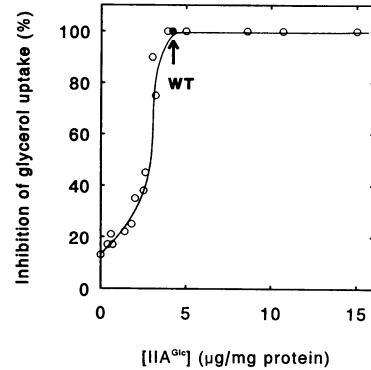


FIG. 4. Inhibition of glycerol uptake by αMG with different amounts of IIA^{Glc}, as determined in PP2005 containing either pBCP206 or pBCP208 (○). The inhibition of glycerol uptake in cells containing no IIA^{Glc} was determined in PP2005/pJFHE118. The inhibition of glycerol uptake and the corresponding amount of IIA^{Glc} in SB3507/pJFHE118 (●) are indicated. The uptake of 0.5 mM [U-¹⁴C]glycerol (specific activity, 210 dpm/nmol) was measured in the presence and absence of 10 mM αMG (added 3 min prior to the start of glycerol uptake). The difference between these two uptake rates (in the presence and absence of 10 mM αMG) was expressed as the percent inhibition of glycerol uptake. The amount of IIA^{Glc} was determined by rocket immunoelectrophoresis as described in the text.

plasmid pBCP208, the amount of IIA^{Glc} could be adjusted from about 5% of the wild-type level with no IPTG present to more than 1,000% the wild-type level in the presence of 60 μM IPTG. The relative activity of IIA^{Glc} varied between 15% (no IPTG present) and more than 1,000% (60 μM IPTG present) of the wild-type level. As was the case with pBCP206, the discrepancy at the very low IPTG concentrations is due to the membrane-bound IIA^{Glc}-like activity mentioned above.

Effect of different levels of IIA^{Glc} on glycerol metabolism. The effects of different levels of IIA^{Glc} on glycerol metabolism were studied in *S. typhimurium* PP2005 (*crr::Tn10*) containing plasmids pJFHE118 (vector without a *crr* gene), pBCP206, or pBCP208. There was no effect on the growth rate (0.38 h⁻¹) or the rate of 0.5 mM glycerol uptake (26 nmol/min/mg [dry weight]) when the amount of IIA^{Glc} was changed from no IIA^{Glc} (PP2005, *crr::Tn10*) to about 10 times the wild-type level (PP2005/pBCP206).

The inhibition of the rate of glycerol uptake by PTS carbohydrates (inducer exclusion) was measured as a function of the amount of IIA^{Glc} (Fig. 4). A sigmoidal relationship between the amount of IIA^{Glc} and the inhibition of the rate of glycerol uptake was found. A 50% inhibition of the rate of glycerol uptake was reached when cells contained about 3.0 μg of IIA^{Glc} per mg of protein, while full inhibition was reached at approximately 3.8 μg of IIA^{Glc} per mg of protein. At higher IIA^{Glc} concentrations, inhibition of the rate of glycerol uptake remained 100%. With no plasmid-encoded IIA^{Glc} present, the rate of glycerol uptake was inhibited about 10%. This inhibition is probably caused by membrane-bound IIA^{Glc}-like activity, which is present in *S. typhimurium* *crr* deletion strains (25).

The cells used in the previous experiments were grown for 1 h in the presence of glucose to induce the other PTS proteins (enzyme I, HPr, and enzyme IICB^{Glc}). It is known that intracellular cAMP levels are low in *crr* strains (10, 18, 25) and that expression of the *ptsHI* genes is affected by the cAMP receptor protein-cAMP complex (8, 9, 11). To rule out the possibility that the expression of the other PTS proteins was influenced by the various levels of IIA^{Glc} and could thus affect

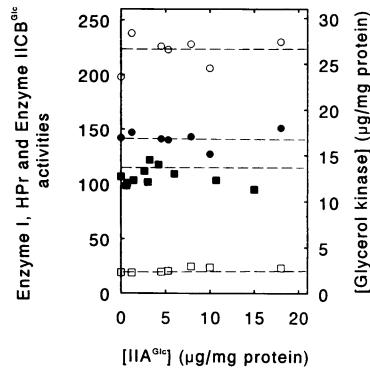


FIG. 5. Amount of glycerol kinase (■) and (relative) activities of enzyme I (○), HPr (●), and enzyme IICB^{Glc} (□) as a function of the amount of IIA^{Glc}, as determined in PP2005/pBCP206 or PP2005/pBCP208. The amount of glycerol kinase and the (relative) activities of enzyme I, HPr, and enzyme IICB^{Glc} in cells containing no IIA^{Glc} were determined in PP2005/pJFHE118. The wild-type value of the amount of glycerol kinase and the (relative) activities of enzyme I, HPr, and enzyme IICB^{Glc} were determined in SB3507/pJFHE118 (dashed lines). Enzyme I and enzyme IICB^{Glc} activities are absolute activities (expressed as nanomoles per minute per milligram of protein), while the HPr activity is relative activity (37); a value of 140 corresponds to an activity of 140 nmol/min/mg of protein, as determined in the wild-type strain under the experimental conditions (see Materials and Methods).

the inhibition of glycerol uptake, their activities were determined as a function of the amount of IIA^{Glc}. The (relative) activities of enzyme I, HPr, and enzyme IICB^{Glc} did not change when the amount of IIA^{Glc} was varied over a broad range (Fig. 5). Since α MG can directly dephosphorylate P-IIA^{Glc} via IICB^{Glc}, we also determined the inhibition of the rate of glycerol uptake as a function of IIA^{Glc}, using as an inhibitor 2-deoxyglucose, which is transported by the mannose PTS and can dephosphorylate P-IIA^{Glc} indirectly via HPr. The inhibition by 2-deoxyglucose was similar to that by α MG (data not shown). We conclude that the inhibition of glycerol uptake is a function of the amount of IIA^{Glc} and independent of the PTS carbohydrate used for inhibition.

Several experiments have suggested that the number of target protein molecules of IIA^{Glc} is important for the extent of inducer exclusion (22, 24, 25, 40). Inhibition of glycerol uptake is caused by the interaction of IIA^{Glc} with glycerol kinase, its target protein (7, 28, 34). Figure 5 shows that the amount of glycerol kinase remained constant when the amount of IIA^{Glc} was changed over a broad range. From the data in Fig. 4 and 5, it was possible to calculate the ratio of the moles of IIA^{Glc} to the moles of glycerol kinase that results in complete inhibition of glycerol uptake. Under those conditions, for each glycerol kinase molecule (tetramer, molecular mass of 220 kDa), 3.7 molecules of IIA^{Glc} (monomer, molecular mass of 18.6 kDa) were present. In the reverse experiment, the amount of IIA^{Glc} was kept constant (chromosomally encoded level) and the amount of glycerol kinase was modulated by growth for different periods of time on glycerol (Fig. 6A). Plotting the percent inhibition of the rate of glycerol uptake by α MG, determined in cells grown as described in the legend to Fig. 6, as a function of the ratio of the moles of IIA^{Glc} to the moles of glycerol kinase (tetramer) showed that 100% inhibition was achieved at ratios higher than approximately 3.5 (Fig. 6B). When the ratio dropped below this value, inhibition of the rate of glycerol uptake was only partial. It can be concluded that when the ratio of the number of IIA^{Glc} and

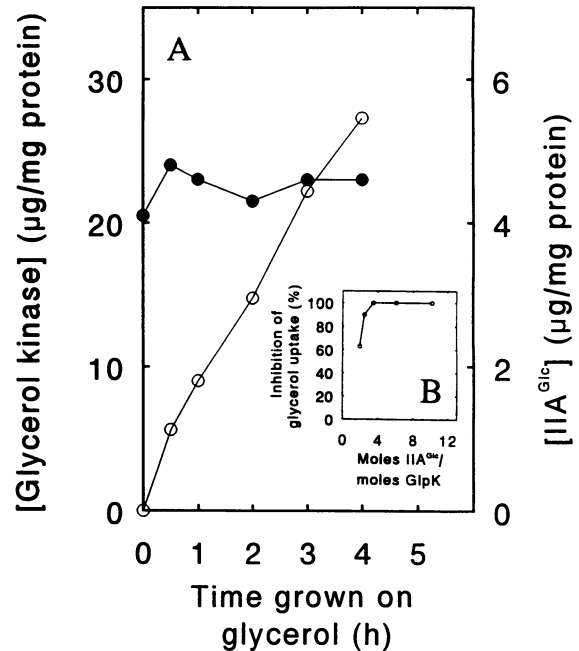


FIG. 6. Inhibition of glycerol uptake by α MG in cells with various amounts of glycerol kinase. (A) Amounts of glycerol kinase (○) and IIA^{Glc} (●) determined in SB3507/pJFHE118 grown for the indicated periods on glycerol. (B) Inset. The inhibition of glycerol uptake (○) plotted as a function of the ratio of moles of IIA^{Glc} to moles of glycerol kinase (GlpK; calculated as a tetramer). Cells were grown overnight on a minimal salts medium supplemented with 0.4% D,L-lactate and other requirements as described in the text. After being washed with minimal salts medium, the cells were diluted in minimal salts medium containing 0.5% glycerol and other required ingredients and allowed to grow on glycerol for the indicated periods of time. Glucose was added to a final concentration of 0.2%, and the cells were allowed to grow for 1 h before they were harvested. The amounts of glycerol kinase and IIA^{Glc} were determined as described in Materials and Methods.

glycerol kinase molecules (tetramer) becomes lower than 3.6, caused either by decreasing the amount of IIA^{Glc} or by increasing the amount of glycerol kinase, inhibition of the rate of glycerol uptake (inducer exclusion) will be incomplete.

Effect of different levels of IIA^{Glc} on maltose metabolism. In contrast to results obtained with glycerol, varying the level of IIA^{Glc} affected the specific growth rate on maltose and the rate of maltose uptake. In the absence of any IIA^{Glc}, the specific growth rate (0.15 h^{-1}) of PP2005 (*crr::Tn10*) was about half of that of the wild type. In PP2005/pBCP206, the growth rate of the wild type was reached when the IIA^{Glc} level was approximately 50% of that of the wild type (Fig. 7A). The rate of maltose uptake increased from about 20% of that of the wild-type level in cells containing no IIA^{Glc} to wild-type levels in cells in which the IIA^{Glc} level was approximately 60% of that of the wild type (Fig. 7B). To quantify the maltose transport system, we measured the amount of MBP synthesized (23). The synthesis of MBP increased, as did the rate of maltose uptake, with increasing amounts of IIA^{Glc} and became maximal when the level of IIA^{Glc} was approximately 90% of that of the wild type (Fig. 7C).

It has been shown previously that adenylate cyclase activity is lower in *crr* strains than in wild-type strains (10, 18, 25). Since the expression of the *mal* regulon is dependent on cAMP (47, 49), this could be a possible explanation for the IIA^{Glc}-

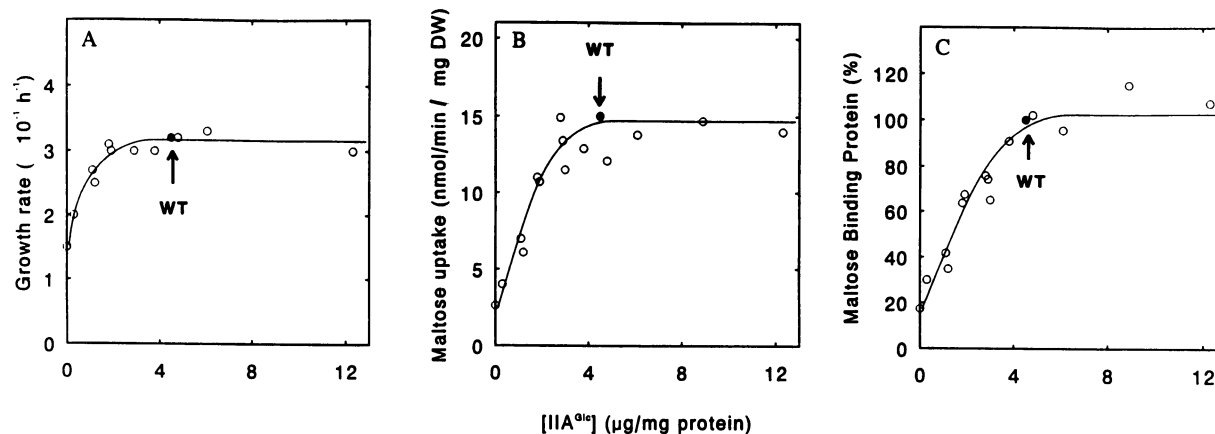


FIG. 7. Growth rate on maltose (A), rate of maltose uptake (B), and relative amount of MBP (C) as a function of the amount of IIA^{Glc} in PP2005/pBCP206 or PP2005/pBCP208 (○). The growth rate, maltose uptake, and amount of MBP in cells containing no IIA^{Glc} were determined in PP2005/pJFHE118. Wild-type (WT) values (●) for the growth rate, maltose uptake, and amount of MBP were determined in SB3507/pJFHE118. DW, dry weight.

dependent synthesis of MBP (Fig. 7). When 5 mM cAMP was added to the growth medium, the specific growth rate on maltose (0.32 h⁻¹), the rate of maltose uptake (14.2 nmol/min/mg [dry weight]), and the amount of MBP became independent of the amount of IIA^{Glc}.

Inhibition of the rate of maltose uptake (by the addition of α MG or 2-deoxyglucose) was sigmoidally related to the amount of IIA^{Glc} (Fig. 8). The rate of maltose uptake was inhibited 50% when the cells contained about 3.0 μ g of IIA^{Glc} per mg of protein, while complete inhibition was reached at about 4.5 μ g of IIA^{Glc} per mg of protein. As was the case with glycerol, maltose uptake was inhibited about 10% in cells lacking IIA^{Glc}, probably because of membrane-bound IIA^{Glc}-like activity, as mentioned previously. Inhibition of maltose uptake, determined in cells grown in the presence of cAMP, was still sigmoidally related to the amount of IIA^{Glc} (Fig. 8).

We investigated whether the synthesis of the MalK protein, the target protein of IIA^{Glc} in the process of inhibiting maltose uptake (6, 16, 33), was affected by different levels of IIA^{Glc}. Figure 9 shows that, in contrast to the amount of MBP, the amount of MalK protein remained constant with increasing levels of IIA^{Glc}. The addition of 5 mM cAMP to the growth medium had only a small stimulatory effect on the amount of MalK protein (Fig. 9). From the data in Fig. 9, we calculated the presence of about 3,000 to 4,000 MalK molecules per cell. These numbers fall in the reported range of 500 to 10,000 MalK molecules per cell (47).

The ratio of the moles of IIA^{Glc} to the moles of MalK protein was calculated at the point at which inhibition of maltose uptake became complete. For each MalK molecule (calculated as a dimer [molecular mass of 81.4 kDa] because two MalK molecules are present in the maltose transport system [5]), about 18 molecules of IIA^{Glc} (calculated as a monomer, molecular mass of 18.6 kDa) were present. The addition of cAMP to the growth medium did not affect this ratio.

DISCUSSION

In this report, we define the role of IIA^{Glc} in PTS-mediated regulation of glycerol and maltose metabolism in *S. typhimurium* in quantitative terms. Using inducible expression plasmids, we were able to vary the intracellular IIA^{Glc} concen-

tration over a broad range and to determine in vivo the effects of different levels of IIA^{Glc} on the inhibition of glycerol and maltose uptake by PTS carbohydrates and, indirectly, on intracellular cAMP levels. We did not observe impairment of the growth rate on glycerol or maltose of *S. typhimurium* cells containing high levels (up to 10 times of that of the wild-type strain) of IIA^{Glc} (this study; 24). In contrast, impairment of the growth rate on glycerol and maltose in a 10-fold-overproducing IIA^{Glc} *E. coli* strain was reported previously (23). Except for the use of different genetic backgrounds and expression plasmids, we do not have an explanation for this discrepancy.

Our results confirm and extend the conclusions drawn previously with respect to inducer exclusion. As described by Nelson and coworkers (24, 25) and others (22, 23, 40), inducer

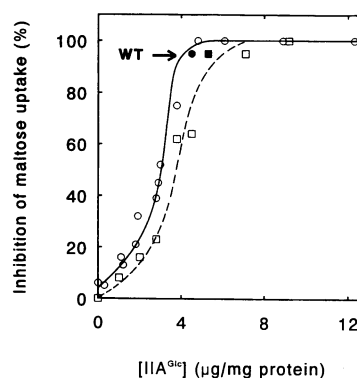


FIG. 8. Inhibition of maltose uptake by α MG in cells with various levels of IIA^{Glc} as determined in PP2005/pBCP206 or PP2005/pBCP208 grown in the absence (○) or presence (□) of 5 mM cAMP. The inhibition of maltose uptake in cells containing no IIA^{Glc} was determined in PP2005/pJFHE118. The wild-type (WT) values of inhibition of maltose uptake, determined in SB3507/pJFHE118 grown in the absence (●) or presence (■) of 5 mM cAMP, are indicated. The uptake of 0.5 mM [U-¹⁴C]maltose (specific activity, 190 dpm/nmol) in the presence and absence of 10 mM α MG (added 3 min prior to the start of maltose uptake) was determined as described in the text. The inhibition of maltose uptake was calculated as described for glycerol in the legend to Fig. 4. The amount of IIA^{Glc} was determined by rocket immunoelectrophoresis as described in the text.

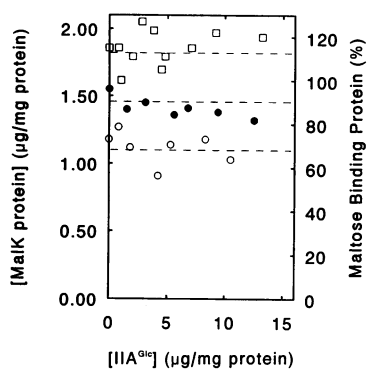


FIG. 9. Relative synthesis of MBP (□) (compared with the wild type grown in the absence of cAMP) and synthesis of MalK as a function of the amount of IIA^{Glc} in PP2005/pBCP206 or PP2005/pBCP208, grown in the presence (●) or absence (○) of 5 mM cAMP. The synthesis of MBP and MalK protein in cells containing no IIA^{Glc} was determined in PP2005/pJFHE118. The levels of MBP and MalK protein in the wild type, as determined in SB3507/pJFHE118, are indicated by dashed lines.

exclusion depends on both the level of IIA^{Glc} and the level of inducer exclusion-sensitive uptake systems. Here we extend this knowledge with the finding that, for complete inhibition of glycerol uptake by a PTS carbohydrate (inducer exclusion) to occur, a minimal ratio of 3.6 moles of IIA^{Glc} to 1 mol of glycerol kinase (tetrameric form) is required. When this ratio is lower, either by decreasing the amount of IIA^{Glc} (an artificial event) or by increasing the amount of glycerol kinase (a natural event), inducer exclusion will be less than complete. If we assume almost complete dephosphorylation of IIA^{Glc} upon the addition of α MG, as described by Nelson et al. (26), approximately four molecules of IIA^{Glc} can bind to one molecule of glycerol kinase. This may explain the cooperative character of the inhibition of glycerol uptake as a function of the amount of IIA^{Glc} (Fig. 4), i.e., the binding of one molecule of IIA^{Glc} to one molecule of glycerol kinase (tetramer) and stimulation of the binding of the next IIA^{Glc} molecule. Our conclusion that four molecules of IIA^{Glc} can bind to one glycerol kinase molecule confirms the results of the experiments reported by Hurley et al. (14), who resolved the crystal structure of IIA^{Glc} in complex with glycerol kinase by means of X-ray diffraction. They reported the binding of four molecules of IIA^{Glc} to each glycerol kinase tetramer (in the presence of ADP and glycerol). Thus, there is a nice fit between the data obtained by measuring inducer exclusion (in vivo) and the crystal structure (in vitro).

Complete inhibition of glycerol and maltose uptake in partially induced wild-type cells, which we report here (Fig. 4 and 8), was not observed in some previous studies (25, 42). This discrepancy may be explained by the correct ratio of IIA^{Glc} and target molecules and by the high level of enzyme IICB^{Glc} which was induced under our experimental conditions by growth for 1 h in the presence of glucose. It has been reported previously (38, 39) that induction of enzyme II can improve the extent of inducer exclusion.

It should be mentioned that the ratio of IIA^{Glc} molecules to glycerol kinase molecules, which gives complete inducer exclusion in vivo, excludes the small contribution of the so-called membrane-bound IIA^{Glc}-like activities still present in *S. typhimurium crr* deletion strains (25, 51). At present, we are investigating the contribution of the IIA^{Glc}-like domain, present in enzyme II^{Nag}, to the process of inducer exclusion.

Recently, Voegelé et al. (50) proposed that glycerol kinase is activated by binding to the glycerol facilitator in *E. coli*. In this activated form, glycerol kinase would be insensitive to the binding of IIA^{Glc} and other effectors. Under our experimental conditions, inhibition of glycerol metabolism is complete. From this we conclude that all glycerol kinase molecules are inactive, presumably by interaction with IIA^{Glc}, and irrespective of the presence of the glycerol facilitator.

The effects of changing levels of IIA^{Glc} on maltose metabolism (i.e., growth rate on maltose, rate of maltose uptake, and synthesis of MBP) were unexpected, because the growth of the *S. typhimurium crr* strain, which we routinely used, is indistinguishable from that of the wild-type strain, as determined on minimal maltose plates. The effects of different levels of IIA^{Glc} on maltose metabolism, in cells grown in liquid media, which we observed here, can be explained by the dependency of the expression of the *mal* genes on cAMP (47, 49). In a *crr* strain, cAMP levels are low (10, 18, 25). When in a *crr* strain IIA^{Glc} is increased gradually, as we report here, one expects the intracellular cAMP concentration also to increase gradually. Most likely, the synthesis of the MBP, the growth rate, and the rate of maltose uptake are sensitive to changes in the intracellular cAMP concentration in this range. However, as shown in Fig. 9, the intracellular concentration of MalK, the target of IIA^{Glc}, is not sensitive to the same changes in the IIA^{Glc} concentration, i.e., the levels are the same in a *crr::Tn10* mutant and the wild-type strain. Full expression of different cAMP-dependent operons can require different amounts of cAMP (15). The isolation of several classes of suppressor mutations in a *cya* strain that restored growth on an increasing number of carbon sources showed that the maltose system is less sensitive to the lack of cAMP than, for example, the succinate system but more sensitive than the glycerol system (36). These different requirements for cAMP by different operons may explain why the synthesis of glycerol kinase, enzyme I, HPr, and enzyme IICB^{Glc} are not affected by low and varying levels of IIA^{Glc}. Although cAMP receptor protein-cAMP complex-binding sites have been reported in the promoter regions of the *glpK* gene (encoding glycerol kinase [53]) and the *ptsHI* operon (9, 11), intracellular cAMP concentrations in the absence of IIA^{Glc} were clearly sufficient for the maximal expression of those genes in our *S. typhimurium* strains. The differences in synthesis of the MalK protein and the MBP, in response to different cAMP levels, may be explained in a similar way since the *malK* gene, encoding the MalK protein, is part of the *malK-lamB-malM* operon, while the *malE* gene, encoding the MBP, is part of the divergently transcribed *malEFG* operon (44, 47).

For complete inhibition of maltose uptake (inducer exclusion) to occur, a minimal ratio of about 18 molecules of IIA^{Glc} per molecule of MalK protein (taken as a dimer, because of the stoichiometry of the proteins present in the maltose transport system [5]) is required. In contrast to the case of glycerol kinase, IIA^{Glc} needs to be in excess of the MalK protein for full inactivation of the maltose transport system. The difference between glycerol kinase and MalK protein for their requirements of IIA^{Glc} can be explained by a much lower affinity of IIA^{Glc} for the MalK protein than for glycerol kinase. The sigmoidal relationship between the amount of IIA^{Glc} and the inhibition of maltose uptake (Fig. 8) can be explained by the assumption that two molecules of IIA^{Glc} are bound to the maltose transport system at the point of complete inhibition of maltose uptake system and that binding of one molecule of IIA^{Glc} stimulates the binding of the second molecule.

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