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Phosphoenolpyruvate:Sugar Phosphotransferase System-Mediated Regulation of Carbohydrate Metabolism in *Salmonella typhimurium*

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The *crr* mutation was shown to affect the phosphoenolpyruvate:sugar phosphotransferase system-mediated transient repression of the *lac* operon, intracellular cAMP levels, and sensitivity to inducer exclusion. Our results indicate that the presumed *crr* gene product, factor III^{Glc}, plays a direct role in the regulation of inducer exclusion. We propose a mechanism in which inducer exclusion depends on both the level and state of phosphorylation of factor III^{Glc} and the level of an inducer exclusion-sensitive transport system. The results of studies on the sensitivity to inducer exclusion of glycerol and maltose in cultures induced for short periods of time on these substrates (resulting in varying degrees of activity of the respective transport systems) support this model of inducer exclusion. Previously, the *crp*-771* mutation has been shown to result in an altered cAMP receptor protein, which has a changed affinity for cAMP, and to affect the sensitivity for inducer exclusion of glycerol. Changes in other functions of the altered cAMP receptor protein were indicated by our results; these changes were in the roles of this protein in (i) the cAMP-dependent initiation of transcription of the *lac* operon and (ii) the regulation of intracellular cAMP levels and the export of cAMP. We propose that the *crp*-771* mutation has an indirect effect in relieving inducer exclusion in repressed or hypersensitive strains, in which the *crp*-771* mutation allows the synthesis of inducer exclusion-sensitive transport systems to higher levels than the levels found in strains containing wild-type cAMP receptor protein.

Salmonella typhimurium and *Escherichia coli* contain closely related phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS), which not only are responsible for the uptake and phosphorylation of a number of sugars, but also are involved in the regulation of cell metabolism (4, 20, 26, 27). The regulatory role of the PTS includes (i) the inhibition of certain non-PTS sugar transport systems by PTS substrates (inducer exclusion) and (ii) the regulation of adenylate cyclase activity by the PTS (10, 18, 29–32). In most cases the regulatory effects have been attributed to changes in the state of phosphorylation of factor III^{Glc} of the glucose-specific PTS pathway (Fig. 1). Inducer exclusion is believed to be the result of inhibition of the PTS-sensitive transport systems for glycerol, maltose, melibiose, and lactose by non-phosphorylated III^{Glc} (4, 26, 32, 33). Adenylate cyclase activity is thought to be stimulated by the phosphorylated form of factor III^{Glc} (6, 21, 29, 35). Most of the evidence for the mediation of PTS regulatory functions by III^{Glc} has been obtained from studies of *crr* mutants, which usually retain less than 10% of the wild-type III^{Glc} activity (21, 31). Strains with the *crr* mutation have lowered adenylate cyclase activity and cAMP levels (6, 28). Some of the growth defects of mutants lacking enzyme I or HPr or both, as well as sensitivity to inducer exclusion in such *pts* mutants, are relieved by the presence of *crr* (21, 29–32, 35).

The relative importance of the two regulatory modes of action of the PTS in *E. coli* and *S. typhimurium* has long been an open question (20). It has been suggested that inducer exclusion is the more important mode (20, 28, 30–33). On the other hand, there is evidence that inducer exclusion may, in turn, be regulated by cAMP levels (4, 35; D. K. Keeler, B. U. Feucht, and M. H. Saier, Jr., Fed. Proc. 36:685, 1977). This implies that cAMP may be the central regulatory factor in the repression of growth on the non-PTS carbohydrates. This conclusion is supported by evidence that permanent repression of growth on glycerol and melibiose in a *ptsHI* deletion strain can be relieved by exogenously added cAMP or by the presence of a *crp*-771*

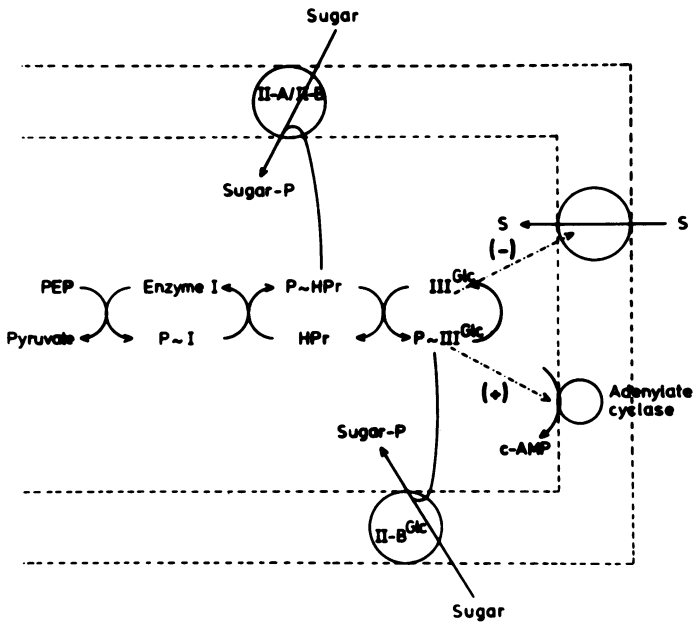

FIG. 1. Schematic representation of the PTS and its regulatory functions. The inhibition of non-PTS transport systems (inducer exclusion) and the activation of adenylate cyclase are indicated by the broken arrows. PEP, Phosphoenolpyruvate.

mutation, which results in an altered cAMP receptor protein (CRP). It has also been shown that hypersensitivity to inducer exclusion of glycerol is relieved by the presence of exogenously added cAMP or by the presence of the *crp*-771* mutation (21, 35).

Clarification of the role of inducer exclusion in regulating cell metabolism is presently hampered by a lack of knowledge of the mechanism of action of this PTS-mediated regulatory function. In this paper we present evidence that inducer exclusion is a function not only of the level and state of phosphorylation of factor III^{Glc} but also of the level of the non-PTS carbohydrate transport system regulated by the PTS. We also present a model for inducer exclusion, in which the formation of a complex between nonphosphorylated III^{Glc} and an inducer exclusion-sensitive transport system is responsible for transport inhibition. We conclude that the effects of added cAMP and of the *crp*-771* mutation on inducer exclusion (21, 35) are indirect and are expressed by relieving the repression of the cAMP-dependent operons for the non-PTS carbohydrates. This allows the PTS-sensitive transport systems to be induced to such levels of activity that they become insensitive to inhibition by the PTS.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Stocks were grown in nutrient broth and stored at 4°C, except for the *lac* episome-containing strains, which were grown in medium A (36) containing 0.2% lactose and were stored at 4°C. Strains *cysA20*/F'*lac* and *crp*-771*/F'*lac* were constructed by conjugation between *E. coli* JL1240/F'*lac* and *cysA20* or PP914 (*crp*-771*). *E. coli* JL1240/F'*lac* was obtained from O. Karlström, Copenhagen, Denmark.

Chemicals. The following radioactive substrates were purchased from Amersham Corp.: [*U*-^{14}C]glycerol (46 mCi/mmol), [*U*-^{14}C]maltose (550 mCi/mmol), [8-^{3}H]cAMP (20 to 30 Ci/mmol), and [α-^{32}P]ATP (2,000 to 3,000 Ci/mmol). [*methyl*-^{14}C]methyl-β-D-thiogalactopyranoside (45 mCi/mmol) and 2-deoxy-D-[1-^{3}H]glucose (20 Ci/mmol) were purchased from New England Nuclear Corp. Agarose was obtained from Merck (electrophoresis grade). All other chemicals were of normal laboratory quality.

Transport rates. The procedure of Postma (19) was used. Washed cells were aerated at 20°C for 2 min (1 to 2 mg [dry weight] per ml of medium A). At zero time a labeled substrate was added, and at different times 100-μl samples were removed, quickly diluted in 10 ml of medium A at room temperature (approximately 20°C), and collected on Whatman GF/F glass fiber filters. The filters were dried by leaving them under suction for 2 min and were counted in 10 ml of Packard Scintillator 299 fluid containing 0.5 ml of water. Background activity was measured by the same procedure but without cells. Results are reported as nanomoles of substrate taken up per minute per milligram (dry weight).

β-Galactosidase assay and repression. Exponentially growing cultures (medium A containing 0.2% galactose) at an optical density at 600 nm of approximately 0.3 were divided into two portions both of which

received inducer (0.5 mM isopropyl-1-thio-β-D-galactopyranoside) and one of which received repressor (0.5 or 10 mM glucose) at the times indicated below (see Fig. 4). At different times, 0.5 ml of each culture was added to 1 drop of toluene in a chilled tube, mixed well, and incubated at 37°C for 15 min. The assay was performed by diluting 150 μl of the toluene-treated cells to a volume of 500 μl with medium A, adding 1 ml of 2 mM *o*-nitrophenyl-β-D-galactopyranoside, and incubating the preparation at 37°C for 15 min. The reaction was stopped by adding 1 ml of ice-cold 1 M Na_2CO_3, and the assay solution was then stored at 4°C for at least 1 h before the absorbance at 420 nm (A_{420}) was measured. The results are reported as units of β-galactosidase activity per A_{600} unit of cells; 1 U of β-galactosidase activity was defined as of 1 μmol of *o*-nitrophenol formed per min, whereas 1 A_{600} unit of cells was defined as the amount of cells which produced an A_{600} of 1 when they were suspended in 1 ml of solution.

Electrophoretic methods. Maltose-binding protein (MBP) and factor III^{Glc} content were determined in cells treated with chloroform and toluene by the method of Lengeler et al. (14). MBP and III^{Glc} were assayed by rocket electrophoresis, as described by Axelsen et al. (1). Anti-III^{Glc} and purified III^{Glc} were prepared as described previously (37). MBP was isolated by the method of Ferenci and Klotz (5), and anti-MBP was prepared as previously described for anti-III^{Glc} (37).

Phosphorylation via the PTS in toluene-treated cells. A modification (36) of the procedure of Kornberg and Reeves (12) was used to measure sugar phosphorylation via the PTS in toluene-treated cells.

Determination of cAMP levels. The procedure of Wayne and Rosen (39) was used to obtain intracellular cAMP and extracellular cAMP by filtering 4 ml of an exponentially growing culture (medium A containing 0.2% galactose; A_{600}, 0.7 to 0.8) and washing the filtered cells with an equal volume of growth medium equilibrated at 37°C (Whatman GF/F glass fiber filters). The filtrate was assayed as obtained, but the cells were extracted by adding the filter to 3 ml of 0.2 N HCOOH and heating for 10 min in a boiling water bath. The extract was centrifuged, and 2 ml was freeze-dried and dissolved in 0.2 ml of water, after which the pH was adjusted to 7. Assays were performed with a cAMP assay kit (Amersham Corp.) according to the procedure suggested by the manufacturer.

TABLE 1. Bacterial strains

Strain	Genotype	Construction	Source or reference[a]
cysA20	*cysA20*		PH
SB3507	*trpB223*		EB
SB1476	*ptsI17*		PH
SB1786	*cya-502*		PH
SB2309	*trpB223* Δ(*cysK-ptsHI*)*41*		JC
SB2950	*trpB223* Δ(*cysK-ptsHI-crr*)*49*		JC
SB3687	*trpB223* Δ(*ptsI-crr*)*167*		PH
NK816	*cysA1539*::Tn*10*		JR
TT2104	*cya-961 argI539 proAB47 amtA1 trpΔ130 zid-2*::Tn*10*		JR
PP378	*trpB223* Δ(*cysK-ptsHI*)*41 crr-303*	Glp^+ in the presence of α-MG,SB2309 (spontaneous)	This paper
PP642	Δ(*cysK-ptsHI*)*41*		22
PP772	*trpB223 crr-303*	PP378 × P22(SB3687)	This paper
PP780	*crr-303*	*cysA20* × P22(PP772)	This paper
PP782	*crr-306*		35
PP800	*trpB223 galP283* Δ(*cysK-ptsHI crr*)*49*		22
PP825	*crr-306 crp*-771*		35
PP844	*cysA1539*::Tn*10 ptsI17*	SB1476 × P22(NK186)	This paper
PP914	*crp*-771*		35
PP916	*crp*-771 cysA1539*::Tn*10*	PP914 × P22(PP844)	This paper
PP930	*cya-502 melP*	Mel^+ SB1786, DES	This paper
PP931	*melP*	PP930 × P22(SB3507)	This paper
PP960	*ptsI17 crp*-771*		This paper
PP967	*melP cysA1539*::Tn*10*	PP931 × P22(NK186)	This paper
PP968	*melP ptsI17*	PP967 × P22(SB1476)	This paper
PP969	*melP* Δ(*cysK-ptsHI*)*41*	PP967 × P22(PP642)	This paper
PP977	*ptsI17*	*cysA20* × P22(SB1476)	This paper
PP994	*crr-307*::Tn*10*		37a
PP1025	*ptsI17 crr-307*::Tn*10*	SB1476 × P22(994)	This paper
	cysA20/F′*lac*	*cysA20* × *E. coli* JL1240/F′*lac*	This paper
	crp-771*/F′*lac*	PP914 × *E. coli* JL1240/F′*lac*	This paper
	crr-303/F′*lac*	*cysA20*/F′*lac* × P22(PP780)	This paper
	crr-306/F′*lac*	*cysA20*/F′*lac* × P22(PP782)	This paper

[a] Abbreviations: PP, P. W. Postma; PH, P. E. Hartman; JR, J. R. Roth; EB, E. Balbinder; JC, J. C. Cordaro.

Adenylate cyclase activity. Adenylate cyclase activity was measured by using a combination of the procedures of Harwood and Peterkofsky (11), Krishna et al. (13), and Salomon et al. (34). Exponentially growing cells in nutrient broth supplemented with 0.2% glucose at an A_{600} of about 0.6 to 1.0 were harvested, washed twice with medium A, and suspended in sufficient buffer (25 mM Tris, 15 mM potassium phosphate, 20 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.5) to give a concentration of 5 mg of protein per ml. The cells were treated with toluene by incubating 1 ml of cell suspension with 10 μl of toluene at room temperature (20 to 25°C) for 5 to 10 min. An ATP-regenerating system was not added in our experiments as this did not yield increased activities and the ATP concentration decreased only about 25% under these conditions, even in the presence of glucose.

The assay was carried out in a total volume of 100 μl containing 25 mM Tris, 15 mM potassium phosphate, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM glucose (when present) (pH 8.6). Toluene-treated cells (50 to 150 μg) were added (final pH, 8.3 to 8.4), and the preparation was equilibrated at 30°C for 1 to 2 min. The assay was begun by adding 10 μl of a solution containing 10 mM [α-^{32}P]ATP (5 to 40 cpm/pmol) and 10 mM $MgCl_2$ (pH 7). The reaction was stopped after 5 to 20 min by adding 100 μl of stop mixture containing 2% sodium dodecyl sulfate, 40 mM ATP, and 1 mM [8-^{3}H]cAMP (as an internal standard to measure the percent recovery of cAMP during chromatography [about 550,000 cpm/100 μl]) and 800 μl of water.

The reaction mixture was chromatographed twice. First, it was passed through a 1-ml column of Dowex AG 50W×4 (Bio-Rad Laboratories; which was regenerated after each experiment by being washed with 10 ml of 1 N HCl and was washed with 10 ml of water before each experiment). This was followed by elution with 3 ml of water, and all eluates were discarded. Then 3 ml of water was added to the column, and the eluate was allowed to pass through a column containing 0.6 g of neutral alumina which had been freshly washed with 10 ml of 0.1 M imidazole hydrochloride (pH 7.5). The column was eluted with 3 ml of imidazole buffer, and the eluate was counted in 17 ml of Packard Scintillator 299 fluid in a Nuclear Chicago Isocap 300 scintillation counter on channel 10 (^{3}H/^{32}P external standard ratio).The results are reported as picomoles formed per minute per milligram of protein.

Protein content. The protein content was determined by the method of Lowry et al. (15), using bovine serum albumin as a standard.

RESULTS

Sensitivity to inducer exclusion is a function of the level of the PTS-sensitive sugar transport system. It was suggested recently that the effects of exogenously added cAMP or the presence of the *crp*-771* mutation on sensitivity to inducer exclusion may be exerted through control of the levels of transport proteins for the PTS-sensitive transport systems (4, 35). If inducer exclusion is the result of the formation of an inactive complex between non-phosphorylated III^{Glc} and a transport protein, the relative amounts of these two proteins should determine the extent of inducer exclusion. Factor III^{Glc} levels, as measured with a specific antibody, are relatively constant in cells (37) and, therefore, are capable of inhibiting only a fixed amount of permease proteins. Permease proteins produced in excess of this inhibitable amount escape regulation by the PTS system. One would predict from this simple model that sensitivity to inducer exclusion should be a function not only of the level and state of phosphorylation of III^{Glc}, but also of the levels of the PTS-sensitive transport proteins. Inducer exclusion is strong when transport protein levels are low and is weak when transport protein levels are high compared with III^{Glc} levels.

This possibility was tested in cells in which all of the relevant factors remained constant, except that the activity of the PTS-sensitive transport system varied. This was accomplished simply by incubating a culture in the presence of an inducer exclusion-sensitive substrate (glycerol or maltose) for short periods of time (partial induction) after pregrowth on a non-PTS (and inducer exclusion-insensitive) substrate, such as galactose, lactate, succinate, or citrate. Thus, we eliminated possible changes in the various PTS proteins which might have confused our results (25).

The results of a typical partial induction experiment are shown in Table 2. We found that inhibition of glycerol uptake by methyl α-glucopyranoside (α-MG) in a strain containing normal levels of the PTS proteins (strain PP916 [*crp*-771*]) was strong only while glycerol uptake activity was low and was not demonstrable when the glycerol uptake activity was fully induced. Sensitivity to inducer exclusion under these conditions seemed to be a function of the activity of the inducer exclusion-sensitive trans-

TABLE 2. Inducer exclusion of glycerol in *S. typhimurium* partially induced for growth on glycerol

Time of induction (min)	Rate of uptake[a]	
	v_o	v_i
0	0	0
20	8.3	0.5
40	9.3	2.3
60	15.0	2.7
100	20.0	10.0
140	30.0	30.0

[a] A culture of strain PP916 grown overnight in medium A containing 0.4% succinate was harvested and washed twice with medium A. The cells were suspended in medium A containing 0.2% glycerol, and at the times indicated samples were harvested, washed, and assayed for [^{14}C]glycerol (0.5 mM; 150 cpm/nmol) uptake activity, as described in the text, in the absence (v_o) and in the presence (v_i) of 1 mM α-MG.

TABLE 3. Inducer exclusion of maltose in *S. typhimurium* partially induced for growth on maltose[a]

Time of induction	Maltose uptake rate[a]		Transport of 2-deoxyglucose[a]	III^{Glc} content[b]
	v_o	v_i		
90 min	0.9	0.3	9.0	2.6
135 min	2.0	0.8	9.6	2.7
160 min	2.8	1.4	11.7	3.1
Overnight	8.5	8.1	12.0	2.5

[a] Overnight cultures of *S. typhimurium cysA20* grown in medium A containing 0.2% citrate were used to inoculate medium A containing 0.2% maltose. After growth for the times indicated, the cultures were harvested and washed, and the uptake of 0.5 mM [^{14}C]maltose (360 cpm/nmol) was measured as described in the text in the absence (v_o) and in the presence (v_i) of 1 mM 2-deoxyglucose. In the same cells transport of 1 mM 2-deoxyglucose (250 cpm/nmol) was measured. The results are expressed as nanomoles of maltose or 2-deoxyglucose taken up per minute per milligram of dry weight.

[b] III^{Glc} contents were determined by using rocket immunoelectrophoresis as described by Scholte et al. (37) and are expressed as micrograms of III^{Glc} per milligram (dry weight).

port system. Other wild-type strains (for example, *cysA20*) exhibited similar sensitivities to inducer exclusion of glycerol. Both *cysA20* and PP916 became relatively insensitive to inducer exclusion as the glycerol transport system approached a level of activity of about 30 nmol of glycerol taken up per min per mg (dry weight). A *crp*-771 ptsI17* strain lacking most of enzyme I activity was much more sensitive to inducer exclusion of glycerol than the two strains having normal enzyme I activity (data not shown).

Partial induction experiments with the maltose transport system yielded results very similar to those obtained with the glycerol system (Table 3 and Fig. 2). These experiments had the following advantage over those with the glycerol transport system: it was possible to measure a component of the maltose transport system, the MBP, by immunological methods and thus to some extent to quantify the amount of transport system synthesized. In each case, measurement of MBP content during partial induction experiments revealed a linear relationship between maltose uptake activity and MBP content, indicating that maltose uptake activity was indeed a reliable measure of the content of maltose transport proteins in *S. typhimurium* (Fig. 3). In addition, these experiments showed that under the conditions used for induction of the maltose transport system both the amount of III^{Glc} and the activity of the enzyme II^{Man} system, as measured by 2-deoxyglucose transport, remained constant. 2-Deoxyglucose was used as a

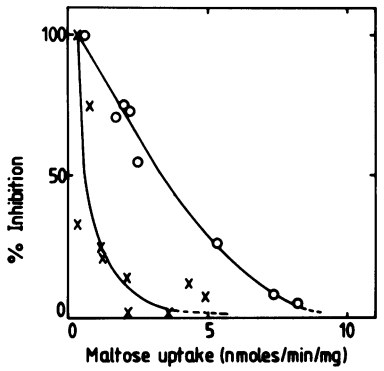

FIG. 2. Maltose inducer exclusion as a function of the maltose uptake activity. *S. typhimurium* strains *cysA20* and PP994 (*crr-307*::Tn*10*) were grown overnight in medium A containing 0.2% citrate and 0.2% lactate, respectively. Fresh medium A containing 0.2% maltose was added, and the cells were allowed to grow for up to 3 h, after which they were harvested and washed and the uptake of 0.5 mM maltose (250 to 360 cpm/nmol) in the presence (v_i^c) and absence (v_o^c) of 1 mM 2-deoxyglucose was measured. The results are expressed as percent inhibition ($100[v_o^c - v_i^c]/v_o^c$) of maltose uptake by the PTS (with 2-deoxyglucose as the substrate) versus the activity of the maltose transport system (v_o^c) (in nanomoles of maltose taken up per minute per milligram of cellular dry weight). The superscript c indicates that a correction was made for aspecific maltose binding by subtracting 0.3 nmol of maltose taken up per min per mg of cellular dry weight from all uptake values; this value was determined by estimating the amount of binding of [^{14}C]maltose to uninduced cells. Symbols: ○, *cysA20*; ×, PP994.

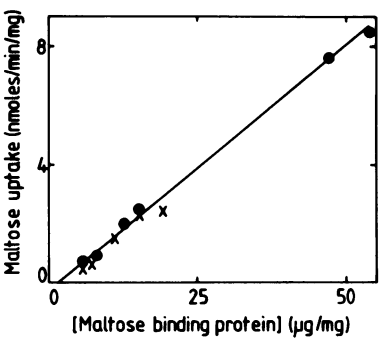

FIG. 3. Maltose uptake activity as a function of the MBP content. *S. typhimurium* strains were grown overnight in medium A containing 0.2% citrate (*cysA20*) (●) or 0.2% lactate (PP994 [*crr-307*::Tn*10*]) (×). Fresh medium A containing 0.2% maltose was added; after incubation at 37°C for short periods of time (up to 3 h), the cells were harvested and washed, and the uptake of 0.5 mM [^{14}C]maltose (250 to 360 cpm/nmol) (in nanomoles of maltose taken up per minute per milligram [dry weight]) and the content of MBP (in micrograms of MBP per milligram [dry weight]) were determined as described in the text.

PTS substrate. The use of α-MG is not always advisable since the membrane-bound IIB^{Glc} enzyme required for phosphorylation of this substrate (20) and for the expression of the PTS-mediated regulatory actions of this substrate (10, 29) is inducible about 50-fold (38). Under conditions in which the IIB^{Glc} activity is low, α-MG may be a relatively inefficient inhibitor of PTS-sensitive transport systems. On the other hand, enzyme II^{Man}, which is responsible for 2-deoxyglucose transport and phosphorylation, is only twofold inducible (25, 38).

The results of experiments in which the maltose transport system was partially induced in both wild-type and *crr* strains are summarized in Fig. 2. It is obvious that sensitivity to inducer exclusion varied in both strains with the activity of the maltose transport system and was highest when the uptake activity was low. Surprisingly, inducer exclusion was even measurable in a *crr* strain (PP994), which lacks cytoplasmic III^{Glc} completely (37a). Inducer exclusion has been reported to be absent in *ptsI crr* strains (29, 30, 32). However, using an antibody against purified soluble III^{Glc}, we have shown recently that some *crr* strains of *S. tphimurium*, including *crr* deletion strains and Tn*10* insertion strains, do not contain any soluble III^{Glc} but still exhibit 10 to 20% III^{Glc} activity, as measured by in vitro phosphorylation of methyl α-glucoside. These *crr* mutants still contain a membrane-bound, III^{Glc}-like activity which is inhibited by antibody against soluble III^{Glc} (37a). The previous failures to demonstrate inducer exclusion in *pts crr* strains were probably due to the fact that inducer exclusion was measured in fully induced cells. A fully induced *crr* strain is especially insensitive to inducer exclusion due to the high induced levels of transport proteins relative to the low factor III^{Glc} content (see below).

Transcription of a cAMP-dependent operon independently of cAMP does not result in relief of sensitivity to inducer exclusion. Previous reports (21, 35) suggested that the presence of the *crp*-771* mutation or of extracellularly added cAMP may relieve sensitivity to inducer exclusion by rendering an operon independent of cellularly produced cAMP for transcription and thus uncoupled from the inhibitory effects of the PTS system on adenylate cyclase. Therefore, it was predicted that a specific mutation in a cAMP-dependent operon rendering it independent of cAMP should have a similar effect. Such a mutation (designated *melP*) was isolated as a specific reversion of the melibiose growth defect of a *cya* strain totally lacking adenylate cyclase activity. (The revertant *melP cya-502* is able to grow on melibiose but still does not grow on any of the other carbon sources that are not able to support growth of the *cya* mutant.) The melibiose operon in *melP* strains is inducible. Strains containing *melP* are able to transport thiomethylgalactoside and oxidize melibiose only after growth on melibiose (data not shown).

In contrast to the specific reversion by *melP* of the melibiose growth defect of a *cya* mutant strain, a *melP* Δ*(cysK-ptsHI)41* double mutant could not be induced to grow with melibiose as the only carbon source. The repression of growth on non-PTS sugar substrates in *ptsHI* deletion strains could be attributed to either or both of the regulatory roles of the PTS (i.e., the regulation of adenylate cyclase activity [cAMP levels] and the regulation of PTS-sensitive transport system activity [inducer exclusion]). Since the *melP* mutation allows transcription of the melibiose operon in the absence of cAMP, the failure of the *melP* Δ*(cysK-ptsHI)41* double mutant to grow on melibiose can be attributed to direct inhibition of the melibiose transport system by the PTS, indicating that the *melP* mutation does not offer protection from PTS inhibition of the PTS-sensitive melibiose transport system. This was confirmed by measuring transport via the melibiose transport system (Table 4). The transport of thiomethylgalactoside via the melibiose transport system was similar in *ptsI17* and *ptsI17 melP* strains, and in both cases inhibition by the PTS substrate (methyl α-glucoside) was very strong.

Transient repression of β-galactosidase activity is relieved by *crp*-771*. Mutation *crp*-771* was isolated as a suppressor of the *crr* phenotype. This mutation allows the growth of *crr* strains on citrate, xylose, succinate, and malate. It also allows growth of *ptsHI* deletion strains on glyc-

TABLE 4. Transport of methyl-1-thio-β-D-galactopyranoside via the melibiose transport system in *S. typhimurium* strains

Strain	Genotype	[^{14}C]TMG uptake rate[a]	
		Uninhibited	Inhibited
cysA20	*cysA20*	5.0	4.0
PP916	*crp*-771 cysA*::Tn*10*	6.3	6.3
PP968	*melP ptsI17*	8.0	1.0
PP977	*ptsI17*	8.1	1.3
SB1476	*ptsI17*	8.1	2.0

[a] Exponentially growing cells in medium A containing 0.2% melibiose were harvested and washed twice with medium A. The initial rate of uptake of 0.5 mM ^{14}C-labeled methyl-1-thio-β-D-galactopyranoside ([^{14}C]TMG; 340 cpm/nmol) was measured in the presence of 10 mM NaCl as described in the text. The inhibited rate of uptake was measured in the presence of 1 mM α-MG. The results are expressed as nanomoles of ^{14}C-labeled methyl-1-thio-β-D-galactopyranoside taken up per minute per milligram (dry weight).

erol and melibiose (21, 35); these strains are normally repressed for growth on these substrates (20). It is thought that *crp*-771* relieves repression of growth on these carbon sources by allowing expression of the cAMP-dependent operons for these substrates independently of cAMP. This is indicated by (i) a change in the affinity of the *crp*-771* gene product for cAMP relative to the affinity of wild-type CRP, (ii) an effect of added cAMP similar to that of the *crp*-771* mutation, and (iii) a partial restoration by *crp*-771* of the growth defects of a *cya* mutant strain which lacks adenylate cyclase activity (21, 35). A fourth piece of evidence is as follows. The PTS-mediated repression by glucose of β-galactosidase synthesis in a *S. typhimurium* strain with a *lac*-containing plasmid was relieved by the *crp*-771* mutation.

Figure 4 shows the sensitivity of β-galactosidase synthesis to repression by glucose in a wild-type strain, a *crp*-771* strain, and a *crr* strain. Synthesis in the wild-type strain was completely inhibited within 2 min of the addition of glucose regardless of whether glucose was added with the inducer (isopropyl-1-thio-β-D-galactopyranoside), 10 min after the inducer was added, or 16 min after the inducer was added.

The total inhibition of β-galactosidase synthesis (transient repression) observed in the wild-type strain was absent in the *crp*-771* strain. Instead, a lowered rate of synthesis occurred after glucose was added, similar to the rate expected for permanent repression. This indicates that, in carrying out its role in the initiation of transcription of the *lac* operon, CRP does not respond to the lowering of cAMP levels expected after the PTS-mediated inhibition of adenylate cyclase activity by glucose. The permanent repression by glucose is still operative. This result and the results described above provide fairly strong evidence that the altered CRP in *crp*-771* is capable of initiating the transcription of some normally cAMP-dependent operons independently of cAMP. This conclusion is consistent with the fact that *crp*-771* can restore some of the growth deficiencies of a *cya* strain that is not capable of producing cAMP (35).

A study of the repression of β-galactosidase synthesis by glucose in *crr* strains (Fig. 4) revealed that transient repression was also absent (or very weak) in these strains. The rate of synthesis in *crr-306*/F'*lac* was very low compared with the rates in the wild-type or *crp*-771* strains and responded very little to the addition of glucose. The rate of synthesis was not completely inhibited but assumed a new course, similar to what would be expected for cAMP-independent permanent repression of β-galactosidase (16). Strain *crr-303*/F'*lac* yielded results almost identical to those obtained with *crr-306*/

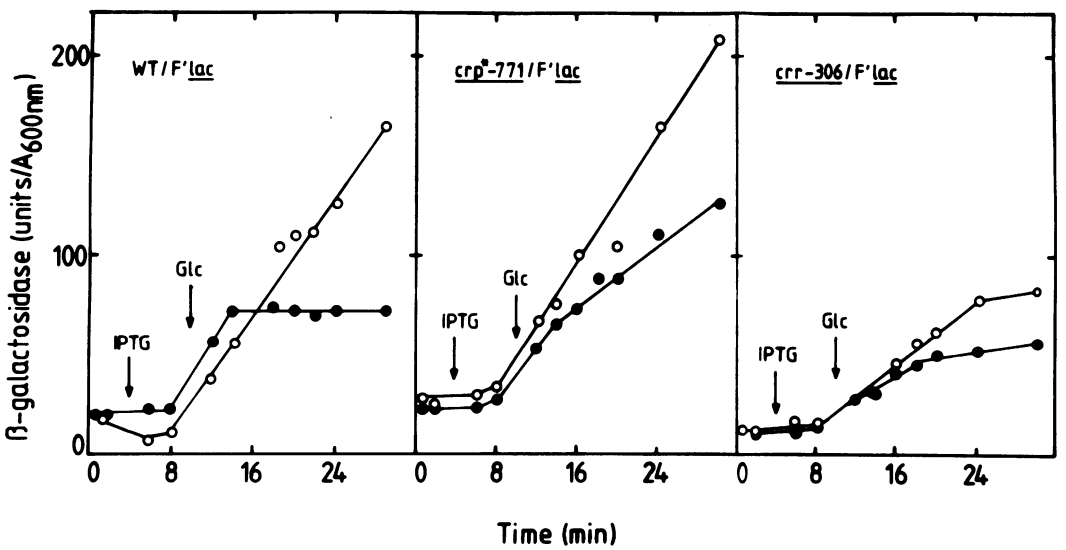

FIG. 4. Repression of β-galactosidase synthesis by glucose in *S. typhimurium* strains containing an F'*lac* plasmid. Cells growing exponentially in medium A supplemented with 0.2% galactose were divided into 20-ml portions and assayed for β-galactosidase activity beginning at zero time, as described in the text. At 4 min, the induction of β-galactosidase was begun by adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Glucose (Glc) repression was tested by adding glucose at 10 min (●). The *cysA20*/F'*lac* strain received 0.5 mM glucose, and the other strains received 10 mM glucose. The results are expressed as units of β-galactosidase activity per A_{600} unit of cells versus the time of incubation. WT, Wild type.

F'*lac* (data not shown). Both *crr* mutations resulted in the complete absence of soluble III^{Glc} (37a). Low cAMP levels were probably the cause of the low unrepressed rates of β-galactosidase synthesis in these strains (Fig. 4). Since the adenylate cyclase activity was permanently unstimulated (or very weakly stimulated) in these strains due to the low III^{Glc} levels (only the membrane-bound III^{Glc}-like protein[s] was still present), the addition of glucose could have very little effect on the basal adenylate cyclase activity remaining and, thus, had little effect on β-galactosidase synthesis (little or no transient repression).

Regulation of cAMP levels by the CRP. CRP involvement in the regulation of cAMP levels has been reported previously (7–9, 23, 24). In the course of our studies, we noticed that the internal cAMP concentration in a *crp*-771* strain was much higher than the concentration in wild-type *Salmonella* (Table 5). In contrast, extracellular cAMP levels were very low in a *crp*-771* strain (Table 5). The adenylate cyclase activity of this strain was normal (Table 5) and was sensitive to PTS-mediated glucose repression. A normal rate of synthesis of cAMP may result in high internal cAMP concentrations if the *crp*-771* mutation causes a reduction in the rate of export of cAMP. In this way, the steady-state internal concentration would be maintained much higher than would be predicted from the initial rate of synthesis.

DISCUSSION

Mechanism of inducer exclusion. In 1959, when the glucose effect was still poorly understood, Cohn and Horibata (3) described the phenomena of heterogeneity and maintenance of *lac* operon expression in *E. coli*. These authors presented evidence that, under conditions in which the inducer concentration was at a suboptimal concentration, a heterogeneous population containing induced and uninduced cells was produced, which could be maintained upon the addition of repressor (glucose). The uninduced cells could not be induced in the presence of glucose regardless of the presence of inducer, and the induced cells were not repressed by glucose as long as inducer was present. Cohn and Horibata suggested that maintenance is a function of lactose permease activity (3), and in this paper we present a mechanistic explanation for the results and conclusions of these authors.

The sensitivity of a PTS-sensitive substrate to inducer exclusion depends on the level of PTS-sensitive transport system activity and the level of III^{Glc} (Fig. 2 and Tables 2 and 3). We describe a simple model, in which we propose that non-phosphorylated III^{Glc} and PTS-sensitive transport systems interact stoichiometrically with each other, forming an inactive complex. According to this simple model, any condition affecting one of the two components of the inactive transport complex also affects sensitivity to inducer exclusion. At low permease levels, the ratio of III^{Glc} to permease is presumably high, and most or all of the permease is inhibitable by non-phosphorylated III^{Glc} (Fig. 2). In contrast, at high permease levels the ratio of III^{Glc} to permease is considerably lower, and the limited amount of III^{Glc} in the cells (37) is not capable of effectively inhibiting all of the per-

TABLE 5. Adenylate cyclase activity and cAMP levels in *S. typhimurium* strains

Expt	Strain	Relevant genotype	Adenylate cyclase[a]		cAMP[b]	
			−Glucose	+Glucose	Intracellular	Medium
1	*cysA20*	Wild type	14.1	1.6	2.6	0.09
	PP914	*crp*-771*	19.4	3.9	7.1	0.01
	PP780	*crr-303*	2.1	1.5	1.0	0.03
	PP782	*crr-306*	4.6	4.6	1.1	0.065
	PP825	*crr-306 crp*-771*	5.7	5.0	ND[c]	ND
	PP642	Δ(*ptsHI*)*41*	2.8	2.1	2.3	0.05
	PP800	Δ(*ptsHI-crr*)*49*	3.4	3.0	ND	ND
2	SB3507	Wild type	8.3	1.2	ND	ND
	SB1476	*ptsI17*	8.7	1.1	ND	ND
	TT2104	*cya-961*	0	ND	ND	ND

[a] Exponentially growing cells in nutrient broth containing 0.2% glucose were harvested, washed, treated with toluene, and assayed for adenylate cyclase as described in the text. The results are expressed as picomoles of cAMP formed per minute per milligram of protein. When present, glucose was added to a final concentration of 10 mM.

[b] Cells were grown exponentially in minimal medium A containing 0.2% galactose, filtered, and washed with warm growth medium. The filtered cells and filtrate were assayed for cAMP content as described in the text. Internal (intracellular) cAMP results are expressed as picomoles of cAMP per microliter of internal volume; the extracellular (medium) cAMP results are expressed as micromoles of cAMP per liter of growth medium.

[c] ND, Not determined.

mease molecules (Fig. 2), resulting in an apparent insensitivity to inducer exclusion.

According to this model, other factors controlling inducer exclusion are the level and state of phosphorylation of factor III^{Glc}. The state of phosphorylation of factor III^{Glc} is determined by the rate of phosphorylation, which depends on the levels of phosphoenolpyruvate, enzyme I, and HPr, and the rate of dephosphorylation, which depends on the presence of a PTS substrate and a corresponding membrane-bound enzyme II (20, 26, 29, 36). Strains containing levels of enzyme I (*ptsI*) or HPr (*ptsH*) lower than the levels in the wild type cannot easily maintain III^{Glc} in the phosphorylated state in the presence of a PTS substrate. Such strains are hypersensitive to inducer exclusion (20, 26). In strains lacking one of the specific enzymes II of the PTS (*ptsG*, *ptsM*, etc.), phosphorylated III^{Glc} cannot be dephosphorylated by the PTS substrates for which the missing enzyme II is specific. Such strains are also insensitive to inducer exclusion and to adenylate cyclase regulation by these substrates (10, 29).

It was concluded recently that the addition of the PTS substrate glucose to a wild-type culture of *S. typhimurium* very likely causes a net dephosphorylation of III^{Glc} (36). Therefore, it follows that inducer exclusion may also occur in wild-type cells, and in this paper we present evidence that, under the proper conditions (low permease activity), this can be demonstrated. It only remained to be determined whether, in cells containing wild-type levels of the general PTS enzymes (enzyme I and HPr), inducer exclusion also depended on the levels of factor III^{Glc}. Our *crr* mutations were selected as suppressors of the *pts* phenotype, as described previously by Saier and Roseman (31). *pts crr* double mutants have been shown previously to be resistant to inducer exclusion. This was attributed to the absence or low level of III^{Glc} (20, 32). Indeed, *crr* mutant strains containing only about 10 to 20% of the wild-type III^{Glc} activity were much less sensitive to inducer exclusion, but under the proper conditions they could still be inhibited by 100% (Fig. 2). This decrease in sensitivity seems to be roughly comparable (at least in *crr-307*::Tn*10*) to the decrease in III^{Glc} activity. The amount of transport activity at which the uptake of maltose is 50% inhibited in *crr-307*::Tn*10* is approximately one-fifth the amount at which maltose uptake is 50% inhibited in the wild-type strain (Fig. 2).

However, as we have shown recently (37a), some of our *crr* strains do not contain any detectable III^{Glc} in the soluble fraction (200,000 × *g* supernatant), as measured with a specific antiserum against purified III^{Glc}. These *crr* strains exhibit some surprising properties; they are able to transport methyl α-glucoside (specific for the III^{Glc}-IIB^{Glc} system) at about 10% of the wild-type rate, and membrane preparations from these *crr* strains catalyze in vitro methyl α-glucoside phosphorylation which depends on enzyme I, HPr, IIB^{Glc}, and phosphoenolpyruvate. This latter activity is inhibited by antiserum against soluble III^{Glc}. The membrane-associated, III^{Glc}-like activity is still present in *crr* deletion strains (SB2950), a *crr*::Tn*10* strain (PP994), and several *crr* point mutants, which all lack soluble III^{Glc} completely. Most likely, inducer exclusion (for example, as measured in partially induced strain PP994 [Fig. 2], which lacks soluble III^{Glc} completely) is mediated by this membrane-associated, III^{Glc}-like protein(s), which thus plays a role similar to that of soluble III^{Glc} in both transport and regulation. It is not known yet whether the gene(s) coding for this membrane-bound III^{Glc} is linked to the known gene affecting the level of soluble III^{Glc}. The latter gene is most likely the structural gene for soluble III^{Glc} since *crr* mutations result in altered III^{Glc} proteins (37a). Furthermore, we have cloned a segment of the *Salmonella* chromosome which complements *crr* and over-produces III^{Glc} 10- to 20-fold (unpublished data).

It has been reported (Keeler et al., Fed. Proc. **36**:686, 1977) that cAMP is involved in the regulation of inducer exclusion. The initial explanation was that this is due to the regulation of a PTS regulatory function by cAMP. This conclusion appears to be supported by the evidence of Saier et al. (29), who showed that repression of the glycerol operon by glucose is accompanied by an increasing sensitivity to glycerol inducer exclusion, suggesting that a PTS regulatory function can be induced by glucose. However, the glucose effect observed by Saier et al. could have resulted from repression of the cAMP-dependent operon for glycerol, causing a gradual decrease in the levels of the glycerol transport system and a concomitant increase in sensitivity to inducer exclusion (29). Another complication in the experiment of these authors was that the level of IIB^{Glc} probably varied under their experimental conditions (38). Our experiments (Fig. 2 and Table 2 and 3) showed the same phenomenon observed by Saier et al. (29), except that the confusing involvement of glucose was avoided in our experiments.

Effect of *crp*-771* on inducer exclusion. Mutation *crp*-771* was isolated as a suppressor of a *crr* mutation permitting growth of *crr* strains on citrate, succinate, malate, and xylose. Transduction of *crp*-771* into other strains revealed that it partially relieves some of the growth defects resulting from a *ptsHI* deletion mutation or the *cya* mutation (21, 35). The growth defects of *cya* strains result from a lack of cAMP,

which, together with the CRP, is required for the initiation of transcription of cAMP-dependent operons. It is thought that in *crp*-771* strains some of these operons can be transcribed in the absence of cAMP as a result of a change in CRP, which is known to have an altered affinity for cAMP in such strains (35). This explanation of the *crp*-771* effect was confirmed by demonstrating that a *crp*-771* strain was insensitive to the PTS-mediated transient repression of β-galactosidase synthesis (Fig. 4). Transient repression is the transient or temporary inhibition of the synthesis of proteins coded for by cAMP-dependent operons. The mechanism of PTS-mediated transient repression involves the inhibition of adenylate cyclase by the PTS, resulting in a decrease in the intracellular cAMP concentration and a concomitant decrease in the expression of cAMP-dependent operons. The insensitivity to transient repression of the normally transient repression-sensitive *lac* operon (Fig. 4) in a *crp*-771* strain indicated that the CRP of this strain was no longer dependent on cAMP for the initiation of transcription of this cAMP-dependent operon.

Explaining the relief by *crp*-771* of the growth defects of mutants totally lacking HPr and enzyme I [Δ(*cysK-ptsHI*)*41*] (21, 35) is more difficult. The failure of Δ(*cysK-ptsHI*)*41* strains to grow on maltose, melibiose, and glycerol can be attributed to both of the regulatory roles of the PTS (inducer exclusion and regulation of adenylate cyclase activity). If the regulation of adenylate cyclase activity is the most important factor, the explanation for the relief of growth defects by *crp*-771* given above could also be used here. Two complications stood in the way of drawing a simple conclusion. In the first place, it is not entirely certain that the regulation of adenylate cyclase activity is the most important factor in the repression of growth on maltose, melibiose, and glycerol, and it has been suggested that in *Salmonella* inducer exclusion may also be an important factor (20, 28, 30–33). Since factor III^{Glc} is expected to be dephosphorylated permanently in Δ(*cysK-ptsHI*)*41* strains, the uptake of an inducer exclusion-sensitive substrate by these strains should be permanently inhibited, even if the relevant operon is expressed in the presence of *crp*-771*. In the second place, it has been discovered that *crp*-771* (and extracellularly added cAMP) could indeed relieve inducer exclusion in Δ(*cysK-ptsHI*)*41*, as well as in *ptsI17* (21, 35; unpublished data). Our finding that inducer exclusion depends on the relative cellular contents of III^{Glc} and the PTS-sensitive transport system proteins offers a solution to this problem. We conclude that the effect of *crp*-771* and the effect of added cAMP on inducer exclusion in Δ(*cysK-ptsHI*)*41* are expressed by allowing the synthesis of an inducer exclusion-sensitive transport system to a cellular concentration higher than that effectively inhibited by the PTS. However, the results with the *melP* mutation, which renders the expression of the *mel* operon independent of cAMP, show that just independence of cAMP is not always sufficient. *melP ptsI17* strains still show inducer exclusion of thiomethylgalactoside and melibiose, in contrast to *crp*-771 ptsI17* strains. This is emphasized by the fact that a *melP* Δ(*cysK-ptsHI*)*41* strain does not grow on melibiose. The most likely explanation for this is that the *melP* mutation does not result in the induction of enough melibiose transport proteins to overcome the inhibitory effects of the permanently non-phosphorylated factor III^{Glc} of this strain.

Effects of *crp*-771* on cAMP levels. The CRP functions in the regulation of cellular cAMP (2, 7, 8, 23, 24). This regulatory role of CRP was also reflected in our results. The altered CRP of *crp*-771* seemed to inhibit the export of cAMP but had almost no effect on adenylate cyclase activity (Table 5). We conclude that the high cAMP levels in *crp*-771* resulted from a lowered rate of export of cAMP, as reflected in the high internal but low external cAMP concentrations (Table 5).

This conclusion is generally consistent with previous reports of the regulation of cAMP efflux by CRP. Several authors have reported increased rates of cAMP efflux in *crp* strains compared with wild-type strains (7–9, 23). In one case, no effect on adenylate cyclase activity was reported in a *crp* strain (23), whereas in another case stimulation of cAMP production was the result of CRP deficiency (24). Potter et al. (23) have reported that the high rates of cAMP efflux in cultures of a *crp* strain correlate with high cAMP levels. Goldenbaum and Hall (9) have shown that inverted membrane vesicles from an *E. coli crp* strain exhibit threefold-higher cAMP transport activity than inverted vesicles from normal cells. It is not clear from these reports whether CRP affects only efflux or whether it also affects synthesis of cAMP, but it is obvious that CRP has several (possibly related) functions in the regulation of cell metabolism. Recently, Majerfeld and co-workers (17) have shown that *crp* strains of *E. coli* contain higher levels of adenylate cyclase than wild-type strains. It is possible that different mutations in the structural gene (*crp*) for CRP have different effects on the various regulatory functions.

As Table 5 shows, adenylate cyclase activity in *ptsHI* deletion mutants is about 20% of the wild-type (pts^+) activity and is roughly similar to the wild-type activity in the presence of a PTS substrate. Introduction of a *crr* mutation does

not alter adenylate cyclase activity. This does not support our previous proposal that non-phosphorylated III^{Glc} is an inhibitor of adenylate cyclase in addition to phosphorylated III^{Glc} being an activator (21, 35). From our data it is clear that the proposal of Peterkofsky and Gazdar (18) that phosphorylated enzyme I activates adenylate cyclase is unlikely. *crr* mutants containing normal levels of enzyme I have equally low adenylate cyclase activity. We do not know whether membrane-bound III^{Glc} regulates adenylate cyclase.

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