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Regulation of Cyclic AMP Synthesis by Enzyme III^{Glc} of the Phosphoenolpyruvate:Sugar Phosphotransferase System in *crp* Strains of *Salmonella typhimurium*

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We investigated the claim (J. Daniel, J. Bacteriol. 157:940–941, 1984) that nonphosphorylated enzyme III^{Glc} of the phosphoenolpyruvate:sugar phosphotransferase system is required for full synthesis of bacterial cyclic AMP (cAMP). In *crp* strains of *Salmonella typhimurium*, cAMP synthesis by intact cells was regulated by the phosphorylation state of enzyme III^{Glc}. Introduction of either a *pstHI* deletion mutation or a *crr*::Tn*10* mutation resulted in a low level of cAMP synthesis. In contrast, *crp* strains containing a leaky *pstI* mutation exhibited a high level of cAMP synthesis which was inhibited by phosphotransferase system carbohydrates. From these results, we conclude that phosphorylated enzyme III^{Glc} rather than nonphosphorylated enzyme III^{Glc} is required for full cAMP synthesis.

Synthesis of cyclic 3′,5′ AMP (cAMP) in *Escherichia coli* and *Salmonella typhimurium* is regulated by the phosphoenolpyruvate:sugar phosphotransferase system (PTS). PTS carbohydrates such as glucose, mannose, and the (nonmetabolizable) analogs α-methylglucoside and 2-deoxyglucose inhibit cAMP synthesis as measured in toluenized cells. *ptsI* mutants, defective in enzyme I of the PTS, or *crr* mutants, defective in enzyme III^{Glc}, have low levels of cAMP synthesis. To explain these phenomena, the hypothesis has been made that the phosphorylated form of the PTS enzyme III^{Glc} acts as an activator of adenylate cyclase (for a review, see references 10 and 13). *ptsI* mutants, in which enzyme III^{Glc} cannot be phosphorylated owing to lack of enzyme I, and *crr* mutants in which enzyme III^{Glc} is absent or inactive, both lack the activator. In wild-type cells, PTS carbohydrates can lower the level of cAMP synthesis if phosphorylated enzyme III^{Glc} is dephosphorylated faster than it can be rephosphorylated by phosphoenolpyruvate via the PTS. Recently, some data were reported by Daniel (5) that seem to contradict the simple hypothesis outlined above. Measurement of the amount of cAMP formed by *crp* mutants of *E. coli* showed that strains containing an additional *ptsI* mutation produced normal amounts, equal to that of the *crp pts*$^+$ strain. In contrast, *crp crr* mutants produced low levels of cAMP. It was concluded (5) that nonphosphorylated enzyme III^{Glc} is required for cAMP synthesis. In this report, we show that different results were obtained with *S. typhimurium crp* strains.

S. typhimurium crp strain TA3302 (*crp-403*) was obtained from B. Ames (2). PP1493 is an isogenic *crp*$^+$ transductant. PP1037 (*crp-773*::Tn*10 trpB223*) was isolated by random insertion of Tn*10* in SB3507 (*trpB223*) and selection of the *crp* phenotype (inability to grow on mannitol and maltose, for instance). The mutation was cotransducible with *cysG*, and the mutant lacked the cAMP binding protein (N. Guiso, unpublished data). Excision of Tn*10* (3) resulted in *crp* deletion strain PP1416. The *ptsI17* and Δ*ptsHI41* mutations (4) were introduced into TA3302 by cotransduction with *cysA1539*::Tn*10*, by using phage P22 and selection for tetracycline resistance. Because *crp* strains are unable to grow on most carbon sources, glucose was used to differentiate the resulting *ptsI17* (PP1693) and Δ*ptsHI* (PP1698) transductants from the isogenic *pts*$^+$ strains (PP1694 and PP1697). The *crr-307*::Tn*10* mutation (11) was introduced into TA3302 by direct selection for tetracycline resistance (PP1701). Similar strains were constructed by using PP1416 (*crp trpB223*) as a recipient. To measure cAMP production, cells were pregrown in Luria broth and diluted 25-fold in minimal medium A (11), containing 0.2% gluconate and 0.1% Casamino Acids (and 25 μg of cysteine per ml when required). When the effect of PTS carbohydrates was measured, 0.2% glucose was added to the Luria broth to induce enzyme II^{Glc}. Growth at 37°C was followed by measurement of the optical density at 600 nm. Samples were taken at certain times (for about one doubling time), and after the reaction was terminated by boiling and removal of cell material, the amount of cAMP formed in the supernatant was determined by the method of Tovey et al. (12). The rate of cAMP production is expressed as nanomoles of cAMP formed per milligram (dry weight) increase.

A *crp* strain of *S. typhimurium* produced and secreted at least 50 times more cAMP than the corresponding *crp*$^+$ strain (Table 1), similar to the results obtained with *E. coli* (5). cAMP synthesis was inhibited by the addition of PTS sugars such as glucose. Introduction of the *ptsHI* deletion (PP1698) or the *crr*::Tn*10* mutation (PP1701) in a *crp* strain lowered the rate of cAMP production to levels approaching those of the *crp*$^+$ parent. These results are similar to those obtained earlier in toluenized *crp*$^+$ cells (7, 9) but differ, in the case of the *ptsHI crp* strain, from the result reported by Daniel (5). We considered the possibility that the *ptsI* mutation used by Daniel was leaky. It is known that, at least in *crp*$^+$ strains, less than 1% residual enzyme I activity is sufficient to yield wild-type, *pts*$^+$, levels of cAMP synthesis (9). We introduced the leaky *ptsI17* mutation into the *crp* strain. PP1693 synthesized cAMP at a high rate which, by the addition of the PTS sugar glucose, was lowered to the level of the *crp* strain containing the *ptsHI* deletion (PP1698) or the *crr*::Tn*10* mutation (PP1701) (Table 1). Similar results were obtained with *pts* and *crr* derivatives from a *crp*

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TABLE 1. cAMP synthesis in *S. typhimurium crp* strains

Strain	Relevant genotype	Generation time (min)	cAMP synthesis[a]		
			−Glucose	+Glucose	+αMG
PP1493	*crp* $^+$	63	0.4	ND	ND
PP1697	*crp*	226	25.8	12.2	ND
PP1698	*crp* Δ(*cysK-ptsHI*)*41*	460	3.5	ND	ND
PP1701	*crp crr-307*::Tn*10*	179	1.6	ND	ND
PP1694	*crp*	201	23.5	13.1	21.7
PP1693	*crp ptsI17*	189	20.2	1.8	6.0

[a] The rate of cAMP synthesis was determined as described in the text. The rate is expressed as nanomoles of cAMP formed per milligram (dry weight) increase. Glucose or α-methylglucoside (αMG), when present, was added to a final concentration of 2 mM. ND, Not determined.

deletion strain, PP1416, which has a different background. Inhibition by α-methylglucoside was somewhat lower. This might have been due to the lower rate of dephosphorylation of phosphorylated enzyme III^{Glc} by α-methylglucoside via its enzyme II^{Glc} compared with glucose.

From these results, we conclude that regulation of cAMP synthesis by the PTS, in particular the role of phosphorylated and nonphosphorylated enzyme III^{Glc}, seems to be the same in *crp*$^+$ and *crp* strains. The conclusion by Daniel (5) that nonphosphorylated enzyme III^{Glc} is required for high levels of cAMP synthesis seems incorrect. The most likely explanation for his result is the use of a *ptsI* mutation that results in a low residual enzyme I level. Unfortunately, the effect of PTS sugars was not tested.

Our results, obtained with intact cells, allow one to consider the proposal, put forward for instance by Dobrogosz and co-workers (6), that the cAMP-binding protein (product of the *crp* gene) can exist in several conformations, one of which can bind to adenylate cyclase and inhibit the enzyme. The role of phosphorylated enzyme III^{Glc} would then be the removal of the inhibitor, the cAMP-binding protein. The results (Table 1) make this proposal less likely, because the *crp* mutants, lacking the cAMP-binding protein, were still regulated by the PTS.

It has long been known that the level of cAMP synthesis in bacterial extracts is very low compared with that in intact or toluenized cells (8). Furthermore, basal activity is not further inhibited by PTS sugars. We have been unable, however, to reconstitute adenylate cyclase activity in bacterial extracts with phosphoenolpyruvate and the purified PTS proteins enzyme I, HPr, and enzyme III^{Glc}, even though we used enzyme III^{Glc} concentrations that occur in the cell (1 to 2 mg of enzyme III^{Glc} per ml; J. L. den Blaauwen and P. W. Postma, unpublished data). Possibly, another protein is required for a high level of cAMP synthesis and its regulation by the PTS. Although adenylate cyclase is considered to be a soluble enzyme (14), one could imagine that it binds in the cell to a membrane protein(s), analogous to the binding of the F_1 ATPase to the F_0 part of the ATPase complex. Based on the sequence analysis of the DNA fragment containing the structural (*cya*) gene for cAMP, it has been suggested that a second gene, *cyaX*, is present, coding for a hydrophobic protein (1). Possibly, this CyaX protein anchors adenylate cyclase to the membrane and is responsible for its regulation by the PTS.

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