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Effects of *crp* Mutations on Adenosine 3',5'-Monophosphate Metabolism in *Salmonella typhimurium*

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Wild-type *Salmonella typhimurium* could not grow with exogenous cyclic adenosine 3',5'-monophosphate (AMP) as the sole source of phosphate, but mutants capable of cyclic AMP utilization could be isolated provided the parental strain contained a functional cyclic AMP phosphodiesterase. All cyclic AMP-utilizing mutants had the growth and fermentation properties of cyclic AMP receptor protein (*crp*) mutants, and some lacked cyclic AMP binding activity in vitro. The genetic defect in each such mutant was due to a single point mutation, which was co-transducible with *cysG*. *crp* mutants isolated by alternative procedures also exhibited the capacity to utilize cyclic AMP. *crp* mutants synthesized cyclic AMP at increased rates and contained enhanced cellular cyclic AMP levels relative to the parental strains, regardless of whether or not cyclic AMP phosphodiesterase was active. Moreover, adenylate cyclase activity in vivo was less sensitive to regulation by glucose, possibly because the enzyme II complexes of the phosphotransferase system, responsible for glucose transport and phosphorylation, could not be induced to maximal levels. This possibility was strengthened by the observation that enzyme II activity (measured both in vitro by sugar phosphorylation and in vivo by sugar transport and chemotaxis) was inducible in the parental strain but not in *crp* mutants. The results suggest that the cyclic AMP receptor protein regulates cyclic AMP metabolism as well as catabolic enzyme synthesis.

As part of a study on the regulation of cyclic adenosine 3',5'-monophosphate (AMP) metabolism in bacteria, we observed that wild-type *Salmonella typhimurium* strain LT-2 could not grow with cyclic AMP as the sole source of carbon, nitrogen, or phosphate. This was surprising because these baceria were known to catalyze the outwardly directed transmembrane transport of cyclic AMP (11) and to possess an intracellular cyclic AMP phosphodiesterase (EC 3.1.1.17), which hydrolyzes cyclic AMP to 5'-AMP (12, 18). The latter compound can be utilized as a source of phosphate for growth. Lack of cyclic AMP utilization, therefore, suggested either that uptake of the cyclic nucleotide occurred at a very slow rate or that the phosphodiesterase was not functional in vivo. Since intracellular cyclic AMP levels were found to be elevated in phosphodiesterase-negative strains (12, 18, 23), we concluded that the intracellular phosphodiesterase was active. This observation led to the possibility that the cyclic AMP transport system catalyzed an essentially unidirectional flux of the cyclic nucleotide across the cell membrane and that the process might be energy dependent (23).

To further elucidate the parameters controlling cyclic AMP metabolism, we attempted to isolate mutant strains capable of cyclic AMP utilization. The isolation and characterization of such mutants are described in the present report. It is shown that cyclic AMP-utilizing strains are defective for the cyclic AMP receptor protein (CR protein) and that loss of this protein alters intracellular cyclic AMP levels, cyclic AMP synthetic rates, and sensitivity of the cyclic AMP metabolic system to regulation. Decreased sensitivity of cyclic AMP metabolism to regulation by sugars such as glucose appears to result secondarily from abnormal induction of the sugar metabolic systems: glucose chemotaxis, transport, and phosphorylation activities were not inducible in *crp* mutants. A preliminary account of some of these results has appeared (M. H. Saier, Jr., R. C. Valentine, and B. U. Feucht, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P205, p. 178).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the present study are listed in Table 1. The complex liquid medium was twofold-concentrated nutrient

TABLE 1. *Bacterial strains*

Strain no.	Genotype	Reference or source
S. typhimurium		
LJ75	*crp-171*	Nitrosoguanidine mutagenesis of LT-2
LJ78	*crp-174*	Nitrosoguanidine mutagenesis of LT-2
LJ83	*cysG439*	B. N. Ames
LJ84	*metC30*	B. N. Ames
LJ95	*metC30 crp-175*[a]	Nitrosoguanidine mutagenesis of LJ84
LJ62	*cpd-401*	Strain LJ62 is the same as strain TA3311 (3)
LJ99	*cpd-401 crp-175*[a]	Transduction of LJ95 to *met*$^+$ with LJ62 as doner
TA3302	*crp-403*	B. N. Ames
MS2073	*cya-407*/F'1905(*fla-1829*)	Contact of TA3336 with MS1905 (25)
MS2083	*crp-401*/F'1905(*fla-1829*)	Contact of TA3339 with MS1905 (25)
E. coli		
1100	*thi*	
5333	*thi crp*	5, 13
158	*thi crp* (TS)	I. Pastan

[a] The *metC30* mutation gave rise to a temperature-sensitive phenotype, which was influenced by the *crp* mutation: methionine was required for growth of strain LJ84 at 37 C but not at 30 C. By contrast, methionine was required for growth of strain LJ95 at 42 C but not at 37 C. Therefore, *met*$^+$ transductants were selected at 42 C.

broth. The minimal phosphate-rich medium was medium 63 without iron (19). The phosphate-deficient salts medium contained, per liter: triethanolamine buffer, pH 7.2, 50 mmol; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and KCl, 5 mmol. Fermentation was estimated on eosin methylene blue indicator plates without lactose (Baltimore Biological Laboratories [BBL]) containing the carbon source at 0.5%. Solid media consisted of the minimal salts media described above supplemented with agar (Difco) (1.5%), a carbon source (0.2%), auxotrophic requirements (20 mg/liter), and a phosphate source when indicated. Aerobic growth of bacterial strains in liquid media was followed by measuring the culture density with a Klett colorimeter. Erlenmeyer flasks equipped with side arms were used for this purpose, with the flasks filled to less than 20% of capacity. Transduction was performed with phage P22 bearing the *int-4* and *cly-1* mutations (7). Manipulations and analyses were as described (4).

Cyclic AMP-utilizing mutants were isolated by the following procedure. *S. typhimurium* cells (2×10^7) were grown in phosphate-deficient medium and spread on solid agar medium containing cyclic AMP as the sole source of phosphate. The medium consisted of the phosphate-deficient salts solution described above supplemented with 0.2% glucose and 1 mM cyclic AMP. Mutagenesis was induced with a crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or 2-aminopurine, and the inverted plates were incubated at 37 C for 3 to 4 days. A ring of mutant colonies appeared around the mutagen. Clones were selected with a sterile loop and isolated. Attempts to apply this procedure to *Escherichia coli* were unsuccessful, because the *E. coli* strains tested could utilize cyclic AMP slowly as a phosphate source.

Assay procedures. Uptake of radioactive substrates was measured as described previously (22, 24). The cyclic AMP binding protein (the CR protein) was assayed essentially as described by Pastan et al. (13). Cyclic AMP was routinely measured by using the Gilman procedure, with the cyclic AMP binding protein from beef muscle purified through the diethylaminoethyl-cellulose step (6). The radioimmuno- and protein kinase assays (26, 27) gave results comparable to those obtained with the Gilman procedure (22).

Adenylate cyclase (EC 4.5.1.1) was assayed in vitro by measuring the formation of [^{32}P]cyclic AMP from α-[^{32}P]adenosine 5'-triphosphate (8). Extracts were derived from cells harvested during exponential growth in medium 63 containing 0.5% glucose as carbon source. Cells were washed three times with medium 63, suspended in 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, and ruptured by passage through a French pressure cell. Cell debris was removed by centrifugation (15,000 $\times$ *g* for 5 min), and the extract was assayed for adenylate cyclase (8). The assay mixture contained in a final volume of 100 μl: tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 9.0, 25 mM; $MgCl_2$, 10 mM; phosphoenolpyruvate, 2 mM; α-[^{32}P]adenosine 5'-triphosphate, 1 mM, 10 μCi/μmol. Incubations were at 37 C for 20 min. Control incubation mixtures either lacked enzyme or were left at 0 C. Enzymes of the phosphotransferase system were assayed as described (9, 22), except that the concentration of the radioactive sugar was 20 μM and the incubation volume was 0.5 ml. Cyclic AMP phosphodiesterase activity in bacterial extracts was assayed as follows. The reaction mixture contained 40 mM tris(hydroxymethyl)-aminomethane-hydrochloride, pH 8.4, 4 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM [^{3}H]cyclic AMP (10 μCi/μmol), and a cell-free extract, to a final protein concentration between 0.1 and 1 mg/ml. After a 30-min incubation at 37 C, the reaction was stopped by addition of 5'-AMP and cyclic AMP (5 μmol/ml), and the tubes were placed in a boiling-water bath for 2 min. Aliquots (50 μl) were spotted onto Whatman 3 MM filter paper moistened with a 1% sodium borate buffer, pH 10. The radioactive compounds

were separated by high-voltage electrophoresis (30 min at 3,000 V) using the 1% sodium borate buffer. This procedure separates adenosine, cyclic AMP, and 5'-AMP, which migrate toward the anode at increasing rates according to their charge. Negligible amounts of adenosine were formed. Cyclic AMP phosphodiesterase activity, measured as the amount of cyclic AMP hydrolyzed to 5'-AMP, was proportional to time and protein concentration under the conditions used.

Measurement of chemotactic capacity. *crp* and *cya* mutant strains used for chemotaxis measurements (strains MS2083 and MS2073, respectively; Table 1) exhibited the growth and fermentation phenotype of "tight" mutants. The genetic defects in these strains have been shown to be due to nonsense mutations (B. N. Ames, personal communication). Both mutants contained an *E. coli* F' episome bearing the *fla-1829* mutation (Table 1), which releases flagellar synthesis from cyclic AMP control (25). The mutants were fully motile under the conditions of the experiment. Whereas strain MS2083 showed elevated cyclic AMP production relative to strain LT-2, strain MS2073 synthesized less than 2% of the wild-type amount. For measurement of chemotaxis, cells were grown at 30 C in medium 63 supplemented with 0.4% D-glucose and 0.5 mM ethylenediaminetetraacetic acid in the presence or absence of cyclic AMP. The cells were harvested during exponential growth, washed free of sugar by repeated centrifugation, and resuspended to a cell density of 10^7 cells/ml in medium 63 supplemented with 0.5 mM ethylenediaminetetraacetic acid, 10 μg of methionine per ml, and 0.2% D,L-lactate (chemotaxis medium). Chemotaxis was estimated by the capillary assay as modified by Aksamit and Koshland (2). Capillary tubes (Drummond Microcaps, 1-μl capacity and sealed at one end) were filled with chemotaxis medium containing the attractant to be tested, usually at a concentration of 1 mM. The open end of the tube was inserted into the bacterial suspension so that a diffusion gradient of the attractant was established. Bacteria were allowed to migrate into the capillary tube during a 40-min incubation period at 30 C. Subsequently, the number of bacteria in the tube was determined (2).

Materials. Radioactive substrates were purchased from New England Nuclear Corp. with the exception of α-[^{32}P]adenosine 5'-triphosphate, which was obtained from ICN, and α-D-[*methyl*-^{14}C]glucoside, which was from Amersham-Searle. Non-radioactive compounds were obtained either from Calbiochem or Sigma Chemical Co. and were of the highest purity available. Sugars were of the D-configuration unless noted otherwise.

RESULTS

Growth and fermentation properties of cyclic AMP-utilizing mutants. Twelve cyclic AMP-utilizing strains were isolated as described above. All 12 mutants exhibited the same qualitative growth and fermentation properties, although quantitative differences were noted. The properties of two such mutants are described: LJ75 exhibited the "tight" phenotype, whereas LJ78 exhibited the "leaky" phenotype.

All mutants tested could utilize cyclic AMP as a phosphate source for growth both in liquid and on solid media. However, a fairly high concentration (0.2 mM) of the cyclic nucleotide was required to support growth (Table 2). Cyclic AMP at a concentration of 2 mM supported growth at the maximal rate. In the presence of this concentration of cyclic AMP, LJ75 grew with a generation time of 3 h, although no detectable growth of strain LT-2 was observed under the same conditions. The leaky mutant, LJ78, grew more slowly than LJ75 (Table 2). Neither mutant could utilize cyclic guanosine 5'-monophosphate as a phosphate source for growth.

Table 3 summarizes the fermentation properties of selected strains. The fermentation phenotype shown for strain LJ75 was that of a mutant defective for cyclic AMP synthesis or action (a *cya* or *crp* mutant, respectively; 5, 14). If the mutants were defective for adenylate cy-

TABLE 2. *Growth of S. typhimurium strains with glucose as carbon source and cyclic AMP as sole phosphate source*

Strain	Cyclic AMP (mM)	Generation time (h)
LT-2	0	∞[a]
LT-2	5	∞
LJ75	0	∞
LJ75	0.1	∞
LJ75	0.25	13
LJ75	0.5	8
LJ75	1.0	4
LJ75	2.5	3
LJ75	5.0	3
LJ78	1.0	7

[a] ∞ signifies a generation time in excess of 20 h.

TABLE 3. *Fermentation of sugars by S. typhimurium strain LT-2 and two cyclic AMP-utilizing mutants*

Sugar	Fermentation response by strain[a]		
	LT-2	LJ78	LJ75
Glucose	+	+	+
Galactose	+	+	+
Mannose	+	±	−
Mannitol	+	±	−
Maltose	+	−	−
Melibiose	+	−	−

[a] Fermentation responses were recorded after a 24-h incubation at 37 C: +, green sheen (strong fermentation); ±, red colonies (weak fermentation); −, pink colonies (little or no fermentation).

clase, inclusion of cyclic AMP in the fermentation agar should reverse the negative fermentation phenotype (14). Cyclic AMP did allow authentic adenylate cyclase-negative mutants to ferment maltose but had no effect on maltose fermentation by any of the 12 cyclic AMP-utilizing strains studied. Thus, the phenotypes of the cyclic AMP-utilizing mutants were as expected for *crp* mutants.

Characterization of cyclic AMP-utilizing strains as *crp* mutants. If cyclic AMP utilization is associated with an abnormal *crp* gene product, two predictions can be made: first, the genetic defect should be co-transducible with *cysG* (7), and, second, at least some of the mutants should be defective for cyclic AMP binding activity in vitro (13). Both predictions were verified. Using four different cyclic AMP-utilizing strains, including LJ75 and LJ78 and phage P22 as carrier, the mutational defects were shown to be about 10% co-transducible with the *cysG439* marker, as is the *crp* gene (7). Moreover, assay of cyclic AMP-utilizing strains for cyclic AMP binding activity revealed that strain LJ78 showed appreciable binding activity, but LJ75 and an independently isolated *crp* mutant, TA3302, were essentially devoid of activity (Table 4). These results suggest that the mutational defects permitting cyclic AMP utilization were in the *crp* gene.

Several *Salmonella crp* mutants, which had been isolated by alternative procedures, were tested for the ability to grow with cyclic AMP as a sole source of phosphate. Among these strains were *crp* mutants in which the genetic lesion had been characterized as a nonsense or frameshift mutation (Ames, personal communication). The mutants showed increased rates of cyclic AMP utilization, suggesting that the absence of a CR protein that could function in ribonucleic acid transcription, rather than the presence of a specifically altered CR protein, was responsible for cyclic AMP utilization. Since all of the mutants isolated could be reverted to the wild-type phenotype, it was presumed that the genetic defects resulted from point mutations.

Dependence of cyclic AMP utilization on cyclic AMP phosphodiesterase. Exposure of *S. typhimurium* strain LJ62 (which lacked cyclic AMP phosphodiesterase activity) to mutagen on phosphate-deficient medium supplemented with cyclic AMP did not give rise to cyclic AMP-utilizing mutants. This fact suggested that the cyclic AMP phosphodiesterase was required for cyclic AMP utilization, a conclusion which was substantiated as follows. The *crp-175* mutation, which allowed cyclic AMP utilization, was introduced into strain LJ84 (*metC30*). Subsequently, the *metC30* auxotrophic marker was eliminated by transduction, using strain LJ62 as donor. Since the *cpd* and *metC* genes are co-transducible (3), double mutants bearing the *crp-175* and *cpd-401* mutations could be isolated. The identity of these strains was established by in vitro enzyme analyses. All of five such transductants tested were incapable of cyclic AMP utilization. It therefore appeared that cyclic AMP utilization depended both on defective CR protein function and normal cyclic AMP phosphodiesterase function. This experiment confirmed the conclusion that cyclic AMP phosphodiesterase is functional in vivo.

TABLE 4. *In vitro activities of the proteins involved in cyclic AMP metabolism and function*

Strain	Relative sp act (%)		
	CR protein	Cyclic AMP phosphodiesterase	Adenylate cyclase
LT-2	100[a]	100[b]	–[c]
LJ78	60	–	–
LJ75	10	–	–
TA3302	10	–	–
LJ84	–	100	–
LJ95	–	70	–
LJ62	100	<2	100[d]
LJ99	60	<2	340

[a] 100% binding activity corresponds to an increase of 70% over the control value. A high concentration of non-radioactive cyclic AMP (10 mM) was included in the control assay tube (but not the experimental sample) to eliminate high affinity binding of the radioactive cyclic nucleotide (13).

[b] 100% cyclic AMP phosphodiesterase activity corresponds to 20 nmol of cyclic AMP hydrolyzed/min per mg of protein.

[c] –, Not determined.

[d] 100% adenylate cyclase activity corresponds to 15 pmol of cyclic AMP formed/min per mg of protein.

Cyclic AMP production by *S. typhimurium* strains. Kinetic studies showed that the rate of cyclic AMP production by a growing bacterial culture was maximal when the cells approached the stationary growth phase and decreased to zero in the late stationary phase of growth (22). The amount of cyclic AMP that accumulated in the growth medium under a given set of conditions was strain dependent. *crp* mutants produced more cyclic AMP than the parental strain, and this effect of the *crp* mutation was not related to the cyclic AMP phosphodiesterase activity of the cell (Table 5). An examination of eight different *crp* mutants revealed a correlation between the maximal concentration of cyclic AMP in the medium and the severity of the *crp* mutation, as judged by fermentation responses. Thus, the tight *crp*

TABLE 5. *Net cyclic AMP production by bacterial cells grown in nutrient broth*[a]

Strain	Genotype	Cyclic AMP produced (μmol/g [dry wt] of cells)
S. typhimurium		
LT-2	Wild type	0.6
LJ78	*crp-174*	4.6
LJ75	*crp-171*	7.4
LJ62	*cpd-401*	1.9
LJ99	*cpd-401 crp-175*	57
E. coli		
1100	*thi*$^-$	0.8
5333	*thi*$^-$ *crp*$^-$	3.5

[a] Cells were grown at 37 C with aeration in 5 ml of nutrient broth. Sixteen hours after inoculation, all cells were in the late stationary growth phase. An aliquot of the total cell suspension was placed in a boiling-water bath for 4 min, cells were removed by centrifugation, and cyclic AMP in the supernatant was measured.

mutants produced more cyclic AMP than the leaky mutants (Table 5).

Glucose and methyl α-glucoside are known to depress rates of cyclic AMP synthesis in wild-type bacteria (11, 15, 21), and the influence of the *crp* mutation on glucose inhibition was studied under carefully defined conditions. Strains were grown in medium 63 in the presence of glucose, lactate, or another carbon source, and the cells were harvested, washed, and resuspended in salts medium 63. The rate of cyclic AMP synthesis was then measured in the presence or absence of sugar. The *crp* mutation enhanced cyclic AMP synthetic rates when measured in vivo or in vitro (Fig. 1; Table 4). It also increased intracellular levels of cyclic AMP and rendered net cyclic AMP synthesis less sensitive to inhibition by sugars such as glucose (Fig. 1). No evidence for defective cyclic AMP transport was obtained (unpublished data).

Lack of induction of glucose chemotaxis, transport, and enzyme II activities in *crp* and *cya* mutants as an explanation for defective regulation of cyclic AMP synthesis by glucose. To explain the observation that cyclic AMP synthesis was less sensitive to glucose regulation in *crp* mutants than in the parental strain, we measured the activities of the components of the phosphotransferase system (9, 10, 20) that have been implicated in the regulation of cyclic AMP production (15, 21, 22). Methyl α-glucoside transport and phosphorylation activities were inducible in strain LJ62 but not in the isogenic *crp* mutant, strain LJ99 (Table 6). The results suggest that the synthesis of the glucose-enzyme II complexes is subject to control by cyclic AMP and the CR protein. Since these enzyme complexes are required for the in vivo inhibition of net cyclic AMP synthesis by glucose (22), lack of induction provides an explanation for the diminished sensitivity of *crp* mutants to glucose regulation.

That a deficiency of the CR protein secondarily influenced the regulatory effect of glucose on cyclic AMP production was further substantiated using a temperature-sensitive *crp* mutant of *E. coli*. Cells were grown at the permissive temperature (28 C) in minimal medium supplemented with glucose and thiamine, harvested during exponential growth, and assayed for induced β-galactosidase synthesis and cyclic AMP production before and after incubation at the nonpermissive temperature (42 C). β-Ga-

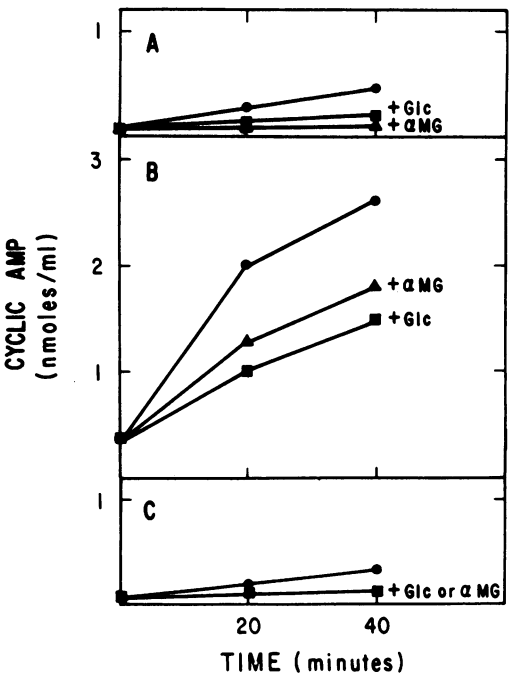

FIG. 1. *Production of cyclic AMP by S. typhimurium strains in the absence and presence of sugars. Cells were grown in nutrient broth supplemented with 1% D,L-lactate, harvested during logarithmic growth, washed with cold medium 63, and resuspended in medium 63 containing 0.2% D,L-lactate to a final cell density of 0.24 mg of dry cells per ml. Glucose (Glc, 0.1%, ■) or methyl α-glucoside (αMG, 0.1%, ▲) was added as indicated, or no addition was made (●). Cells were incubated at 37 C for 0, 20, or 40 min. Subsequently cyclic AMP was extracted and measured as described in the text. The figure shows cyclic AMP production by S. typhimurium strain LJ62(A); strain LJ99(B); and a crp*$^+$ *revertant of strain LJ99(C).*

lactosidase induction was used as a measure of functional CR protein activity in vivo. The results are summarized in Table 7. The parental strain (1100) synthesized β-galactosidase regardless of the growth temperature, and production of cyclic AMP was strongly inhibited by glucose after growth at either 28 or 42 C. By contrast, the tight *crp* mutant (strain 5333) could not be induced for β-galactosidase synthesis after growth at either temperature, and glucose exerted a stimulatory rather than an inhibitory effect on net cyclic AMP synthesis under the conditions used. The temperature-sensitive *crp* mutant (strain 158) synthesized β-galactosidase after growth at the permissive temperature (28 C), and cyclic AMP production was strongly inhibited by glucose. Heat destruction of the CR protein, as assayed by β-galactosidase inducibility, did not alter sensitivity of cyclic AMP synthesis to glucose inhibition. However, glucose stimulated cyclic AMP synthesis when the cells had been grown at the nonpermissive temperature. These results support the conclusion that the CR protein is indirectly involved in the regulation of cyclic AMP production, possibly because of its regulatory effect on the synthesis of the glucose-enzyme II complexes of the phosphotransferase system.

Adler and Epstein have presented evidence suggesting that, in *E. coli*, the enzymes II of the phosphotransferase system mediate chemotaxis of the bacterium toward sugar substrates of this enzyme system (1). In view of the observation (Table 6) that glucose-enzyme II activity is inducible in the parental strain, but not in a *crp* mutant, it would be predicted that a *crp* (or *cya*) mutation would depress glucose chemotaxis relative to that observed in the induced parental strain. Although synthesis of flagellar components is normally subject to cyclic AMP control, this hypothesis could be tested by using strains capable of synthesizing functional bacterial flagella in the absence of cyclic AMP or the CR protein (25). The chemotactic responses observed with these strains verified this prediction (Table 8). All strains exhibited normal chemotaxis toward L-aspartate, but the *crp* and the *cya* mutations specifically depressed chemotactic activity toward D-glucose. In the *cya* mutant, but not in the *crp* mutant, glucose taxis was restored by inclusion of cyclic AMP in the medium during growth.

TABLE 6. *Activities of the methyl α-glucoside enzyme II measured in vivo and in vitro*

Strain	Carbon source for growth	Rate of methyl α-glucoside	
		Transport (μmol/min per g [dry wt])	Phosphorylation[a] (nmol/min/mg of protein)
LJ62	Lactate	0.61	0.03
LJ62	Glucose	2.3	0.12
LJ99	Lactate	0.43	0.02
LJ99	Glucose	0.51	0.03

[a] Methyl α-glucoside phosphorylation was measured in the presence of excess enzyme I and HPr so that the enzyme II complex (10) was limiting. Additional assays showed that neither enzyme I nor HPr activities (9) were inducible in strain LJ99. In some *crp* and *cya* mutants of *S. typhimurium*, the in vitro activities of the various enzyme II complexes of the PTS were depressed two- to fourfold below the uninduced activities of the wild-type parental strain.

TABLE 7. *Dependence of cyclic AMP production on the cyclic AMP receptor protein in E. coli*

Strain	Growth temp (C)[a]	Heated[b]	β-galactosidase induction[c]	Cyclic AMP production[d]	
				− Glucose	+ Glucose
1100	26	−	49	42	0
1100	26	+	40	58	0
1100	42	−	75	42	0
158	26	−	20	42	7
158	26	+	3	35	3
158	42	−	4	17	62
5333	26	−	0.2	44	89
5333	42	−	0.1	23	87

[a] Cells were grown in medium 63 containing 0.1% glucose and 10 μg of thiamine per ml.

[b] The incubation period was 90 min at 42 C.

[c] Assayed in toluenized cells at 37 C and expressed as nanomoles of *o*-nitrophenyl-β-galactoside hydrolyzed per minute per milligram of dry cells.

[d] Expressed as nanomoles of cyclic AMP produced per minute per gram (dry weight) of cells at 28 C.

DISCUSSION

S. typhimurium strain LT-2 cannot utilize cyclic AMP as a source of phosphate for growth, but loss of the cyclic AMP receptor protein permits utilization of this nucleotide. Growth of a *crp* mutant on cyclic AMP requires a functional cyclic AMP phosphodiesterase, suggesting that utilization of the cyclic nucleotide involves its cleavage to 5'-AMP. By contrast, the periplasmic acid and alkaline phosphatases appear to be non-essential (unpublished data).

Both nonsense and frameshift mutations in the *crp* gene were found to allow utilization of cyclic AMP. This observation suggested that the absence of a CR protein that can function in stimulating transcription, rather than the presence of a specifically altered CR protein, was required for cyclic AMP utilization. Consequently, the CR protein probably controls cyclic

TABLE 8. *Chemotaxis of S. typhimurium strains toward* D-*glucose and* L-*aspartate*

Strain	Cyclic AMP during growth (10 mM)	Chemotactic attractant (1 mM)	Bacteria accumulated[a] (× 10^{-3})
LT-2[b] (wild type)	–	None	5
	–	Glucose	>100
	–	Aspartate	>100
MS2073[c]	–	None	4
[*cya-407*/F'1905	–	Glucose	8
(*fla-1829*)]	–	Aspartate	>100
	+	None	6
	+	Glucose	>100
	+	Aspartate	>100
MS2083[c]	–	None	6
[*crp-401*/F'1905	–	Glucose	10
(*fla-1829*)]	–	Aspartate	>100
	+	None	4
	+	Glucose	3
	+	Aspartate	>100

[a] Values (averages of duplicate determinations) represent the number of bacteria that accumulated in the capillary tube after a 40-min incubation period at 30 C.

[b] Strain LT-2 showed optimal chemotactic responses at attractant concentrations near 1 mM.

[c] These strains showed little or no chemotactic response toward glucose at sugar concentrations between 10^{-6} and 10^{-3} M. Inclusion of 5 mM cyclic AMP in the medium during the chemotaxis experiment did not promote chemotaxis toward glucose appreciably.

AMP utilization secondarily by influencing the synthesis of some cellular constituent. However, the nature of this constituent is not known. Thus, the activity of cyclic AMP phosphodiesterase was comparable in parental and *crp* mutant strains, and periplasmic cyclic AMP phosphodiesterase activity was not detected in either strain (unpublished data). Moreover, the *crp* mutation had no apparent effect on the excretion of cellular cyclic AMP, suggesting that the cyclic AMP transport system (11, 23) was normal.

The *crp* mutation, although permitting cyclic AMP utilization, also enhanced the rate of net cyclic AMP synthesis, as reported by others (16, 28). Enhanced adenylate cyclase activity could be demonstrated both in vivo and in vitro, leading to the possibility that synthesis of the enzyme is subject to negative control by the CR protein. Negative control of the synthesis of other enzymes by the CR protein has been reported (17). However, our studies do not allow the conclusion that the CR protein directly controls synthesis of adenylate cyclase; the functioning of the CR protein might influence the activity of the enzyme, either directly or secondarily as a result of its effect on the synthesis of another cellular constituent.

Of particular interest was the observation that cyclic AMP synthesis was less sensitive to inhibition by glucose in *crp* mutants relative to the parental strain. Our results with a temperature-sensitive *crp* mutant provide evidence that the CR protein is only secondarily involved. When cells were induced for the glucose-enzyme II at the permissive temperature and the CR protein was subsequently destroyed at the nonpermissive temperature, cyclic AMP synthesis remained sensitive to glucose inhibition. Moreover, it could be shown that the glucose-enzyme II complex was inducible in wild-type strains but not in tight *crp* mutants. This result demonstrated the dependence of induced synthesis of the glucose-enzyme II complex on the CR protein and provides an explanation for the altered sensitivity of adenylate cyclase activity in vivo to glucose inhibition.

Adler and Epstein have presented evidence that the enzyme II complexes of the phosphotransferase system function as chemoreceptors (1). If this suggestion is correct, then glucose chemotaxis should be dependent on cyclic AMP and the CR protein. Our results verified this prediction. Motile *cya* and *crp* mutants of *Salmonella* exhibited deficient chemotaxis toward glucose, although they were normally chemotactic toward L-aspartate. Restoration of glucose chemotaxis to the adenylate cyclase-deficient strain required that the cells be grown in the presence of cyclic AMP, suggesting that synthesis, rather than the activity of a cell constituent, was the target of cyclic AMP action. In view of the enzyme analyses reported and the studies of Adler and Epstein (1), this cell constituent is likely to be the glucose-enzyme II complex of the phosphotransferase system (9, 10, 20).

The present study leads to the suggestion that cyclic AMP metabolism in *S. typhimurium* is subject to regulation by several interrelated mechanisms. Controls appear to exist over the utilization, synthesis, degradation, and excretion of cytoplasmic cyclic AMP. Moreover, cellular constituents regulating or directly involved in carbon and energy metabolism appear to be implicated. It seems that complex feedback control mechanisms influencing cellular cyclic AMP levels have evolved to insure that cellular energy and carbon metabolism will occur at rates that meet, but do not exceed, the requirements of the bacterial cell.

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