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Fatty Acid Degradation in *Escherichia coli*: Requirement of Cyclic Adenosine Monophosphate and Cyclic Adenosine Monophosphate Receptor Protein for Enzyme Synthesis

GEORG PAULI,[1] RUTH EHRING, AND PETER OVERATH[2]

Institut für Genetik der Universität zu Köln, 5 Köln 41, Federal Republic of Germany

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The strong repression of inducible synthesis of the enzymes of fatty acid degradation by glucose can be partially relieved by the addition of cyclic adenosine 3′,5′ monophosphate (cyclic AMP) to the growth medium. This reversal of the glucose effect by cyclic AMP is *not* observed in a mutant (K29) that is unable to grow on fatty acids as sole carbon source and that was found to synthesize low levels of several enzymes specified by the *fad* regulon. In a revertant selected for the ability to grow on oleate these effects are concomitantly relieved. By both genetic (co-transduction of the mutation with the *strA* locus) and biochemical experiments (an extract of the mutant strain does not show the cyclic AMP-dependent stimulation of the deoxyribonucleic acid-directed in vitro synthesis of the enzymes of the *gal* operon), it is demonstrated that the mutant lacks functional cyclic AMP receptor protein (CR protein). It is concluded that, like many other inducible enzyme systems, expression of the enzymes of the *fad* system requires cyclic AMP and the CR protein.

The synthesis of the enzymes of fatty acid degradation in *Escherichia coli* is strongly repressed by the presence of glucose in the growth medium. Addition of an inducer, e.g., oleate, cannot relieve this repressing effect (7, 9, 10, 19). It has been shown for a variety of inducible enzyme systems that glucose exerts its repressing effect via a lowering of the intracellular level of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) which, in combination with a cyclic AMP receptor protein (CR protein), is required for maximal expression of the corresponding genes (3, 4, 8, 12, 14, 18). An influence of cyclic AMP on the synthesis of the enzymes of fatty acid degradation has been described by Weeks et al. (19): cells grown on glucose plus palmitate and cyclic AMP oxidized palmitate five times faster than cells grown in the absence of the nucleotide.

From 21 mutants unable to grow on fatty acids as sole carbon source (Fad mutants), two had both an unknown enzymatic defect and an undefined mutation that could not be assigned to one of the genes of the *fad* regulon (7). The mutation of one of these strains (K29) could now be mapped close to the *strA* locus. Because the structural gene of the CR protein is also closely linked to *strA*, it appeared likely that the mutation affected the gene specifying the CR protein. This study indicates that mutant K29 lacks a functional CR protein, suggesting that the *fad* regulon, like many other inducible systems, requires cyclic AMP for expression and that the stimulatory effect of cyclic AMP is mediated by the same CR protein.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1 (for genetic nomenclature see ref. 13 and 17). Strain K29 has been isolated as an oleate-negative strain from the prototrophic wild type K12Ymel after nitrous acid mutagenesis and penicillin selection (9). Anticipating the results of this study, the mutation of strain K29 will be referred to as *crp-29*. Co-transduction with the *strA* locus by phage P1 was used to transfer mutation *crp-29* from a *strA* derivative of strain K29 into different strains. Transfer of the mutation *crp-29* into a *fadR16* derivative of strain K12Ymel (9), a mutant showing constitutive synthesis of the enzymes of fatty acid degradation, enabled us to study the behavior of *crp-29* under conditions of derepressed enzyme synthesis. By using strain Fi165 (6) carrying a deletion of the *gal* operon as recipient, a strain (Fi165 *crp-29*) was constructed, extracts of which could be assayed for the presence of the CR protein in an in vitro system for the synthesis of the galactose enzymes. Similarly, the *cya* mutation was introduced from strain CA 7902 (16) by co-trans-

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[1] Present address: Robert-Koch-Institut, 1 Berlin 65, Nordufer 20, Federal Republic of Germany.

[2] Present address: Max-Planck-Institut für Biologie, 74 Tübingen, Corrensstrasse 38, Federal Republic of Germany.

Table 1. *Bacteria and bacteriophages*

Strain	Characteristics	Sex	Origin
K12Ymel	Prototrophic	F$^-$	U. Henning
fadR16	Derivative of K12Ymel, constitutive for the synthesis of the enzymes of the β-oxidation pathway	F$^-$	(9)
K29	Fad-derivative of K12Ymel carrying the mutation *crp-29*	F$^-$	This study
Fi165	*trp, gal* (deletion)	F$^-$	(6)
fadR16 crp-29	Derivative of *fadR16*	F$^-$	This study
Fi165 *crp-29*	Derivative of Fi165	F$^-$	This study
Fi165 *crp-29* (Rev.)	Fad$^+$ revertant of Fi165 *crp-29*	F$^-$	This study
CA7902	*cya*, derivative of a prototrophic HfrH	Hfr	(16)
cya	Derivative of K12Ymel	F$^-$	This study
crp-29 cya	Derivative of K29	F$^-$	This study
fadR16 cya	Derivative of *fadR16*	F$^-$	This study
AT700	*argG, his, aroE, gal, lac, malB, mtl, xyl, thi, strA, tsx*	F$^-$	A. Taylor
AT2455	Derivative of HfrH, *cysG, thi, mal*	Hfr	A. Taylor
P1	P1kc		U. Henning

duction with an *ilv* marker into the *fadR16* and *crp-29* derivatives of strain K12Ymel. A spontaneous Fad$^+$ revertant referred to as Fi165 *crp-29* (Rev.) was selected from the *crp-29* derivative of strain Fi165 by spreading cells on agar plates containing oleate as sole carbon source.

Growth conditions. Media for growing cells have been described before (9, 10). For the experiments shown in Table 2, the cells were inoculated into mineral salts medium with 0.5% glucose or 0.5% tryptone with or without 0.1% oleate plus 1% Brij35 and grown overnight. The cultures were then diluted and incubated with shaking at 37 C. The tryptone-containing cultures were harvested at an absorbance at 420 nm (optical density [OD] at 420 nm with a Gilford 240 spectrophotometer; one unit of absorbance corresponds to 0.107 mg of protein/ml) of 1.6 to 1.8. The glucose- or glucose plus oleate-containing cultures were divided into two parts at an OD$_{420}$ of 0.35. One part received cyclic AMP at 5×10^{-3} M final concentration. All cultures were then grown for another two generations before the cells were harvested by centrifugation.

Assay of the enzymes of the fad regulon and the CR protein. Acyl-coenzyme A (CoA) synthetase (acid:CoA ligase [AMP-forming], EC 6.2.1.3), hydroxy-acyl-CoA dehydrogenase (L-3-hydroxyacyl-CoA:NAD oxidoreductase, EC 1.1.1.35), and thiolase (acyl-CoA:acetyl-CoA *C*-acyltransferase, EC 2.3.1.16, also termed thiolase I in ref. 13) were determined in cell-free extracts as described previously (9, 10).

CR protein was assayed in the deoxyribonucleic acid (DNA)-directed in vitro system for the synthesis of the enzymes of the *gal* operon (galactokinase or adenosine 5′-triphosphate [ATP]:D-galactose 1-phosphotransferase, EC 2.7.1.6, and uridyltransferase or uridine 5′-diphosphate [UDP] glucose:α-D-galactose-1-phosphate uridylyltransferase, EC 2.7.7.12) as previously described (20). Crude extracts (20, 21) which contain ribosomes and protein factors required for protein synthesis were prepared from strains Fi165 as well as from its *crp-29* derivative and the Fad$^+$ revertant Fi165 *crp-29* (Rev.). Conditions for protein synthesis and assays of galactokinase and uridyltransferase were as described before (20, 21). A sample of CR protein purified through the phosphocellulose step according to W. B. Anderson et al. (1) was kindly provided by R. Willmund.

RESULTS AND DISCUSSION

Effect of cyclic AMP on the synthesis of the enzymes of fatty acid oxidation. Table 2 shows the effect of glucose and cyclic AMP on the enzyme levels of three enzymes involved in the oxidation of fatty acids. The synthesis of all three enzymes is induced by a long-chain-length fatty acid like oleate (lines 1 and 2, Table 2, and ref. 9, 10, and 19). The structural genes for thiolase and β-hydroxy-acyl-CoA dehydrogenase are part of an operon close to the *metE* gene near 77 min on the *E. coli* chromosome (9). Acyl-CoA synthetase is encoded by the *fadD* gene, which lies near 35 min (9, 17). The expression of both loci is under the control of the product of the *fadR* gene. Growth on glucose strongly represses the synthesis of these enzymes (line 3, and ref. 9). Cyclic AMP (line 4) and oleate (line 5) when added separately to the glucose-containing medium do not lead to an increase in enzyme synthesis. However, a combination of both (line 6) results in a 10- to 15-fold increase in the specific activity of thiolase and β-hydroxyacyl-CoA dehydrogenase and a threefold increase in the level of the acyl-CoA synthetase. The incomplete reversal of the glucose effect by cyclic AMP (compare lines 6 and 1) may be due to an insufficient intracellular level of cyclic AMP. An insufficient concentration of inducer (oleyl-CoA? [7]) appears to be ruled out by experiments with the constitutive mutant *fadR16* (lines 18 to 22). This strain also shows a strong "glucose effect" which is only

TABLE 2. *Effect of cyclic AMP on enzyme levels of fatty acid oxidation*

Line	Strain	Medium	Relative specific activity[a]		
			Thiolase	β-Hydroxyacyl-CoA dehydrogenase	Acyl-CoA synthetase
1	K12Ymel	Tryptone oleate	100	100	100
2	K12Ymel	Tryptone	12	14	37
3	K12Ymel	Glucose	1	1	8
4	K12Ymel	Glucose + cyclic AMP	0.9	0.9	10
5	K12Ymel	Glucose + oleate	1	1	11
6	K12Ymel	Glucose + oleate + cyclic AMP	10	15	33
7	K29	Tryptone + oleate	13	10	13
8	K29	Tryptone	3	2	7
9	K29	Glucose	0.4	1	5
10	K29	Glucose + oleate	0.5	0.8	5
11	K29	Glucose + oleate + cyclic AMP	0.7	1	6
12	*cya*[b]	Glucose	1	0.9	7
13	*cya*	Glucose + oleate	1	0.8	9
14	*cya*	Glucose + oleate + cyclic AMP	11	11	29
15	*crp-29-cya*[c]	Glucose	0.4	0.1	6
16	*crp-29 cya*	Glucose + oleate	0.5	0.2	7
17	*crp-29 cya*	Glucose + oleate + cyclic AMP	0.6	0.4	8
18	*fadR16*[d]	Tryptone	152	146	112
19	*fadR16*	Glucose	3	4	7
20	*fadR16*	Glucose + cyclic AMP	11	10	22
21	*fadR16*	Glucose + oleate	3	4	7
22	*fadR16*	Glucose + oleate + cyclic AMP	30	40	35
23	*fadR16 crp-29*	Tryptone + oleate	20	18	18
24	*fadR16 crp-29*	Tryptone	21	21	16
25	*fadR16 crp-29*	Glucose	3	2	6
26	*fadR16 crp-29*	Glucose + oleate	3	3	4
27	*fadR16 crp-29*	Glucose + oleate + cyclic AMP	3	3	5
28	*fadR16 cya*	Glucose	2	1	11
29	*fadR16 cya*	Glucose + oleate	2	1	9
30	*fadR16 cya*	Glucose + oleate + cyclic AMP	16	15	37
31	Fi165 *crp-29* (Rev.)[e]	Tryptone + oleate	54	58	43
32	Fi165 *crp-29* (Rev.)	Tryptone	9	11	27
33	Fi165 *crp-29* (Rev.)	Glucose	0.6	1	2
34	Fi165 *crp-29* (Rev.)	Glucose + oleate	0.7	1	3
35	Fi165 *crp-29* (Rev.)	Glucose + oleate + cyclic AMP	9	12	19

[a] The absolute specific activities for strain K12Ymel grown on tryptone + oleate in micromoles per minute per milligram of protein are: thiolase, 0.24; β-hydroxyacyl-CoA dehydrogenase, 2.7; acyl-CoA synthetase, 0.0033.

[b] When grown on tryptone plus oleate or tryptone, strain *cya* shows enzyme levels similar to strain K29 (lines 7 and 8).

[c] When grown on tryptone + oleate or tryptone, strain *crp-29 cya* shows enzyme levels similar to strain K29 (lines 7 and 8).

[d] It has been shown before (9) that enzyme synthesis in this strain is constitutive, i.e., not dependent on the addition of oleate to the medium.

[e] Strains Fi165 and Fi165 *crp-29* show enzyme levels similar to strains K12Ymel and K29, respectively.

partially relieved by cyclic AMP. Although in the absence of glucose, inducer has no effect on the enzyme levels of mutant *fadR16* (9), oleate in the presence of glucose and cyclic AMP results in a further two- to fourfold stimulation of enzyme synthesis in this strain (compare lines 20 to 22). This behavior is not understood at present. An explanation requires more knowledge of the function of the *fadR* gene product (repressor?).

Strain K29 has a pleiotropic defect on the synthesis of the three enzymes tested (lines 7 and 8). Therefore, this mutation could either be the result of a mutation in the *fadR* gene (similar to an i^s mutation of the *lac* system [21a]) or it may interfere with a different control element, e.g., the action of the cyclic AMP-CR protein complex. Strain K29 indeed does not respond to cyclic AMP (lines 9 to 11), suggesting that it may carry a mutation in the gene (*crp*) specifying the CR protein. The mutation in strain K29 is therefore referred to as *crp-29*. As expected (15, 16), a lesion in the adenyl-cyclase gene (*cya*) when introduced into strain K12Ymel can be compensated for by the addition of cyclic AMP (lines 12 to 14). In the double mutant *crp-29 cya*, enzyme synthesis is not stimulated by cyclic AMP (lines 15 to 17). The experiments reported on lines 23 through 30 with *crp-29* and *cya* in a *fadR* background are in accordance with the results obtained with the inducible strains. Strains Fi165 and Fi165 *crp-29*, which were used for the biochemical experiments (cf. Tables 4 and 5), show enzyme levels similar to those of strains K12Ymel and K29, respectively.

Mapping of the mutation of strain K29. Strain K29 was isolated as a mutant unable to use oleate as sole carbon source, but which was able to grow on acetate, succinate, and glucose. Although completely negative on oleate, it showed at least moderate growth on lactose, galactose, and glycerol, and thus did not reveal the pleiotropic negative phenotype on several carbon sources typical for *crp* mutants (16). This strange behavior is probably caused by the selection procedure used. The expected pleiotropic phenotype on lactose, galactose, and other carbon sources became evident after transfer of mutation *crp-29* by co-transduction with *strA* from strain K29 into either the wild-type strain K12Ymel or into strains *fadR16* and Fi165 (note that the latter carries a *gal* deletion). Despite the incomplete manifestation of the *crp* mutation in strain K29 with respect to the other carbon sources, this strain does not grow on oleate. Thus, the strong "glucose effect" observed for the *fad* system (Table 2) is in accordance with a particular sensitivity of the Fad phenotype for revealing alterations in genes involved in catabolic repression.

Table 3 shows that *crp-29* is 40 to 80% co-transducible with *strA*. Furthermore, *crp-29* is co-inherited with the *aroE* and *cysG* markers (transduction 5 and 6), which suggests the gene order *cysG*. . .*crp-29*. . .*strA*. . .*aroE*. The mutation of strain K29 thus shows the genetic properties expected for a *crp* mutation (5, 17).

Synthesis of the galactose enzymes in extracts. Biochemical evidence that *crp-29* affects the CR protein comes from the experiments reported in Tables 4 and 5. It has been shown for several operons (11, 20, 22) that CR protein and cyclic AMP stimulate the DNA-directed in vitro synthesis of active enzymes. An extract from the wild type (Fi165) stimulates, in the presence of cyclic AMP, the synthesis of galactokinase and uridyltransferase by a factor of 10 to 20 (Table 4). Fi165 *crp-29* extracts give only a two- to threefold stimulation under these conditions. Control experiments containing extracts from both strains demonstrate that the Fi165 *crp-29* extract does not contain an inhibitor. Finally, the addition of purified CR protein to the Fi165 *crp-29* extract restores the cyclic AMP-dependent stimulation of enzyme synthesis (Table 5).

A revertant Fi165 *crp-29* (Rev.) has been isolated from strain Fi165 *crp-29* by selection of

TABLE 3. *Mapping of the mutation of strain K29*

Transduction	P1-donor	Recipient	No. of colonies scored	Selected marker	Transductants that score as (%)
1	*strA*[a]	K29	1,345	*strA*	Fad$^+$ (34)
2	K29 *strA*[b]	K12Ymel	2,460	*strA*	Fad$^-$ (39)
3	Fi165 *strA*[c]	K29	414	*strA*	Fad$^+$ (82)
4	K29 *strA*	Fi165	212	*strA*	Fad$^-$ (80)
5	K29	AT700	210	*aroE*	Str$^+$ (55); Fad$^-$ (15); Str$^+$ Fad$^-$ (10)
6	K29 *strA*	AT2455	280	*cysG*	StrA (26); Fad$^-$ (31); StrA Fad$^-$ (19)

[a] Derivative of strain K12Ymel.

[b] Derivative of strain K29.

[c] Derivative of strain Fi165.

TABLE 4. *Prevention of stimulation by crp-29 of in vitro synthesis of the galactose enzymes by cyclic AMP*

Extract prepared from cells	Galactokinase synthesis (units/ml)[a]			Uridyltransferase synthesis (units/ml)[a]		
	+ Cyclic AMP (0.5 mM)	− Cyclic AMP	Stimulation factor	+ Cyclic AMP (0.5 mM)	− Cyclic AMP	Stimulation factor
Expt. 1						
Fi 165	6.2	0.7	8.9	6.9	0.4	17.3
Fi 165 *crp-29* (1)[b]	1.7	0.7	2.4	2.2	0.8	2.8
Fi 165 *crp-29* (2)[b]	1.3	0.4	3.3	0.5	0.2	2.5
Fi 165 + Fi 165 *crp-29* (1)[c]	6.0	0.7	8.6	6.4	0.5	12.8
Fi 165 + Fi 165 *crp-29* (2)[c]	5.9	0.6	9.8	5.6	0.3	18.7
Expt. 2						
Fi 165	7.6	0.7	10.9	5.3	0.2	26.5
Fi 165 *crp-29* (1)[b]	0.6	0.4	1.5	0.3	0.2	1.5
Fi 165 *crp-29* (Rev.)	4.3	0.5	8.6	2.6	0.2	13.0

[a] The activities of galactokinase and uridyltransferase shown in the table represent the synthesis obtained in 1 ml of undiluted synthesis reaction mixture after incubation for 30 min at 37 C with 50 μg of λ*dgal* DNA and 6.5 mg of extract protein per ml (final concentrations). One unit of galactokinase activity is defined as the amount of enzyme that phosphorylates 1 μmol of galactose per hour (20). One unit of transferase activity is defined as the amount of enzyme that converts 1 μmol of galactose-1-phosphate per hour to uridine diphosphategalactose (20). The values have been corrected by subtracting blank values obtained from control incubations with DNA which does not contain the *gal* operon.

[b] (1) and (2) are two independent transductants.

[c] Both extracts were used at a concentration of 3.25 mg/ml (total protein concentration 6.5 mg/ml).

TABLE 5. *Restoration of stimulatory effect of cyclic AMP on enzyme synthesis in extracts from crp-29-cells by addition of CR protein*[a]

Extract prepared from cells	Addition of CR protein (25 μg/ml)[b]	Galactokinase synthesis (units/ml)		
		+ Cyclic AMP (0.5 mM)	− Cyclic AMP	Stimulation factor
Fi165 *crp-29*(1)	−	0.4	0.1	4.0
	+	2.1	0.2	10.5
Fi165 *crp-29* (2)	−	0.3	0.2	1.5
	+	1.5	0.1	15.0
Fi 165	−	6.6	0.3	22.0
	+	6.5	0.2	32.0

[a] Unless stated, all conditions were similar to the experiments described in Table 4.

[b] CR protein had an activity of 2,000 units/mg as defined in (1). The controls received the same amount of heat-inactivated CR protein (5 min, 100 C).

a Fad$^+$ colony on mineral salts-oleate plates. This revertant regained at the same time the ability to grow on other carbon sources like lactose which strain Fi165 *crp-29* had lost. The restoration of the activity of the CR protein, seen both from the in vivo data of Table 2 (lines 31 and 35) and the in vitro experiments of Table 4, appears incomplete. By suitable backcrosses to the parent (Fi165) it could be shown that Fi165 *crp-29* (Rev.) still carries the original *crp-29* mutation closely linked to a second mutation, presumably in the *crp*-gene. Such reversions may become important for structure-function relationships of the CR protein (2).

It may be noted that the main conclusions of this study are supported by experiments with bacterial strains that had received mutation *crp-29* by P1 transduction from strain K29. These derivatives express the typical phenotype of a *strA*-linked *crp* mutation and they fail to respond to cyclic-AMP in vivo and in vitro. Our conclusions are therefore not affected by any uncertainty about the phenotype of original mutant K29 which may carry additional mutations.

In summary, it may be inferred from the data presented in this paper that the synthesis of the enzymes of fatty acid degradation is under control of cyclic AMP and cyclic AMP receptor-protein. Because the expression of the *fad* regulon is strongly affected by glucose, this system may be suitable as a model for further studies of the "glucose effect."

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