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Regulation by Cyclic AMP of the *ilvB*-Encoded Biosynthetic Acetohydroxy Acid Synthase in *Escherichia coli* K-12

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Summary. The biosynthetic acetohydroxy acid synthase activities of *E. coli* K12 are encoded by three genetic loci namely, *ilvB* (acetohydroxy acid synthase I), *ilvG* (acetohydroxy acid synthase II) and *ilvHI* (acetohydroxy acid synthase III). The previously reported involvement of cyclic AMP in the regulation of the biosynthetic acetohydroxy acid synthase isozymes in *E. coli* K-12 was found to be due to the effect of this nucleotide on the expression of *ilvB*. Cyclic AMP had no effect on acetohydroxy acid synthase activity in strains lacking wild-type *ilvB* activity but containing the remaining isozymes. Very little activity of acetohydroxy acid synthase coded for by *ilvB* was found when ppGpp and cyclic AMP were severely limited. Addition of cyclic AMP under these conditions increased *ilvB* expression 24-fold. The data suggest that in addition to multivalent repression and ppGpp, cyclic AMP plays a major role in the regulation of the *ilvB* biosynthetic operon.

Introduction

In *E. coli* K-12 and other enteric bacteria, amino acid operons appear to be regulated by two mechanisms. In the first of these the endproduct or a derivative acts to control operon function. This control may be mediated by an attenuator system, by an operator-repressor mechanism, or by a combination of both (Umbarger, 1978). The second type of control acts in a positive fashion and is thought to involve guanosine 5′-diphosphate 3′-diphosphate (ppGpp). This nucleotide is synthesized by the product of the *relA* gene in response to amino acid starvation (Block and Hazeltine, 1974). It has been suggested that ppGpp serves as a general regulatory signal to help bacteria adjust to proper levels of amino acid supply (Stephens et al., 1975).

Regulation of the isoleucine and valine enzymes in *E. coli* K-12 involves both endproduct repression (Umbarger, 1978) and ppGpp (Freundlich, 1977). In addition, recent data suggest that the ppGpp requirement for normal expression of one of the isoleucine and valine enzyme activities, acetohydroxy acid synthase, can be replaced by cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) (Freundlich, 1977). Cyclic AMP is thought to be a signal molecule that indicates a fuel or carbon deficiency to the cell (Alper and Ames, 1978). It is usually involved in regulation of degradative operons (Pastan and Adhya, 1976). Therefore, the participation of cyclic AMP in the control of acetohydroxy acid synthase is highly unusual since biosynthetic enzymes account for this activity in *E. coli* K-12 (Guardiola et al., 1974). These isozymes, termed acetohydroxy acid synthase I, II and III are coded for by the *ilvB, ilvG* and *ilvHI* genes (Guardiola et al., 1977).

In the present paper we have extended our observations on the role of cyclic AMP in the expression of acetohydroxy acid synthase. We show that cyclic AMP is involved in the regulation of *ilvB* only and that the two remaining isozymes are not controlled by cyclic AMP.

Materials and Methods

Reagents and Media. Sodium α, β-dihydroxy isovalerate was a gift from H.E. Umbarger. All other chemicals were obtained from Sigma Chemical Company. The minimal medium used was that of Davis and Mingioli (1950), except that citrate was omitted. The medium was supplemented with glucose or other carbon sources (0.5%). In addition to the minimal medium, the amino acid rich medium contained 17 L-amino acids each at a concentration of 0.2 mM and L-isoleucine (0.5 mM), L-valine (1.0 mM) and L-leucine (0.5 mM). Thiamine, when added, was at 2 μg per ml.

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Table 1. Bacterial strains

Strain	Genotype	Source or reference
MF810	F⁻, *ara*, *Δ(lac)*$_{x74}$, *ilvH612*, *ilvI614*, *thi-1*, *relA1*, *strA220*, *λ*−	Leu⁺, IlvHI⁻ transductant of RFS817 using P1 grown on MI253C
MI158	HfrH, *pyrA53*, *thi-1*, *λ*⁻	(Iaccarino and Berg, 1971)
MI253C	HfrH, *ilvH612*, *ilvI614*, *thi-1*, *ara*, *su*⁻, *λ*⁻	M. Iaccarino
MI257	*F*⁻, *thr-4*, *leu-8*, *pdx-A1 Mu*⁺ *proA2*, *ilvH612*, *ilvI614*, *lac Y-1*, *gal K2*, *xyl–5*, *mtl-1*, *thi-1*, *strA20*, *ϕ80psu3*	M. Iaccarino
MI261c	HfrH, *ilvO603*, *ilvG605* *ilvH612*, *ilvI614* *thi-1*, *glyA*, *ara*, *bgl*, *su*⁻, *λ*⁻	M. Iaccarino
MI262e	HfrH, *ilvO603*, *ilvB619* *ilvH512*, *ilvI614*, *thi-1*, *glyA*, *ara*, *su*⁻, *λ*⁻	M. Iaccarino
MI263	HfrH, *ilvO603*, *ilvG605*, *ilvB619*, *thi-1*, *su*⁻, *λ*⁻	M. Iaccarino
MI263D	HfrH, *ilvO603*, *ilvG605* *ilvH612*, *ilvB619*, *thi-1*, *λ*⁻	(DeFelice et al., 1974)
PS1426	F⁻, *Δ(ara leu ilvHI)*$_{863}$, *xyl-7*, *lacY1*, *mglP-1*, *cya 283*	T. Newman
PS1436	F⁻, *Δ(ara leu ilvH1)*$_{863}$, *xyl-7*, *lacY-1*, *mglP-1*	T. Newman
RFS817	F⁻, *Δ(ara-leu)*, *Δ(lac)*$_{x74}$, *thi-1*, *relA1*, *strA220*, *λ*⁻	(Schleif, 1972)
SA1913	F⁻, *his*⁻, *pro*⁻, *str*^{r}, *su*⁻	S.Adhya
SA500	F⁻, *his*⁻, *str*^{r}, *rel-1*, *su*⁻	S. Adhya
SA1040	F⁻, *his*⁻, *str*^{r}, *rel-1*, *su*⁻, *crp** contains an altered *crp* which responds to exongenous cAMP	S. Adhya

Growth of Bacteria and Enzyme Assays. Unless otherwise noted, cells were grown overnight at 37° C, centrifuged at room temperature, washed in minimal medium, and resuspend at a Klett-Summerson colorimeter reading of 25–30 (blue filter), in media as described in the text. The cells were grown for at least two doublings, and cell extracts for enzyme assays were prepared as previously described (Rizzino et al., 1974). The following enzyme assays were performed as described previously: threonine deaminase (Freundlich and Umbarger, 1963), acetohydroxy acid synthase (Stormer and Umbarger, 1964), dihydroxy acid dehydrase (Freundlich et al., 1962). Protein was measured by the method of Lowry et al. (1951). Enzyme specific activity is expressed as μmole of product formed per mg of protein per h.

Results

Effect of Carbon Source and Cyclic AMP on Acetohydroxy Acid Synthase in Wild Type E. coli K-12.

Table 2. Effect of carbon source and cyclic AMP on acetohydroxy acid synthase in wild-type *E. coli* K-12

Strain	Growth media[a]	Specific activity	
		Threonine deaminase	Acetohydroxy acid synthase
MI158	Glucose	3.0	1.9
	Glucose+cAMP	5.0	5.3
	Glycerol	2.9	3.8
	Succinate	2.7	2.9
SA1913	Glucose	6.6	1.3
	Glucose+cAMP	9.0	3.7
	Succinate	6.0	3.7
	Lactate	3.5	3.6

[a] Cells were grown overnight at 37° C in minimal medium containing 0.5% of the indicated carbon source. Where noted cyclic AMP (cAMP) was 2 mM. In the morning the cells were diluted in the same medium to a Klett-Summerson colorimeter reading of 25 (blue filter) and harvested during late exponential growth

The levels of some of the isoleucine and valine enzymes were examined in several wild type *E. coli* K-12 strains grown in minimal medium with a variety of carbon sources. In most cases acetohydroxy acid synthase activity was elevated 2 to 3-fold, as compared to glucose, when the cells were grown with a poor carbon source (Table 2). The level of the other isoleucine and valine enzymes measured remained fairly constant under these conditions. The increased activity of acetohydroxy acid synthase during growth on poor carbon sources could be duplicated by the addition of cyclic AMP to the minimal-glucose medium. Comparable data have been reported by Poglase and coworkers using a number of strains of *E. coli* (Coukell and Polglase, 1969; Whitlow and Polglase, 1974). The significance of these small stimulatory effects on acetohydroxy acid synthase activity is difficult to assess. Slight differences in biosynthetic enzyme activities in wild type cells have been found during numerous environmental and nutritional changes. We have previously found that cyclic AMP can replace ppGpp for normal derepression of acetohydroxy acid synthase in *relA* strains of *E. coli* K-12 (Freundlich, 1977). In these experiments a 15 to 30-fold increase in enzyme activity was observed with added cyclic AMP indicating a significant role for this nucleotide in acetohydroxy acid synthase expression.

Effect of Branched-Chain Amino Acid Limitation on Cyclic AMP Stimulation of Acetohydroxy Acid Synthase. Each of the acetohydroxy acid synthase isozymes in *E. coli* K-12 shows a different pattern of regulation. Leucine and valine are required for repression of *ilvB* while leucine alone represses *ilvHI*

Table 3. Effect of branched-chain amino acid limitation on cyclic AMP stimulation of acetohydroxy acid synthase

Growth limitation after shift[a]	Specific activity		
	Acetohydroxy acid synthase	Threonine deaminase	Dihydroxy acid dehydrase
None	0.4	2.0	1.1
None + cAMP	1.2	2.4	1.2
Isoleucine	0.4	2.2	1.4
Isoleucine + cAMP	0.8	3.7	1.2
Valine	0.9	3.2	2.4
Valine + cAMP	9.4	1.8	1.7
Leucine	1.1	2.2	1.6
Leucine + cAMP	29.0	1.6	1.8

[a] Strain SA1040 (*relA crp**) was grown overnight at 37° C in minimal medium containing 17 amino acids at 0.2 mM each and L-isoleucine (0.5 mM), L-valine (1.0 mM) and L-leucine (0.5 mM). In the morning the cells were centrifuged, washed, and resuspended in the same medium or with growth limiting amount of a branched-chain amino acid. Growth limitation was accomplished by using the glycyl peptides of isoleucine, valine or leucine at 0.05 mM. Where indicated cyclic AMP (cAMP) was added at 2 mM. The cells were grown for 2 doublings and enzyme levels determined as described in Materials and Methods

Table 4. Effect of cyclic AMP on acetohydroxy acid synthase activity in mutants containing a single isozyme

Strain and gene expressed[a]	Cyclic AMP	Specific activity	
		Threonine deaminase[b]	Acetohydroxy acid synthase
MI253C (*ilvB*$^+$)	–	3.7	1.6
	+	5.6	8.3
MI257 (*ilvB*$^+$)	–	4.2	1.3
	+	5.0	4.8
MF810 (*ilvB*$^+$)	–	1.6	2.1
	+	2.0	8.4
MI261C (*ilvB*$^+$)	–	11.2	0.8
	+	7.4	3.6
MI262E (*ilvG*$^+$)	–	9.5	0.31
	+	10.5	0.29
MI263 (*ilvHI*$^+$)	–	12.1	1.6
	+	11.4	1.4
MI263D (*ilvI*$^+$)	–	9.5	1.8
	+	9.7	1.7

[a] Each of these strains expresses only the indicated gene coding for one of the acetohydroxy acid synthase isozymes. The cells were grown at 30° C. For other conditions see Table 2

[b] Differences in threonine deaminase levels are due to the *ilvO603* mutation in some of these strains. For complete genotype of these strains see Table 1

(DeFelice et al., 1978; DeFelice and Levinthal, 1977). Acetohydroxy acid synthase II, coded for by *ilvG*, is not normally expressed in *E. coli* K-12 (O'Neill and Freundlich, 1972). Repression of this enzyme may be mediated by valine and isoleucine (Umbarger, 1978). We used these differences in control in an attempt to determine which of the acetohydroxy acid synthase isozymes is regulated by cyclic AMP. In these experiments a *relA crp* strain that responds to exogenous cyclic AMP (Freundlich, 1977) was grown overnight in a mixture of 20 amino acids with glucose as carbon source. In the morning the cells were washed and resuspended in the same medium with growth limiting amounts of one of the branched-chain amino acids. A phenotypic amino acid auxotrophy is obtained under these downshift conditions due to the *relA* mutation (Neidhardt and Eidlic, 1963). No derepression of acetohydroxy acid synthase was found when this strain was downshifted into the amino acid rich medium with growth limiting amounts of either isoleucine, valine or leucine (Table 3). The addition of cyclic AMP resulted in a 10 to 25-fold increase in acetohydroxy acid synthase activity during leucine or valine starvation. The nucleotide did not allow derepression during isoleucine starvation. The other isoleucine and valine enzymes tested failed to derepress with or without the addition of cyclic AMP. The ability of cyclic AMP to allow derepression of acetohydroxy acid synthase during valine and leucine but not isoleucine starvation suggests that the nucleotide is involved in the regulation of *ilvB*. However, since *ilvHI* is repressed by leucine, cyclic AMP may also control these genes.

Effect of Cyclic AMP on Strains Containing Single Acetohydroxy Acid Synthase Isozymes. To more definitively study the effect of cyclic AMP on the expression of acetohydroxy acid synthase we used mutants of *E. coli* K-12 that express only one of each of the three isozymes. These strains were grown in minimal medium with glucose with or without cyclic AMP. The data in Table 4 show that only those strains containing a wild type *ilvB* gene had increased 4 to 6-fold) acetohydroxy acid synthase activity during conditions allowing relief of catabolite repression.

Cyclic AMP or ppGpp is Required for Minimal in vivo Expression of ilvB. Previous results from our laboratory showed that cyclic AMP could replace the ppGpp requirement for derepression of total acetohydroxy acid synthase activity in *relA* strains of *E. coli* K-12 (Freundlich, 1977). Data in the present paper suggest that the involvement of cyclic AMP in the regulation of acetohydroxy acid synthase is due to

Table 5. Effect of cyclic AMP in PS1426 (*cya*, *ilvB*$^+$)

Strain[a]	Cyclic AMP	Specific activity	
		Threonine deaminase	Acetohydroxy acid synthase
PS1436 (*cya*$^+$)	–	2.3	0.4
	+	2.1	1.9
PS1426 (*cya*$^-$)	–	2.0	0.05
	+	2.1	1.2

[a] Strain PS1426 has a probable deletion in the *cya* gene and expresses only the *ilvB* acetohydroxy acid synthase isozyme. Strain PS1436 is the isogenic *cya*$^+$ parent strain. Cells were grown overnight in glucose-minimal medium with a mixture of 20 amino acids. Where indicated cyclic AMP was added at 2 mM. In the morning the cells were diluted in the same medium to a Klett-Summerson colorimeter reading of 25 (blue filter) and harvested after 2 doublings. Enzyme levels were determined as described in Materials and Methods

effects of this nucleotide on *ilvB* expression. To investigate this further, conditions were employed that severely limited the levels of cyclic AMP and ppGpp in a strain containing only the acetohydroxy acid synthase coded for by *ilvB*. For these studies strain PS1426 was used. This strain expresses only the *ilvB* isozyme and has a probable deletion of the *cya* gene (T. Newman, personal communication). Cells were grown in glucose-minimal medium with a mixture of 20 amino acids. These conditions result in an almost undetectable level of ppGpp (Cashel, 1969). Growth of strain PS1426 in this medium produced almost no acetohydroxy acid synthase activity (Table 5). This activity is only twice as much as that reported for a strain that lacks all of the acetohydroxy acid synthase isozymes (Guardiola, et al., 1974). The *cya*$^+$ parent strain showed a 9-fold higher expression of *ilvB* during growth in the amino acid rich medium as compared to the *cya* strain (Table 5). The addition of cyclic AMP to strain PS1426 resulted in a 24-fold increase in acetohydroxy acid synthase activity. Expression of *ilvB* was elevated 4.5-fold during growth of the *cya*$^+$ parent strain with cyclic AMP.

Discussion

The data in this paper confirm our previous observations that cyclic AMP plays a major role in the expression of acetohydroxy acid synthase in *E. coli* K-12 (Freundlich, 1977). In addition, we show that this nucleotide controls only the acetohydroxy acid synthase I isozyme coded for by *ilvB*. The remaining two isozymes do not appear to be regulated by cyclic AMP. This conclusion is based on the following observations. First, the same branched-chain amino acids that participate in endproduct inhibition and repression of the *ilvB* acetohydroxy acid synthase regulate the enzyme that responds to cyclic AMP. Second, only those strains with a wild-type *ilvB* gene had increased acetohydroxy acid synthase activity during growth with a poor carbon source or with added cyclic AMP. Third, very little expression of acetohydroxy acid synthase was found in a strain containing only acetohydroxy acid synthase I during growth conditions that severely limited cyclic AMP and ppGpp. The addition of cyclic AMP to these cells increased acetohydroxy acid synthase 24 fold. These same observations effectively rule out the possibility that the increase in acetohydroxy acid synthase caused by cyclic AMP is due to the activation of a degradative form of the enzyme. A number of enteric bacteria contain degradative acetohydroxy acid synthase (Halpern and Umbarger, 1959; Malthe-Sorrensen and Stormer, 1970) but this enzyme has not been found in *E. coli* K-12 (Guardiola et al., 1974).

The involvement of cyclic AMP in the control of *ilvB* is highly unusual. This nucleotide normally is a positive regulator of degradative operons concerned with carbon and energy metabolism (Pastan and Adhya, 1976). A possible role for cyclic AMP in the control of some of the arginine biosynthetic enzymes has been reported (Gorini and Gunderson, 1961; Kelln and O'Donovan, 1976). In addition, Polglase and his collaborators have suggested that acetohydroxy acid synthase in *E. coli* is regulated by cyclic AMP (Coukell and Polglase, 1969; Whitlow and Polglase, 1974). However, in these investigations the effects of cyclic AMP on enzyme levels were small and the data could be interpreted in a variety of ways. The present report appears to be the most definitive example of the participation of cyclic AMP in the control of an enzyme concerned with a major biosynthetic pathway of intermediary metabolism. The physiological reason for this regulation is not apparent. Perhaps this is a vestigial remnant of a role of the ilvB gene that has been lost in evolution. Alternatively, the *ilvB* gene product may participate in other cell functions in addition to the biosynthesis of the branched-chain amino acids. In this regard, several observations indicate a possible connection between *ilvB* and the E. coli K-12 cell envelope (DeFelice et al., 1977). It has been suggested that the ilvB product has a regulatory role in the synthesis of diaminopimelic acid (DeFelice et al., 1977). Preliminary studies in our laboratory indicate that a number of cell envelope proteins are greatly reduced in a strain of *E. coli* K-12 containing a mutation in *ilvB* (A. Sutton, R. Movva and M. Freundlich, unpublished observations). In addition, these proteins appear to be regulated by cyclic AMP.

The *ilvB* gene has recently been shown to be located at approximately minute 81.5 on the *E. coli* K-12 chromosome (T. Newman and M. Levinthal, personal communication). This position is different from that previously reported which placed *ilvB* within the *ilv* gene cluster at minute 83.5 (Ramakrishnan and Adelberg, 1965). Data in the present paper show that *ilvB* differs from the other *ilv* genes in being regulated by cyclic AMP in addition to control by multivalent repression (DeFelice et al., 1978) and ppGpp (Freundlich, 1977). The relationship between the positive regulation exerted by cyclic AMP and ppGpp has been studied in the *lac* operon (Primakoff and Artz, 1979). *In vitro* expression of this operon requires cyclic AMP but this expression is increased 20-fold with added ppGpp. Data suggest that these effectors may act at different but overlapping sites at the *lac* promoter (Primakoff and Artz, 1979). In the expression of *ilvB* it is not clear if these nucleotides exert their effects through independent or concerted action. An in vitro study of *ilvB* expression is required to understand the relationships between cyclic AMP and ppGpp and how these nucleotides interact on the molecular level.

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