Cyclic AMP and anaerobic gene expression in *E. coli*

Gottfried Unden and John R. Guest*

Department of Microbiology, Sheffield University, Western Bank, Sheffield S10 2TN, England

Received 30 March 1984

The expression of the fumarate reductase system of *Escherichia coli* is completely dependent on the presence of adenylate cyclase or cyclic AMP, but the cyclic AMP receptor protein (CRP) is not required. This suggests that cyclic AMP may function as an effector for a second gene activator protein, possibly Fnr, and thus form part of a redox-sensitive regulatory mechanism controlling the expression of anaerobic respiratory functions.

Cyclic AMP *Gene expression* fnr *gene* *Adenylate cyclase* crp *gene* *Anaerobic respiration*

1. INTRODUCTION

A major unsolved problem in microbial biochemistry concerns the nature of the mechanisms that control the switch between aerobic and anaerobic metabolic pathways. Facultative anaerobes such as *Escherichia coli* can derive energy for growth from aerobic or anaerobic respiration or from fermentation. The respiratory pathways depend upon the availability of different terminal electron acceptors such as oxygen, fumarate or nitrate [1]. Previous studies on the expression of anaerobic respiratory systems have suggested that they are under dual control, involving specific repressor proteins responding to the terminal electron acceptor (fumarate or nitrate) and a general regulatory protein responding to an effector that signals redox state [2–5]. The general control appears to be mediated by Fnr, the product of the *fnr* gene, which is essential for the expression of diverse anaerobic functions such as the reduction of fumarate and nitrate, and the oxidation of hydrogen and formate [6].

The mechanism by which Fnr controls anaerobic metabolism is not known. However, the *fnr* gene has been cloned, and the primary structure of the Fnr protein deduced from the nucleotide sequence [7,8]. This has revealed a striking degree of homology between Fnr and CRP, the cyclic AMP receptor protein, which mediates catabolite repression by binding to specific regulatory sites of catabolite-sensitive genes [9]. The regions of homology are found in both the DNA- and nucleotide-binding domains of CRP although the amino acid residues that make specific contacts with cyclic AMP are not conserved in Fnr [8,10]. The homologies suggest that Fnr may function like CRP to activate the transcription of genes concerned with anaerobic metabolism. If so, interaction with DNA could involve Fnr binding an unidentified effector or effectors that signal the redox and/or energy states of the cell. This paper describes evidence that adenylate cyclase (EC 4.6.1.1) and cyclic AMP, but not CRP, are essential for the expression of the fumarate reductase system (EC 1.3.99.1) of *E. coli*.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

Deletion strains lacking adenylate cyclase or the cyclic AMP receptor protein (CRP) and the corresponding parental strains were kindly provided by Dr J. Beckwith: CA8306 (*Δcya-854*) derived from CA8000 (Hfr, *thi*) and EC84-54

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

(*Δcrp-45Δ(lac-pro)*XIII *rpsL*) from EC52 (Hfr, *Δ(lac-pro)*XIII *supE mal*) [11,12].

2.2. *Media*

The glycerol plus fumarate medium [6] containing thiamine (5 mg/l), L-tryptophan (75 mg/l), vitamin-free casamino acids (0.5 g/l) and Bacto Nutrient Broth (16 mg/l) was used, and the glucose medium was identical except that glycerol plus fumarate were replaced by glucose (11 mM). A peptone-yeast extract broth plus glucose (55 mM) [13] served as the complex medium. Cyclic AMP was added to the autoclaved medium as the free acid and subsequently neutralized with equimolar amounts of sterile NaOH.

2.3. *Growth experiments and enzymology*

Cultures (50 ml) were grown anaerobically in 100-ml conical flasks fitted with side arms for optical measurements. The medium was inoculated with an aerobically-grown subculture (3–5%), sealed, and the air replaced by a mixture of H_2 plus CO_2 (95:5). Growth at 37°C was estimated by measuring the absorbance at 650 nm. For enzymology the cultures were harvested in late exponential phase and cell-free extracts prepared by ultrasonic disruption [13]. The reduction of fumarate was assayed by measuring the fumarate-dependent oxidation of reduced benzylviologen [14]. Protein was determined as in [15] and specific activities are quoted as units (μmol/min) of fumarate reductase (EC 1.3.99.1) per g protein.

3. RESULTS

To investigate the possible involvement of cyclic AMP in the expression of anaerobic electron transport systems, deletion mutants of *E. coli* lacking either adenylate cyclase (*cya*) or the cyclic AMP receptor protein CRP (*crp*) were grown anaerobically on a glycerol plus fumarate medium (GF). Under these conditions *E. coli* derives energy for growth from anaerobic phosphorylation by coupling glycerol oxidation to the reduction of fumarate.

The parental strain (CA8000) grew with a doubling time of about 4 h, whereas the *cya* mutant (CA8306) grew only very poorly (fig.1A). However, the growth of the mutant was restored by addition of cyclic AMP (5 mM). Growth of the parental strain was not significantly influenced by the presence of cyclic AMP (fig.1A). The growth rate of the *crp* mutant (EC84-54) was reduced to about one half the rate of the corresponding parental strain (EC52), but neither was affected by the addition of cyclic AMP (fig.1B).

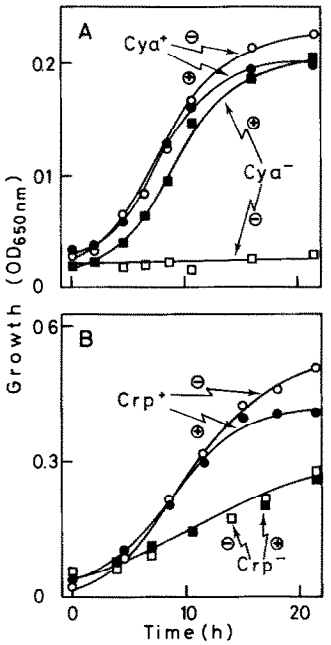

Fig.1. Effect of cyclic AMP on the anaerobic growth of (A) the *cya* mutant CA8306 and its parental strain CA8000, and (B) the *crp* mutant EC84-54 and its parent EC52. The strains were grown on the minimal glycerol plus fumarate medium as described in section 2. Each strain was grown without (open symbols) and with (close symbols) cyclic AMP (5 mM) as indicated.

These results show that a functional adenylate cyclase is essential when the energy for growth is derived from anaerobic respiration. This contrasts with the observation that under the same conditions CRP is not essential, although the *crp* deletion does impair growth to some extent. In order to investigate whether the effects of cyclic AMP and the *cya* lesion are due to changes in the expression of the anaerobic electron transport system or of some other metabolic process, the fumarate reductase activities of the mutant and parental strains were measured after growth under different conditions (table 1). After growth in GF medium, cell-free extracts of the *cya* mutant (CA8306) reduced fumarate at only 7% of the parental rate.

The same decrease in enzyme activity has been observed with *fnr* mutants [6]. However, the fumarate reductase activity of the *cya* mutant could be restored by growth in the presence of 5 mM cyclic AMP (table 1). Addition of cyclic AMP had no effect on the fumarate reductase activity of the parental strain.

In the case of the *crp* mutant and its parent, the fumarate reductase activities were similar and not significantly affected by growth in the presence of cyclic AMP (table 1). It would therefore appear that the lower growth rate of the *crp* mutant (EC84-54) is probably not due to impaired electron transport but to some secondary function that requires CRP for full expression.

To exclude the possibility that the low fumarate reductase activity of the *cya* mutant is a secondary consequence of its poor growth (fig.1A), the strains were grown anaerobically with glucose as the energy source. Under these permissive conditions the bacteria derive their energy from fermentation and anaerobic respiration, and it was found that the growth rate of the *cya* mutant (doubling time = 2.2 h) was only slightly lower than that of the parental strain (doubling time = 1.6 h). However, the fumarate reductase activity of the *cya* mutant remained at the same low level observed after anaerobic growth on GF medium (table 1). This clearly demonstrates that the slow rate of growth on glycerol plus fumarate is due to a lack of energy from electron transport phosphorylation. The fumarate reductase activity of the parental strain (CA8000) grown on glucose was almost 5 times greater than that of the *cya* mutant (table 1). It did not reach the levels observed on glycerol plus fumarate but the expression of fumarate reductase is not normally fully induced on glucose or in the absence of exogenous fumarate.

Table 1

Fumarate reductase activities in cell free extracts of *E. coli* strains grown anaerobically on glycerol plus fumarate (GF) or glucose (Glc) medium with and without cyclic AMP

Strain	Relevant phenotype	Medium	Cyclic AMP (mM)	Fumarate reductase (μmol/min per g protein)
CA8000	Cya^+	GF	0	1080
		GF	5	1000
CA8306	Cya^-	GF	0	80
		GF	5	1100
EC52	Crp^+	GF	0	710
		GF	5	840
EC84-54	Crp^-	GF	0	870
		GF	5	890
CA8000	Cya^+	Glc	0	470
CA8306	Cya^-	Glc	0	100

The effects of the *cya* mutation and cyclic AMP on growth rate and fumarate reductase were not observed in complex media, where fumarate reductase was fully expressed in all of the strains. It was also found that the decreased growth rate and fumarate reductase activity (fig.2) could be overcome by increasing the concentration of casein hydrolysate in the GF medium. In view of the high concentrations that are required, it seems unlikely that the casein is satisfying a growth requirement. This effect could be analogous to the relief of catabolite repression by certain amino acids [16] or the phenomenon of metabolite gene regulation [17].

The involvement of other potential effectors of the expression of anaerobic electron transport systems has been investigated in the same way using appropriate mutants, e.g., *relA relX* double mutants which lack the effector of the stringent response, ppGpp [18], but no decrease in fumarate reductase was detected.

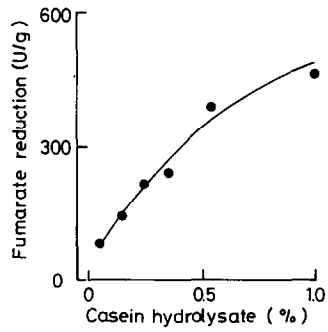

Fig.2. Effect of casein hydrolysate on the expression of fumarate reductase activity by the *cya* mutant CA8306. The mutant was grown anaerobically on glycerol plus fumarate medium with different concentrations of casein hydrolysate and the fumarate reductase activities (μmol/min per g protein) were determined as described in section 2.

4. DISCUSSION

This study has shown that when *cya* and *crp* mutants of *E. coli* are grown anaerobically in a glycerol plus fumarate medium the synthesis of fumarate reductase, a representative anaerobic electron transport complex, is completely dependent on the presence of a functional adenylate cyclase or exogenous cyclic AMP. However, unlike other proteins whose expression is controlled by the cellular level of cyclic AMP [9], the expression of fumarate reductase is not dependent on the presence of CRP. The results suggest that unless adenylate cyclase or cyclic AMP are acting indirectly, there is a second regulatory protein different from CRP which mediates cyclic AMP-dependent control of anaerobic electron transport functions. An obvious candidate is the Fnr protein. The *fnr* gene has already been recognised as encoding a positive regulator of anaerobic electron transport systems [2], and the homology between the CRP and Fnr proteins suggests that Fnr may contain a nucleotide binding domain similar to that of CRP even though the residues making contact with cyclic AMP are not conserved [8]. The present results support the view that cyclic AMP is an effector of the Fnr protein. Since the Fnr protein and cyclic AMP both function in a positive mode, it is possible that they could combine to activate transcription as a cyclic AMP–Fnr complex. Such a mechanism would depend on there being relatively high concentrations of cyclic AMP during anaerobic growth, but this has not been investigated. Under aerobic conditions the cellular concentration of cyclic AMP is closely related to the energy state of the cell. Energy-rich carbon sources result in low levels whereas poor carbon sources induce high levels of cyclic AMP [19], and because the transmembrane electrochemical proton gradient is reduced under anaerobic conditions [20], it is possible that the anaerobic cyclic AMP concentration may be relatively high.

The proposed model suggests that high cellular concentrations of cyclic AMP activate the transcription of genes controlled by CRP and Fnr. However, it does not explain how redox control is mediated because cyclic AMP appears only to signal energy state, and even when the energy state is low, anaerobic functions are not normally derepressed under aerobic conditions. A solution to this problem would be to postulate the existence of an additional control system that responds to the redox state. This could be achieved by a second effector or by the Fnr protein being itself redox-sensitive. In this connection it should be noted that Fnr differs from CRP in possessing a small N-terminal domain containing a cluster of 3 cysteine residues. This lends support to the view that the Fnr protein may be redox-sensitive. There is evidence for redox-control of other anaerobically-induced enzymes in the enterobacteria [21] and their expression could involve a comparable regulatory mechanism.

ACKNOWLEDGEMENTS

We are very grateful to Dr B.A. Fry for good advice and encouragement. The work was supported by the Science and Engineering Council (GR/A99879) and by an Ausbildungstipendium of the Deutsche Forschungsgemeinschaft.

REFERENCES

[1] Haddock, B.A. and Jones, C.W. (1977) Bacteriol. Rev. 41, 47–99.
[2] Shaw, D.J. and Guest, J.R. (1981) Mol. Gen. Genet. 181, 95–100.
[3] Shaw, D.J. and Guest, J.R. (1982) J. Gen. Microbiol. 128, 2221–2228.
[4] Cole, S.T. and Guest, J.R. (1979) FEMS Microbiol. Lett. 5, 65–67.
[5] Chippaux, M., Bonnefoy-Orth, V., Ratouchniak, J. and Pascal, M.C. (1981) Mol. Gen. Genet. 182, 477–479.
[6] Lambden, P.R. and Guest, J.R. (1976) J. Gen. Microbiol. 97, 145–160.
[7] Shaw, D.J. and Guest, J.R. (1982) Nucleic Acids Res. 10, 6119–6130.
[8] Shaw, D.J., Rice, D.W. and Guest, J.R. (1983) J. Mol. Biol. 166, 241–247.
[9] Pastan, I. and Adhya, S. (1976) Bacteriol. Rev. 40, 527–551.
[10] McKay, D.B., Weber, I.T. and Steitz, T.A. (1982) J. Biol. Chem. 257, 9518–9524.
[11] Brickman, E., Soll, L. and Beckwith, J. (1973) J. Bacteriol. 116, 582–587.
[12] Sabourin, D. and Beckwith, J. (1975) J. Bacteriol. 122, 338–340.
[13] Spencer, M.E. and Guest, J.R. (1973) J. Bacteriol. 114, 563–570.

[14] Kröger, A. and Innerhofer, A. (1976) Eur. J. Biochem. 69, 487–495.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Broman, R.L., Goldenbaum, P.E. and Dobrogosz, W.J. (1970) Biochem. Biophys. Res. Commun. 39, 401–406.
[17] Kline, E.L., Bankaitis, V.A., Brown, C.S. and Montefiori, D.C. (1980) J. Bacteriol. 141, 770–778.
[18] Pao, C.C. and Gallant, J. (1978) Mol. Gen. Genet. 158, 271–277.
[19] Epstein, W., Rothman-Denes, L.B. and Hesse, J. (1975) Proc. Natl. Acad. Sci. USA 72, 2300–2303.
[20] Hellingwerf, K.J., Bolscher, J.G.M. and Konings, W.N. (1981) Eur. J. Biochem. 113, 369–374.
[21] Pecher, A., Zinoni, F., Jatisatienr, C., Wirth, R., Hennecke, H. and Böck, A. (1983) Arch. Microbiol. 136, 131–136.