Energy Coupling to Active Transport in Anaerobically Grown Mutants of *Escherichia coli* K12

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1. Anaerobic uptake of proline requires either the presence of a coupled Mg^{2+} -stimulated adenosine triphosphatase or anaerobic electron transport. 2. Anaerobic uptake of glutamine does not require anaerobic electron transport even in the absence of a coupled Mg^{2+} -stimulated adenosine triphosphatase. 3. These results support previous suggestions [Berger (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 1514–1518; Berger & Heppel (1974) *J. Biol. Chem.* 249, 7747–7755; Kobayashi, Kin & Anraku (1974) *J. Biochem. (Tokyo)* 76, 251–261] that two distinct mechanisms of energy coupling to active transport exist in *Escherichia coli* in that energization of anaerobic proline uptake requires the 'high-energy membrane state', whereas the energization of anaerobic glutamine uptake does not.

In *Escherichia coli* the transport of some solutes requires the production of a high-energy membrane state (Harold, 1972; Klein & Boyer, 1972; Simoni & Postma, 1975) and may be energized in three ways; under aerobic conditions by coupling to the electron flow to oxygen (Barnes & Kaback, 1970), and under anaerobic conditions either by coupling to the electron flow to other acceptors [nitrate or fumarate (Konings & Kaback, 1973)] or to available ATP, the utilization of which requires a coupled intact Mg^{2+} -stimulated ATPase* (Schairer & Haddock, 1972; Or *et al.*, 1973; Schairer & Gruber, 1973; van Thienen & Postma, 1973).

Recently it has been observed that the transport of some solutes showed resistance to uncouplers and proceeded under anaerobic conditions in mutants defective in the Mg^{2+} -stimulated ATPase (Berger, 1973; Kobayashi *et al.*, 1974; Curtis, 1974; Cowell, 1974; Kadner & Winkler, 1975). From this it has been concluded that the transport of some substances does not require the 'high-energy membrane state' and is energized directly by phosphate-bond energy (Berger, 1973; Berger & Heppel, 1974; Kobayashi *et al.*, 1974). However, two objections can be levelled at this interpretation. First, the uncoupler evidence is not clear-cut in that the systems described were only partially resistant. Secondly, the experiments with anaerobic transport in Mg^{2+} -stimulated ATPase-deficient mutants did not rigorously exclude the possible contribution of low levels of anaerobic electron transport with fumarate as electron acceptor to the energization of solute uptake either via the formation of a 'high-energy membrane state' or by some other means.

* Abbreviation: ATPase, adenosine triphosphatase.

We therefore studied anaerobic glutamine and proline uptake by anaerobically-grown cells of mutants of *E. coli* defective in the coupling ability of the Mg^{2+} -stimulated ATPase (*uncB*), or in fumarate reductase (*frd-1*) ability or both.

Experimental

Materials

Chemicals. Chemicals were of the purest grade available commercially and were not further purified. L-[U- ^{14}C]Glutamine (49mCi/mmol) and L-[U- ^{14}C]proline (290mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and were diluted appropriately with carrier before use. N_2 was passed through two scrubbing towers containing 5% (w/v) pyrogallol in 14M-KOH before use. Ground firefly lanterns (Sigma Chemical Company, St. Louis, MO, U.S.A.) (100mg) were extracted in 10ml of buffer (50mM-sodium arsenate/5mM-potassium phosphate/5mM- $MgCl_2$, pH 7.3). The extract was filtered before use (Berger & Heppel, 1974).

Organisms. All organisms used were kindly given by Dr G. B. Cox of this Department. They were derived from *E. coli* K12 and are described in Table 1.

Methods

Media and growth of organisms. The medium used for growth of cells was half-strength medium 56 (Monod *et al.*, 1951), pH 7.0. Where appropriate, sterile solutions of growth supplements were added to the sterilized mineral-salts base to the following final concentrations: arginine, 1 mM; isoleucine and

Table 1. *Strains of E. coli used in this study*

Genes coding for enzymes in various biosynthetic pathways are denoted as follows: *ilv*, isoleucine, valine; *arg*, arginine; *ent*, enterochelin. The *unc* genes code for factors required in the coupling of phosphorylation to electron transport and the *frd* gene for the enzyme fumarate reductase.

Strain	Relevant genetic loci	Other information
AN259	<i>argH</i> ⁻ , <i>entA</i> ⁻	Butlin <i>et al.</i> (1973)
AN283	<i>argH</i> ⁻ , <i>entA</i> ⁻ , <i>uncB401</i>	Butlin <i>et al.</i> (1973)
AN472	<i>ilvC-7</i> , <i>frd-1</i> , <i>entA</i> ⁻	Rosenberg <i>et al.</i> (1975)
AN480	<i>entA</i> ⁻ , <i>frd-1</i> , <i>uncB401</i>	Rosenberg <i>et al.</i> (1975)

valine, 0.5 mM; uracil, 0.2 mM; thiamin, 3.3 μ M; 2,3-dihydroxybenzoate, 10 μ M. Glucose was added at the growth-limiting concentration of 5 mM. Nitrate (75 mM) and fumarate (5 mM) were added to the growth medium for anaerobic growth of strains AN480 (*uncB401*, *frd-1*) and AN283 (*uncB401*) respectively.

Cells were grown at 37°C, anaerobically in filled screw-capped glass bottles, or aerobically in one-third filled conical flasks shaken at 300 rev./min in an orbital shaking water bath. Cell densities were measured turbidimetrically. Cell growth ceased, as a result of glucose limitation, approx. 3 h before harvest.

Cultures of *unc* mutants were checked daily for reversion to *succ*⁺. Experiments presented in this study were done only with cultures in which no revertants were detected.

Measurement of uptake. The medium used for measurement of uptake (uptake medium) was the same as that used for growth except that chloramphenicol (50 μ g/ml) was added. Cells were washed three times in uptake medium and resuspended in the same medium at a density of 0.12–0.15 mg dry wt./ml for determinations of glutamine uptake, and at a density of 0.35–0.45 mg dry wt./ml for determinations of proline uptake.

For the assays, cell suspensions (5 ml) at 37°C were either shaken at 300 rev./min in 25 ml flasks for 6 min (aerobic conditions) or were purged with N₂ for 15 min in 10 ml capped tubes (anaerobic conditions). Glucose (20 mM), nitrate (5 mM), fumarate (5 mM) and carbonyl cyanide *m*-chlorophenylhydrazine (20 μ M) were added where appropriate at the start of incubation. Assays were initiated by the addition of a nitrogen-purged solution of [¹⁴C]glutamine or [¹⁴C]proline at final concentrations of 10 μ M and 100 μ M respectively. Samples (0.5 ml) were withdrawn at the appropriate times, filtered, washed, counted for radioactivity and the results expressed as described previously (Rosenberg *et al.*, 1975).

Measurement of intracellular ATP concentrations. Cells were harvested, washed three times with 100 mM-KCl/2 mM-glycylglycine, pH 7.0, containing 50 μ g of chloramphenicol/ml, and resuspended in 20 ml of the same medium to a density of 2–5 mg dry wt./ml. Glucose (20 mM), specific growth supplements and, where appropriate, potassium fumarate (5 mM) and potassium nitrate (5 mM) were added at the start of incubation.

The cell suspensions were incubated anaerobically at 37°C for 20 min and were then extracted as described by Berger & Heppel (1974).

The extracts were freeze-dried, and the dried residue was suspended in 5 ml of water and filtered. ATP was assayed in samples (0.5 ml) of the filtrate by the chemiluminescence method as described by Berger & Heppel (1974) by using appropriately prepared standard ATP samples.

Results were expressed as nmol of ATP/mg dry wt.

Results and Discussion

Aerobic uptakes of glutamine (Fig. 1*a*) and proline (Fig. 1*b*) in the presence of glucose are similar in all strains tested. It is thus unlikely that the mutations present in any of the strains tested have any effect *per se* on the levels or activities of the proline and glutamine transport systems.

The curves shown in Figs. 1*a*) and 1*b*) are also qualitatively representative of the anaerobic uptakes observed. The rates deviate slightly from linearity over the first 30 s of glutamine uptake or the first 1 min of proline uptake; uptake still proceeds at 4 min, when the last reading was taken. In order to avoid unnecessary repetition of figures, data on anaerobic uptake have been tabulated.

It can be seen (Table 2 and Fig. 1) that the rates of anaerobic uptake of proline in the presence of glucose are lower than the corresponding rates of aerobic uptake in all strains tested. Under anaerobic conditions, and in the presence of glucose, strain AN472 (*frd-1*) takes up proline faster than the wild-type, whereas in strain AN480 (*uncB401*, *frd-1*) glucose produces no stimulation of uptake over endogenous rates. Nitrate stimulates anaerobic uptake of proline in strain AN480 (*uncB401*, *frd-1*) about threefold (Table 2). In strain AN283 (*uncB401*) glucose alone stimulates anaerobic proline uptake to slightly above endogenous levels. Fumarate causes a further slight stimulation (see particularly the 4 min result, Table 2). This is consistent with the ability of mutants bearing the *uncB* allele to grow anaerobically on glucose in the absence of fumarate (Butlin *et al.*, 1973), and with the observation (S. J. Gutowski, unpublished work) that this strain, when grown anaerobically on glucose, produces succinate in similar quantities to the wild-type. Thus

Fig. 1. Aerobic uptake of glutamine (a) and proline (b) by mutants of *E. coli* K12

○, Strain AN259; □, strain AN283; ●, strain AN472; ■, strain AN480. Cells were grown, prepared and assayed aerobically as described under 'Methods'.

Table 2. Anaerobic uptake of glutamine and proline by mutants of *E. coli* K12 defective in aspects of anaerobic energy metabolism

Strains were grown anaerobically, prepared and assayed anaerobically as described under 'Methods'. At the start of incubation additions were made as shown to the following final concentrations: glucose, 20 mM; fumarate, 5 mM; nitrate, 5 mM; carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 20 μ M.

Strain and relevant genetic loci	Addition	Glutamine uptake (nmol/mg dry wt.)		Proline uptake (nmol/mg dry wt.)	
		15 s	4 min	30 s	4 min
AN259 (wild-type)	0	0.0	0.0	0.1	0.4
	Glucose	2.0	17.7	1.0	4.7
	Glucose + fumarate	2.2	18.8	1.1	4.4
	Glucose + CCCP	0.6	7.1	0.2	0.2
AN472 (<i>frd-1</i>)	0	0.0	0.0	0.3	0.7
	Glucose	1.8	17.5	2.6	9.7
	Glucose + fumarate	1.7	18.2	2.4	8.9
	Glucose + CCCP	0.9	7.2	0.0	0.2
AN283 (<i>uncB401</i>)	0	0.0	0.0	0.2	0.6
	Glucose	2.8	22.0	0.2	1.0
	Glucose + fumarate	2.9	22.6	0.3	1.3
	Glucose + fumarate + CCCP	0.5	4.9	0.0	0.1
AN480 (<i>uncB401, frd-1</i>)	0	0.0	0.0	0.15	0.5
	Glucose	2.2	13.8	0.25	0.5
	Glucose + nitrate	3.0	21.5	0.75	2.0
	Glucose + nitrate + CCCP	0.6	4.6	0.2	0.1

anaerobic proline uptake requires the presence of either a functional Mg^{2+} -stimulated ATPase or anaerobic electron transport, and in the absence of both systems is abolished. This confirms that anaerobic proline transport is coupled to the 'high-energy membrane state'.

Rates of anaerobic glutamine uptake in the presence of glucose alone are similar to the corresponding rates of aerobic uptake in all strains used in

this study (Table 2 and Fig. 1). Fumarate does not stimulate anaerobic glutamine uptake in any of the strains tested. Nitrate stimulates the rate of anaerobic glutamine uptake in strain AN480 (*uncB401, frd-1*) approx. 50% (as compared with threefold in the case of proline, Table 2). Thus glutamine is taken up anaerobically under conditions where both coupled Mg^{2+} -stimulated ATPase activity and anaerobic electron transport have been rigorously excluded. Its

Table 3. Intracellular ATP concentrations in the mutants under various conditions

Cells were grown anaerobically on glucose as described under 'Methods'. Fumarate (5mM) was added to the growth medium for strain AN283. Nitrate (75mM) was added to the growth medium for strain AN480. Cells were harvested, washed and incubated as described under 'Methods'. Fumarate or nitrate (5mM) was added to the incubation media as indicated. Extracts were prepared and assayed as described under 'Methods'.

Strain	Addition	ATP concentration (nmol/mg dry wt.)
AN259	0	0.48
	Fumarate	0.83
AN283	0	0.50
	Fumarate	1.33
AN480	0	0.37
	Nitrate	1.02
AN472	0	0.52
	Fumarate	0.60

uptake therefore does not require the presence of a 'high-energy membrane state'.

Our results confirm the suggestion by Berger (1973), Berger & Heppel (1974) and Kobayashi *et al.* (1974) that two distinct mechanisms of energy coupling to active transport exist in *E. coli* and extend the validity of this suggestion to anaerobically-grown cells.

Several observations remain to be explained. First, energy-dependent glutamine uptake is inhibited by 60–80% by carbonyl cyanide *m*-chlorophenylhydrazine (under similar conditions energy-dependent proline uptake is inhibited by 85–100%) (Table 2). Since the uncoupler inhibits anaerobic glutamine uptake in the presence of glucose in strain AN480 (*uncB401*, *frd-1*) (Table 2), it is likely that the uncoupler does not merely dissipate the 'high-energy membrane state', but also has secondary inhibitory effects, which may possibly be related to the ability of carbonyl cyanide *m*-chlorophenylhydrazine to form complexes with thiol groups (Kaback *et al.*, 1974). Secondly, nitrate stimulates anaerobic glutamine uptake in strain AN480 (*uncB401*, *frd-1*). In an attempt to explain this finding we determined the intracellular concentration of ATP in the mutants used in this study under various incubation conditions (Table 3). It can be seen (Table 3) that nitrate increases the intracellular ATP concentration in strain AN480 (*uncB401*, *frd-1*). Table 3, however, highlights a second point. Several previous authors

have shown a correlation between intracellular ATP concentrations and the rates of transport of some solutes (Berger, 1973; Berger & Heppel, 1974; Kobayashi *et al.*, 1974). However, although there is a correlation (Tables 2 and 3) between the effect of nitrate on anaerobic glutamine uptake and its effect on intracellular ATP concentrations (strain AN480), no similar correlation is found for fumarate (strain AN283). It is thus questionable whether the intracellular concentration of ATP alone can be correlated with the energization of glutamine uptake; the mechanism by which nitrate stimulates anaerobic glutamine uptake thus remains unclear.

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References

- Barnes, E. M. & Kaback, H. R. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 1190–1198
- Berger, E. A. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1514–1518
- Berger, E. A. & Heppel, L. A. (1974) *J. Biol. Chem.* **249**, 7747–7755
- Butlin, J. D., Cox, G. B. & Gibson, F. (1973) *Biochim. Biophys. Acta* **292**, 366–375
- Cowell, J. L. (1974) *J. Bacteriol.* **120**, 139–146
- Curtis, S. J. (1974) *J. Bacteriol.* **120**, 295–303
- Harold, F. M. (1972) *Bacteriol. Rev.* **36**, 172–230
- Kaback, H. R., Reeves, J. P., Short, S. A. & Lombardi, F. J. (1974) *Arch. Biochem. Biophys.* **160**, 215–222
- Kadner, R. J. & Winkler, H. H. (1975) *J. Bacteriol.* **123**, 985–991
- Klein, W. L. & Boyer, P. D. (1972) *J. Biol. Chem.* **247**, 7257–7265
- Kobayashi, H., Kin, E. & Anraku, Y. (1974) *J. Biochem. (Tokyo)* **76**, 251–261
- Konings, W. N. & Kaback, H. R. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3376–3381
- Monod, J., Cohen-Bazire, G. & Cohn, M. (1951) *Biochim. Biophys. Acta* **7**, 585–599
- Or, A., Kanner, B. & Gutnick, D. L. (1973) *FEBS Lett.* **35**, 217–219
- Rosenberg, H., Cox, G. B., Butlin, J. D. & Gutowski, S. J. (1975) *Biochem. J.* **146**, 417–423
- Schairer, H. U. & Gruber, D. (1973) *Eur. J. Biochem.* **37**, 282–286
- Schairer, H. U. & Haddock, B. A. (1972) *Biochem. Biophys. Res. Commun.* **48**, 544–551
- Simoni, R. D. & Postma, P. W. (1975) *Annu. Rev. Biochem.* **44**, 523–554
- van Thienen, G. & Postma, P. W. (1973) *Biochim. Biophys. Acta* **323**, 429–440