Metabolite Transport in Mutants of *Escherichia coli* K12 Defective in Electron Transport and Coupled Phosphorylation

By HARRY ROSENBERG, GRAEME B. COX, JANET D. BUTLIN and
STEPHEN J. GUTOWSKI

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia

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1. The uptakes of P_i and serine by whole cells of mutant strains of *Escherichia coli* K12, grown under both aerobic and anaerobic conditions, were studied. 2. Uptake by aerobic cells was low in a ubiquinone-less mutant but normal in two mutant strains unable to couple phosphorylation to electron transport. 3. One of these uncoupled strains, carrying the *unc-405* allele, does not form a membrane-bound Mg^{2+} -stimulated adenosine triphosphatase aggregate, and it is concluded that the Mg^{2+} -stimulated adenosine triphosphatase does not serve a structural role in the aerobic active transport of P_i or serine. 4. The other uncoupled strain, in which aerobic uptake is unaffected, carries a mutation in the *uncB* gene, thus distinguishing this gene from the *etc* gene, previously shown to be concerned with the coupling of electron transport to active transport. 5. The uptakes of P_i and serine by anaerobic cells were normal in the ubiquinone-less mutant, but defective in both the uncoupled strains. 6. The uptake of P_i and serine by anaerobic cells of the *uncB* mutant could be increased by the addition of fumarate to the uptake medium. The *unc-405* mutant, however, required the addition of fumarate for growth and for uptake. 7. The *uncB* mutant, unlike the *unc-405* mutant, is able to grow anaerobically in a minimal medium with glucose as sole source of carbon. Similarly a strain carrying a mutation in the *frd* gene, which is the structural gene for the enzyme fumarate reductase, is able to grow anaerobically in a glucose-minimal medium. However, a mutant strain carrying mutations in both the *uncB* and *frd* genes resembles the *unc-405* mutant in not being able to grow under these conditions.

The accumulation of a solute within a cell, against a concentration gradient, requires the utilization of metabolic energy. Mutant strains of *Escherichia coli* affected in either electron transport or in oxidative phosphorylation have been used to study the mechanism of this energy utilization (Schairer & Haddock, 1972; Simoni & Shallenberger, 1972; Hong & Kaback, 1972; Prezioso *et al.*, 1973; Berger, 1973; Parnes & Boos, 1973; Reeves *et al.*, 1973; Rosen, 1973; Schairer & Gruber, 1973; Or *et al.*, 1973). It would appear that, in general, the active transport of a number of amino acids, sugars and other metabolites, under aerobic conditions, is coupled directly to electron transport without the intermediate formation of ATP. One of the Mg -ATPase*-deficient mutants isolated (NR70) showed a decreased ability to accumulate amino acids and sugars under aerobic conditions, but the activity in these mutants could be partially restored to normal by the addition of *NN'*-dicyclohexylcarbodi-imide (Rosen, 1973). It was concluded that the Mg -ATPase has a structural function in the aerobic uptake of solutes. Another

* Abbreviation: Mg -ATPase, Mg^{2+} -stimulated adenosine triphosphatase (EC 3.6.1.3).

Mg -ATPase-deficient mutant (DL-54) also showed a decreased ability to accumulate amino acids under aerobic conditions (Simoni & Shallenberger, 1972), but this strain was later shown to have normal activity if D-lactate rather than glucose was used as the energy source (Berger, 1973).

In the present paper we report results of studies on the uptake of P_i and of serine, under both aerobic and anaerobic conditions, by strains carrying one or two of the following mutant alleles: *ubiB*⁻, *ubiD*⁻, *menA401*, *unc-405*, *uncB401*, *frd-1*.

Experimental

Materials

Chemicals. Chemicals were of the highest purity available commercially and were not further purified. [$3\text{-}^{14}\text{C}$]Serine was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The radiochemical purity of the product was checked by t.l.c. in solvent systems I and II described by Rosenberg & Ennor (1961). Labelled orthophosphate (carrier-free) was

Table 1. *Strains of E. coli K12 used*

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

Strain	Relevant genetic loci	Other information
AN248	<i>ilvC-7, argH⁻, entA⁻</i>	Butlin <i>et al.</i> (1973)
AN259	<i>argH⁻, entA⁻</i>	Butlin <i>et al.</i> (1973)
AN285	<i>unc-405, argH⁻, entA⁻</i>	Cox <i>et al.</i> (1974)
AN283	<i>uncB401, argH⁻, entA⁻</i>	Butlin <i>et al.</i> (1973)
AN98	<i>argE⁻, metE⁻</i>	Newton <i>et al.</i> (1971)
AN99	<i>menA⁻, metE⁻</i>	Newton <i>et al.</i> (1971)
AN293	<i>argE⁻, ubiB⁻</i>	Isolated after transduction with strain AB3283 (Cox <i>et al.</i> , 1969) as donor and strain AN98 as recipient
AN318	<i>argE⁻, ubiD⁻</i>	Isolated after transduction with strain AN66 (Cox <i>et al.</i> , 1969) as donor and strain AN98 as recipient
R-4, frd-1	<i>met⁻, frd-1</i>	Obtained from J. R. Guest (Spencer & Guest, 1973)
AN472	<i>ilvC-7, frd-1, entA⁻</i>	Isolated after mating between R-4, frd-1 and AN248
AN480	<i>uncB401, frd-1, entA⁻</i>	Isolated after transduction with AN283 as donor and AN472 as recipient

obtained from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia, and used after appropriate dilution with carrier.

Organisms. All the bacterial strains used 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 described by Monod *et al.* (1951). Growth supplements were added as sterile solutions to the sterilized mineral-salts base, to the following final concentra-

tions: L-amino acids, 0.2mM; thiamin, 0.2 μ M; 2,3-dihydroxybenzoate, 40 μ M. Glucose was added at the growth-limiting concentration of 5mM.

Cells were grown aerobically at 37°C in 40ml of medium in 150ml flasks, shaken at 300 rev./min in an orbital shaking water bath.

Cells were grown anaerobically in Pyrex screw-capped bottles. After each bottle was inoculated, it was loosely capped and then placed in an anaerobic culture jar containing a 'cold' catalyst. The jar was partially evacuated, filled with hydrogen containing 5% CO₂, sealed, and then incubated at 37°C.

Cell densities were measured as extinction at 660nm in a Gilford 600 spectrophotometer, and expressed as mg dry wt./ml by using an experimentally derived factor ($E_{660}^{1.0\text{cm}}$ of 1.0 = 0.43 mg dry wt./ml). The desired cell density was usually reached overnight, but in some slower-growing mutants longer periods were required.

Measurement of uptake. Serine-uptake measurements were performed in the same medium as that used for growth, except that glucose was at 20mM. The cells were washed twice in the uptake medium suspended at a density of 0.1–0.15 mg dry wt./ml and kept on ice until used.

The medium used for measurement of phosphate uptake contained 50mM-triethanolamine-HCl buffer, pH 7.0, 15mM-KCl, 10mM-(NH₄)₂SO₄ and 1mM-MgCl₂. It was supplemented with glucose, thiamin and specific growth supplements as in the growth media. The cells, grown as described above, were harvested at a density of 0.2–0.3 mg dry wt./ml and were centrifuged and washed twice with 'uptake medium'. They were then resuspended in the uptake medium and incubated in the same atmosphere in which they were grown for sufficient time (2–4h) to evoke the high-rate initial uptake (Medveczky & Rosenberg, 1971). The cells were suspended at a density of about 0.1 mg dry wt./ml, and usually increased in density by about 25% over the P_i-deprivation period. The density was then adjusted to about 0.15 mg dry wt./ml and the cells were stored on ice until required.

For assay, cell suspensions (5.0ml) were adjusted to 37°C and the uptake experiment was commenced by the addition of 100 μ l of 2.5mM-³²P_i or of 50 μ l of 5mM-[¹⁴C]serine to the suspension. The suspension was either shaken at 300 gyrations/min (aerobic conditions), or stirred by bubbling with N₂ which had been passed through KOH-pyrogallol (anaerobic conditions). Four 0.5 ml samples were withdrawn over a period of 1 min (for phosphate) or 8 min (for serine) and passed through filter membranes of pore size 0.45 μ m, which were immediately washed with two 2ml lots of wash solution at 20°C. The 'wash solution' contained 10mM-triethanolamine-HCl, pH 7, 100mM-KCl, 10mM-(NH₄)₂SO₄, 1mM-MgSO₄ and 5mM-K₂HPO₄ buffer, pH 7.0. The washed damp mem-

branes containing ^{32}P -labelled cells were removed from the filters with the vacuum on, glued to aluminium planchets and counted for radioactivity in a Nuclear-Chicago gas-flow counter.

Membranes containing ^{14}C -labelled cells were thoroughly dried at 100°C and placed in vials each containing 10 ml of Bray's (1960) scintillation mixture in which the fluors were replaced by 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) (Scales, 1967) at 0.6% (w/v) final concentration. The radioactivity was counted in a Packard liquid-scintillation spectrometer, model 3320.

Standard counts were carried out with each batch on stock solutions, and background counts on membranes through which standard dilutions (without bacteria) had been passed and which had been washed under the usual conditions. All counts were accordingly corrected, and ^{32}P counts were also corrected for decay. The densities of the bacterial suspensions were measured directly after completion of the uptake. All results were expressed as nmol of substrate taken up/mg dry wt. of bacteria.

In control experiments, where the samples of cell suspensions were extracted with 5% (w/v) trichloroacetic acid before filtration, it was found that about 60% of the serine and about 98% of the P_i taken up

by the cell was acid-soluble. The latter is in agreement with previous observations on the fate of P_i taken up during the first 1 min by *E. coli* (Medveczky & Rosenberg, 1971).

Results

Preparation of strains

The two ubiquinone-deficient strains, AN293 (*ubiB*⁻) and AN318 (*ubiD*⁻) were derived by transduction from strain AN98. A mutation in the *ubiB* gene causes the accumulation of the biosynthetic intermediate 2-octaprenylphenol and a mutation in the *ubiD* gene causes the accumulation of 2-octaprenyl-4-hydroxybenzoate. The mutation in the *ubiD* gene is 'leaky' and allows the formation of about 20% of the normal content of ubiquinone. Strain AN99 (*menA*⁻) was also derived by transduction from strain AN98. The uncoupled strains, AN285 (*unc-405*) and AN283 (*uncB401*) were derived by transduction from strain AN248. The membrane-bound Mg-ATPase aggregate is not present in membranes prepared from strain AN285 (*unc-405*), whereas a normal Mg-ATPase is present in mem-

Fig. 1. Rates of uptake of orthophosphate (a) and serine (b) in respiratory mutants grown and assayed under aerobic conditions

●, Strain AN98 (parent); ■, strain AN99 (*men*⁻); ▼, strain AN318 (*ubiD*⁻); ▲, strain AN293 (*ubiB*⁻). For experimental details see under 'Methods'.

branes prepared from strain AN283 (*uncB401*). Strain AN259 is an *ilv*⁺ transductant, also derived from strain AN248, and is used as the normal control strain for the uncoupled mutants. Strain AN480 (*uncB401*, *frd-1*) was prepared by transduction from strain AN472 (*frd-1*), the presence of the *frd-1* allele causing a loss of fumarate reductase activity.

Uptake under aerobic conditions

The uptake of P_i under aerobic conditions by the ubiquinone- or menaquinone-deficient strains with glucose as energy source is shown in Fig. 1(a). The loss of menaquinone has no effect on the aerobic uptake of P_i , but the loss of ubiquinone decreases the rate of uptake of P_i to about 10% of the control rate. The mutation affecting ubiquinone biosynthesis in strain AN318 (*ubiD*⁻) is somewhat 'leaky' and decreases the NADH oxidase rate to about 40% of the control rate (Newton *et al.*, 1972). The uptake of P_i in this strain is decreased to about 60% of the rate found in the normal cells. The uptake of serine was also tested (Fig. 1b) and results essentially similar to those for P_i uptake were obtained.

The uptake of P_i by the uncoupled strains AN285 (*unc-405*) and AN283 (*uncB*⁻), under aerobic conditions with glucose as energy source, is shown in Fig. 2(a). The lack of the membrane-bound Mg-ATP-

ase aggregate in strain AN285 (*unc-405*) has no effect on the uptake of P_i under these conditions. Similarly, a mutation in the *uncB* gene has no effect on the rate of uptake of P_i . The results on the uptake of serine (Fig. 2b) are similar to those obtained for P_i uptake.

Uptake under anaerobic conditions

The rate of P_i uptake by normal cells under anaerobic conditions was similar to that obtained for the normal strain under aerobic conditions. The uptake of P_i under anaerobic conditions by the quinone-deficient strains grown anaerobically is shown in Fig. 3(a). In contrast with the results obtained with aerobic cells, there is no significant difference in the initial rates of P_i uptake by the ubiquinone-deficient strain and the normal strain. The lack of menaquinone, as was the case for aerobic cells, has no effect on the rate of P_i uptake by anaerobic cells. The rate of uptake of serine anaerobically (Fig. 3b) in normal cells is only about 30% of the rate obtained under aerobic conditions, but comparatively the results obtained with the quinone-deficient strains for serine uptake are analogous to those obtained for P_i uptake.

Whereas the rates of uptake by the uncoupled strains and the normal strain are similar under aerobic conditions, under anaerobic conditions the mutant

Fig. 2. Rates of uptake of orthophosphate (a) and serine (b) in uncoupled mutants grown and assayed under aerobic conditions

●, Strain AN259 (parent); ▲, strain AN283 (*uncB*⁻); ■, strain AN285 (*unc-405*). For experimental details see under 'Methods'.

Fig. 3. Rates of uptake of orthophosphate (a) and serine (b) in respiratory mutants grown and assayed under anaerobic conditions

●, Strain AN98 (parent); ■, strain 99 (*men*⁻); ▲, strain AN293 (*ubiB*⁻). For experimental details see under 'Methods'.

Fig. 4. Rates of uptake of orthophosphate (a) and serine (b) in uncoupled mutants grown and assayed under anaerobic conditions

●, Strain AN259 (parent); ▲, strain AN283 (*uncB*⁻); ■, strain AN285 (*unc-405*). Corresponding opening symbols refer to uptake in the presence of 10 mM-fumarate. For experimental details see under 'Methods'.

strains are defective in the uptake of P_i and serine (Fig. 4). The *uncB* mutant takes up P_i at a rate less than 10% of that in the normal cells. However, if fumarate is added to the uptake medium then the rate of uptake of P_i by the *uncB* mutant is about 60% of that found in normal cells (Fig. 4a). The *unc-405* mutant lacks the membrane-bound Mg-ATPase and requires the addition of fumarate to a glucose-minimal medium to enable growth to occur under anaerobic conditions. However, if fumarate is not included in the uptake medium then the *unc-405* mutant takes up P_i at a rate less than 3% of that found for normal cells. If fumarate is added to the uptake medium then the rate of uptake of P_i is about 50% of that found in normal cells.

The rate of uptake of serine by the normal strain under anaerobic conditions is much lower than that found under aerobic conditions (see Figs. 2b and 4b)

and the effect of the *uncB* and *unc-405* mutations is less. The addition of fumarate to the uptake medium for the *uncB* mutant and the addition of fumarate to both the growth medium and the uptake medium for the *unc-405* mutant increase the rate of uptake of serine in the mutant strains to that found in the normal cells.

The addition of fumarate to the growth and/or uptake medium has little effect on the rate of uptake of either P_i or serine by normal cells under anaerobic conditions.

Anaerobic growth of the *uncB* mutant

It would appear from the results described above that mutations affecting the coupling process also have some effect on the formation of fumarate from glucose under anaerobic conditions. The differences

Table 2. Doubling times of various *E. coli* mutants grown under anaerobic conditions

Strain	Relevant genetic markers	Supplement	Doubling time (h)
AN259	—	—	1.4
AN472	<i>frd</i> ⁻	—	2.3
AN283	<i>uncB</i> ⁻	—	3.8
AN283	<i>uncB</i> ⁻	Sodium fumarate (5 mM)	2.5
AN480	<i>uncB</i> ⁻ , <i>frd</i> ⁻	—	>13
AN480	<i>uncB</i> ⁻ , <i>frd</i> ⁻	NaNO ₃ (50 mM)	2.3

Fig. 5. Rate of uptake of orthophosphate in uncoupled and fumarate reductase mutants grown and assayed under anaerobic conditions

●, Strain AN259; ■, strain AN472 (*frd*⁻); strain AN480 (*uncB*⁻, *frd*⁻) in the absence (▲) and in the presence (△) of nitrate. For experimental details see under 'Methods'.

in growth between the *uncB* and *unc-405* mutants may be due to a difference in the degree of fumarate limitation. Strains were therefore prepared to determine whether a mutation in the *uncB* gene inserted into a strain already carrying a mutation in the *frd* gene, the structural gene for fumarate reductase (Spencer & Guest, 1973), would prevent growth anaerobically on glucose-minimal medium. Comparison of doubling times for strains AN472 (*frd*⁻) and AN283 (*uncB*⁻), growing anaerobically on glucose-minimal medium (Table 2), indicates that the loss of fumarate reductase activity alone has little effect on the rate of growth under these conditions and, although the *uncB* mutant is able to grow, its growth is stimulated by the addition of fumarate. The presence of both mutations (in strain AN480) abolishes growth under anaerobic conditions; growth is restored if nitrate is added to the medium.

The relative rates of uptake of P_i under anaerobic conditions by these strains (Fig. 5) reflect the relative

growth rates, although the *uncB* mutation affects the uptake of P_i somewhat more severely than it does the growth rate.

Discussion

The results described above indicate that, under aerobic conditions, ubiquinone is required for the uptake of P_i and of serine, presumably reflecting a requirement of ubiquinone for electron transport (Cox *et al.*, 1970). The coupling of aerobic electron transport to the uptake of P_i or serine does not, however, require either the *uncB* protein or the Mg-ATPase aggregate, both of which are required for the coupling of electron transport to ATP synthesis (Butlin *et al.*, 1973; Cox *et al.*, 1974). In this respect, the uptake of P_i or serine under aerobic conditions is similar to another energy-linked reaction, the electron-transport-dependent transhydrogenase (Cox *et al.*, 1973, 1974). The *etc* mutants described by Hong & Kaback (1972) are phenotypically similar to the *uncB* mutant with respect to growth yields, oxidase activities and Mg-ATPase activities. However, *etc* mutants have lost the ability to couple amino acid uptake to electron transport, whereas the *uncB* mutant has not. The *etc* gene and the *uncB* gene therefore appear to be different genes. Two ATPase-deficient mutant strains (DL-54, NR70) have been described in which aerobic active transport of amino acids has been affected (Simoni & Shallenberger, 1972; Rosen, 1973). In one of these strains (DL-54) further work indicated that uptake of amino acids with D-lactate as the oxidizable substrate was the same as for the parent strain (Berger, 1973). The defect in the mutant strain NR70 could be partially overcome by the addition of *NN'*-dicyclohexylcarbodi-imide. It was concluded that the ATPase may have a structural role as well as an enzymic one. A similar conclusion had been reached by Bragg & Hou (1973) as a result of experiments with strain DL-54, in which they showed that the electron-transport-dependent transhydrogenase activity, which was absent from this mutant, could be reactivated by the addition of *NN'*-dicyclohexylcarbodi-imide. Altendorf *et al.* (1974) demonstrated, using membrane vesicles of strain DL-54, that the lesion in this strain resulted in an increased permeability of the vesicular membrane to protons and an inability to maintain a potential across the membrane. The lesion, apparent in the vesicles, could be repaired by *NN'*-dicyclohexylcarbodi-imide. It should be noted that whole cells of strain DL-54 were still capable of active transport (Berger, 1973). Moreover, the present results obtained with the *unc-405* mutant suggest that the ATPase does not in fact play a structural role in either of these energy-requiring reactions.

Under anaerobic conditions, the loss of either ubiquinone or menaquinone had no effect on the

transport of serine or P_i . However, the question of an absolute quinone requirement for either of these uptakes can only be resolved by using a double quinone mutant, as ubiquinone has been shown capable of partially replacing menaquinone in a menaquinone-deficient mutant strain (Newton *et al.*, 1971).

In contrast with the effect under aerobic conditions, the uncoupled mutants are defective in the uptake of P_i and serine under anaerobic conditions. The *unc-405* mutant strain requires the addition of fumarate to the glucose-minimal medium for growth under anaerobic conditions. The *uncB* mutant strain is able to grow under these conditions in the absence of fumarate, but significant uptake of P_i and serine by both mutants requires the addition of fumarate to the uptake medium.

It appears then that mutations affecting the coupling of electron transport to oxidative phosphorylation have some effect on the formation of fumarate from glucose under anaerobic conditions, with the *unc-405* mutation having the more severe effect. Normally, fumarate reductase is induced in anaerobically grown cells of *E. coli*, which produce enough fumarate from glucose for the induction to occur (Spencer & Guest, 1973). It should be noted that both whole-cell suspensions of anaerobically grown *E. coli* and membrane vesicles from such cells have been shown to couple lactose uptake to electron flow from either D-lactate or α -glycerophosphate to fumarate (Konings & Kaback, 1973). The fumarate reductase system may play an important role in the supply of energy for active transport under anaerobic conditions and the lack of uptake by the uncoupled mutants might be attributed entirely to an indirect effect on this system. However, the uptake of P_i by the fumarate reductase mutant and the effect of an *uncB* mutation inserted into this mutant strain would suggest that a reversal of the coupling process does play some part in the energization of uptake under anaerobic conditions.

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