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ANAEROBIC TRANSPORT OF AMINO ACIDS COUPLED TO THE GLYCEROL-3-PHOSPHATE-FUMARATE OXIDOREDUCTASE SYSTEM IN A CYTOCHROME-DEFICIENT MUTANT OF *ESCHERICHIA COLI*

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SUMMARY

The uptake of proline and glutamine by cytochrome-deficient cells of *Escherichia coli* SASX76 grown aerobically on glucose or anaerobically on pyruvate was stimulated by these two substrates. Pyruvate could not stimulate transport in the glucose-grown cells. Uptake of these amino acids energized by glucose was inhibited by inhibitors of the Ca^{2+}, Mg^{2+}-stimulated ATPase such as DCCD, pyrophosphate, and azide, and by the uncouplers CCCP and 2,4-dinitrophenol. Glycerol (or glycerol 3-phosphate) in the presence of fumarate stimulated the transport of proline and glutamine under anaerobic conditions in cytochrome-deficient cells but not in membrane vesicles prepared from these cells although glycerol 3-phosphate-fumarate oxidoreductase activity could be demonstrated in the vesicle preparation. In contrast, in vesicles prepared from cytochrome-containing cells of *E. coli* SASX76 amino acid transport was energized under anaerobic conditions by this system. Inhibitors of the Ca^{2+}, Mg^{2+}-activated ATPase and uncoupling agents inhibited the uptake of proline and glutamine in cytochrome-deficient cells dependent on the glycerol-fumarate oxidoreductase system. Ferricyanide could replace fumarate as an electron acceptor to permit transport of phenylalanine in cytochrome-deficient or cytochrome- containing cells under anaerobic conditions. It is concluded that in cytochrome-deficient cells using glucose, pyruvate, or glycerol in the presence of fumarate, transport of both proline and glutamine under anaerobic conditions is energized by ATP through the Ca^{2+}, Mg^{2+}-activated ATPase. In cytochrome-containing cells under anaerobic conditions electron transfer between glycerol and fumarate can also drive transport of these amino acids.

INTRODUCTION

Over the past few years evidence has been accumulated which suggests that the active transport in *E. coli* of many sugars and amino acids is coupled to an energized

Abbreviations: CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

state of the cell membrane which may be generated either by substrate oxidation through the respiratory chain or by hydrolysis of ATP [1]. When electron transport is blocked, as in cells treated with cyanide [2–4], or incubated under anaerobic conditions [5], active transport of the sugars and amino acids continues if the cells have an active Ca^{2+}, Mg^{2+}-activated ATPase but is abolished in mutants lacking this activity or in cells treated with inhibitors of the ATPase, such as DCCD [2, 3, 6]. Recently, Berger and Heppel [4, 7] have shown that the transport of a group of amino acids, exemplified by glutamine, is driven by ATP but does not involve the generation of an energized state of the membrane through the ATPase. However, independent of the mechanism, the transport in *E. coli* of amino acids and of some sugars under anaerobic conditions appears to be driven by ATP [1].

When facultative anaerobes such as *E. coli* are grown anaerobically on glycerol in the presence of fumarate the formation of an NAD^+-independent glycerol 3-phosphate dehydrogenase and of fumarate reductase is induced [8]. Since, this system in basically an electron transfer system in which reducing equivalents can be transferred from glycerol to fumarate even in the absence of cytochromes [9], it is conceivable that electron flow through this system might be able to drive transport of amino acids and sugars without the involvement of ATP. From their studies with whole cells and membrane vesicles of wild-type *E. coli*, Konings and Kaback [10] found that under anaerobic conditions active transport of lactose driven by the glycerol-fumarate system was coupled to electron flow. The role of ATP in this process was discounted since their membrane vesicles did not contain ATP, and even after incorporating ATP into the vesicles there was no uptake of lactose by the vesicles. Similar results have been obtained for serine and phosphate transport in *E. coli* by Rosenberg et al. [11].

We have re-examined the role of electron transfer and of ATP as the energy sources under anaerobic conditions for amino acid and succinate transport coupled to the glycerol-fumarate system. We have used a mutant, *E. coli* SASX76, which is unable to form cytochromes in the absence of added 5-aminolevulinic acid [12, 13] so that substrate oxidation through the cytochrome pathway would not complicate the results. The transport of proline and of glutamine was studied since these represent the two classes of amino acids which can be distinguished on the basis of the mechanism by which their transport is energized by ATP [4, 7]. Our results suggest that in cytochrome-deficient cells growing anaerobically on glycerol in the presence of fumarate, ATP, not electron transfer, is used to energize the transport of proline, glutamine and succinate. However, in the presence of cytochrome electron transfer between glycerol and fumarate will drive amino acid transport. Furthermore, in contrast to the results of Berger and Heppel [4, 7] the energization of glutamine transport by ATP involves the Ca^{2+}, Mg^{2+}-activated ATPase and an energized state of the membrane [1].

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade purity. Dicumarol, D-lactate (lithium salt), and chloramphenicol were purchased from Calbiochem. DL-α-Glycerophosphate (disodium salt), and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide were obtained from

Sigma Chemical Co. Lysozyme (Worthington Biochemical Corp.), 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) (C.G. Frederick Smith Chemical Co.), 2,6-dichlorophenolindophenol and *N,N'*-dicyclohexylcarbodiimide (Eastman Organic Chemicals) were purchased as indicated.

L-[U-^{14}C]phenylalanine (522 mCi/mmol) and L-[U-^{14}C]proline (290 mCi/mmol) were obtained from Amersham-Searle Corporation. L-[U-^{14}C]glutamine (235 mCi/mmol) and [2,3-^{14}C]succinate (12.99 mCi/mmol) were purchased from New England Nuclear.

Organism and growth

E. coli SASX76 (formerly SHSP18) (*F*$^-$, *hemA*$^-$, *met*$^-$, *trp*$^-$, *lac*$^-$, *str*$^-$) [12], a generous gift of Dr. S. Sasarman (University of Montreal, Canada) was used in this study. Cells were grown anaerobically at 22 °C or 37 °C in 4 l flasks (filled to the top) in a minimal salts-glycerol medium [14] containing L-methionine, L-tryptophan, and 5-aminolevulinic acid (if present), all at a concentration of 25 mg/l. Vitamin-free casamino acids (2 g/l) or bacto-tryptone (5 g/l), and fumarate (2.3 g/l) were also present. Dissolved oxygen was removed from the flasks by flushing with nitrogen for 20 min before sealing with a fermentation lock. The cells were harvested either in the middle or at the end of the exponential phase of growth. In some experiments, cells were grown anaerobically in 1-l flasks on trypticase soy broth or on a minimal-salts medium containing 0.5 % bactopeptone and 50 mM sodium pyruvate.

Preparation of membrane vesicles

Membrane vesicles were prepared from cells harvested at the end of the exponential phase by the method of Konings and Kaback [10] with the following modifications: (a) the cells were washed once with 30 mM Tris · HCl buffer, pH 7.5, containing 20 % (w/v) sucrose instead of sucrose-free buffer, (b) following treatment with lysozyme plus EDTA, and lysis, whole cells were removed from the lysate by centrifugation at 2500 $\times g$ for 20 min, and the membrane vesicles then sedimented by centrifugation at 100 000 $\times g$ for 30 min [15]. The pellet was suspended in 50 mM potassium phosphate buffer, pH 6.6, at a protein concentration of 5–10 mg/ml. Experiments using these vesicles were performed on the day of the preparation because the vesicles lost 80 % of their transport activity for proline or phenylalanine if stored overnight at 0 °C or at −80 °C.

Uptake studies with whole cells and vesicles

Uptake of amino acids and succinate under aerobic conditions was measured at 22 °C by the method of Cox et al. [16] using cells harvested at room temperature in the mid exponential phase of growth. The cells were washed twice in a minimal salts medium, and resuspended in the same medium at a cell density giving 2.8 mg/protein/ml. Each incubation mixture contained in a total volume of 0.2 ml, 0.02 ml cell suspension, 0.1 ml minimal salts medium containing 200 μg/ml chloramphenicol, and the energy source at the concentration indicated. The cells were preincubated in the minimal medium without the energy source for 10 min at 37 °C, then cooled to 22 °C, and the energy source added. After incubation for a further 5 min at 22 °C, the radioactive amino acid or succinate diluted with the unlabelled substance was added to a final concentration of 12 μM (or 100 μM for succinate). The concentrations of

[^{14}C]proline, [^{14}C]glutamine, [^{14}C]phenylalanine, and [^{14}C]succinate in the assay mixture were 0.43, 0.53, 0.26, and 7.6 μM, respectively. Uptake was terminated by the addition of 3 ml 200 mM LiCl immediately followed by filtration through a Millipore membrane filter (25 mm diameter; pore size, 0.45 μm). The cells were washed twice with 3 ml 100 mM LiCl. After drying, the filters were dissolved in Bray's scintillation fluid and the radioactivity measured with a Packard Tri-Carb liquid scintillation spectrophotometer, model 2425.

For inhibition studies the cells were preincubated at 37 °C for 10 min with the indicated concentration of the inhibitor prior to the addition of the energy source. When pyrophosphate and arsenate were used the cells were washed twice with 10 mM Tris · HCl buffer, pH 7.5, containing 0.5 mM $MgCl_2$ and 150 mM NaCl, and resuspended in the same buffer for the transport studies. The water insoluble inhibitors were added as ethanolic solutions. At the level used, the ethanol did not affect the uptake of the amino acids.

For transport experiments under anaerobic conditions the reaction mixture was based on that used for the aerobic experiments except that a larger volume was used. The cell samples in buffer (4 ml) were placed in 15 ml test tubes fitted with rubber serum stoppers and flushed with nitrogen for 10 min via syringe needles to remove oxygen. The other reagents were also flushed with nitrogen. The reaction was started as before by the addition of the labelled amino acid to the cell suspension under nitrogen. Samples (0.5 ml) (0.2 mg protein/ml) were removed with a hypodermic syringe, care being taken to avoid the introduction of air into the sample or into the incubation mixture. The samples were rapidly filtered, washed with oxygen-free buffer, and the radioactivity determined as above.

The uptake of [^{14}C]proline by membrane vesicles under aerobic and anaerobic conditions was measured by the method of Kaback [17].

Enzyme assays

D-lactate, DL-glycerol 3-phosphate, and NADH dehydrogenase activities were measured as described previously [9]. Fumarate reductase, glycerol 3-phosphate-fumarate oxidoreductase, and Ca^{2+}-activated ATPase were measured by the methods of Spencer and Guest [18], Miki and Lin [19], and Davies and Bragg [20], respectively. All assays were carried out in cuvettes of 1 cm light path. Protein was determined by the Folin method [21].

RESULTS

Energization of amino acid and succinate transport by glucose and pyruvate in cytochrome-deficient cells

As previously shown [9, 13], *E, coli* SASX76 grown either on glucose or on glycerol with fumarate, aerobically or anaerobically, did not form cytochromes in the absence of 5-aminolevulinic acid. Similar results were obtained for cells grown anaerobically on pyruvate as the carbon source.

In agreement with our previous results with phenylalanine [13], glucose could also stimulate the uptake of proline, glutamine and succinate by cytochrome-deficient cells under aerobic conditions (Fig. 1). These results suggested that ATP generated by glycolysis was involved in the transport process. However, NADH is also formed

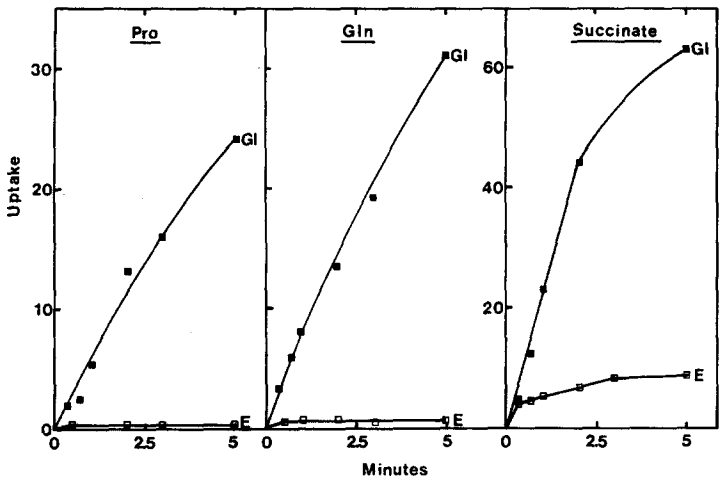

Fig. 1. Uptake of proline, glutamine and succinate by cytochrome-deficient cells under aerobic conditions in the presence of 50 mM glucose (Gl) or in its absence (E). *E. coli* SASX76 was grown aerobically at 37 °C to the mid-exponential phase in a minimal salts/tryptone/glucose medium in the absence of 5-aminolevulinic acid. Uptake was measured at 22 °C and is expressed as nmol/mg cell protein.

during glycolysis and we have recently shown that it can be oxidized even in cytochrome-deficient cells to energize the cell membrane as demonstrated by the quenching of the fluorescence of the acridine dye, atebrin [9]. Therefore, we examined the ability of pyruvate to energize the transport of proline and glutamine in cytochrome-deficient cells grown anaerobically on pyruvate to induce the phosphoroclastic system [22, 23]. Phosphoroclastic cleavage of pyruvate under anaerobic conditions yields ATP without the formation of NADH. As shown in Table I, uptake of proline and glutamine was stimulated two to three-fold under aerobic and anaerobic conditions. We were not able to lower the rather high endogenous rate of amino acid uptake in these cells by shaking in air in the absence of substrate or by repeated washings. Pyruvate could not energize the uptake of these amino acids under aerobic or an-

TABLE I

ENERGIZATION BY PYRUVATE OF PROLINE AND GLUTAMINE UPTAKE UNDER AEROBIC AND ANAEROBIC CONDITIONS IN CYTOCHROME-DEFICIENT CELLS

E. coli SASX76 was grown anaerobically without 5-aminolevulinic acid on tryptone broth containing 0.25 % sodium pyruvate. Uptake was measured as described in Materials and Methods using 50 mM sodium pyruvate as the energy source.

Energy source	Initial rate of uptake (nmol/min/mg protein)			
	Proline		Glutamine	
	Aerobic	Anaerobic	Aerobic	Anaerobic
None	0.38	0.18	0.70	0.33
Pyruvate	0.61	0.43	1.75	0.73

aerobic conditions in cells grown anaerobically on glucose without 5-aminolevulinic acid demonstrating the need for induction of the phosphoroclastic system.

The above results suggested that ATP was involved in energy-coupling to proline, glutamine and succinate transport in cytochrome-deficient cells. The involvement of the Ca^{2+}, Mg^{2+}-activated ATPase and of an energized state of the membrane in this process was examined by the use of inhibitors of the ATPase (azide, DCCD, pyrophosphate) [24] and of uncoupling agents (CCCP, 2,4-dinitrophenol) [25]. Proline, glutamine and succinate transport energized by glucose in cytochrome-deficient cells grown on glucose was inhibited 98 to 99 % by 10 mM sodium azide, 0.5 mM DCCD, 0.05 mM CCCP, and 1 mM 2,4-dinitrophenol. Sodium arsenate (2 mM) inhibited glutamine, proline and succinate uptake by 61 %, 60 %, and 34 %, respectively. Sodium pyrophosphate (5 mM) inhibited the uptake of these substances by 72, 52 and 48 %, respectively. The involvement of ATP and of an energized state of the membrane in the transport of glutamine, proline, and succinate by these cells is suggested by these results.

Energization of amino acid transport by glycerol in the presence of fumarate in cytochrome-deficient cells

As shown in Fig. 2 the uptake of proline, phenylalanine, and glutamine was stimulated by 14-, 9-, and 215-fold over endogenous rates by glycerol with fumarate in cytochrome-deficient cells under anaerobic conditions. Glycerol and fumarate alone were ineffective. D-Lactate in the presence or absence of fumarate was unable to support the uptake of phenylalanine (Fig. 2). Since D-lactate dehydrogenase and fumarate reductase were present (Table II), the absence of cytochrome must have been responsible for the lack of transport as Konings and Kaback [10] have observed

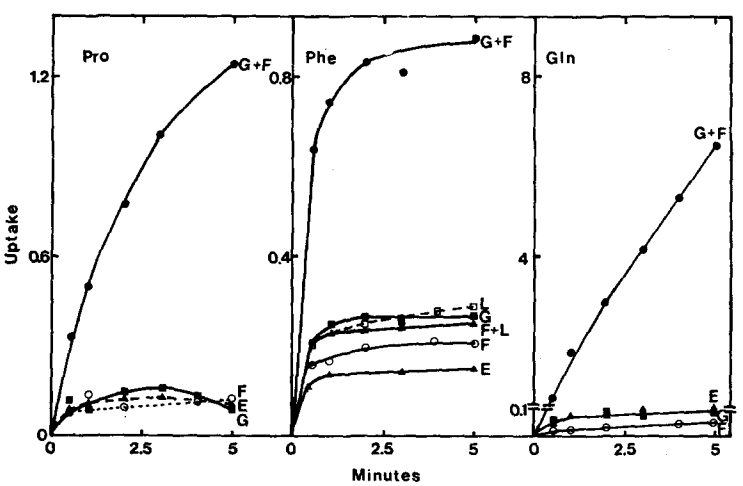

Fig. 2. Uptake of proline, phenylalanine and glutamine by cytochrome-deficient cells under anaerobic conditions in the absence of added substrate (E), or in the presence of 50 mM glycerol (G), 20 mM fumarate (F), or 20 mM D-lactate (L), alone or in combination. *E. coli* SASX76 was grown anaerobically at 37 °C to the mid-exponential phase in a glycerol-fumarate medium in the absence of 5-aminolevulinic acid. Uptake was measured at 22 °C and is expressed as nmol/mg protein.

TABLE II

SPECIFIC ACTIVITIES OF ENZYMES IN MEMBRANE VESICLES FROM CYTOCHROME-CONTAINING AND -DEFICIENT CELLS

E. coli SASX76 was grown anaerobically at 37 °C, with or without 5-aminolevulinic acid (ALA), in a glycerol-fumarate medium. Membrane vesicles were prepared and enzymes were assayed at 37 °C as described in Materials and Methods.

Enzyme	Spec. act. (nmol/min/mg protein)	
	−ALA	+ALA
DL-Glycerol 3-phosphate dehydrogenase	11.2	6.3
D-lactate dehydrogenase	39.2	18.0
NADH dehydrogenase	143	36
DL-Glycerol 3-phosphate-fumarate oxidoreductase	8.0	12.0
Fumarate reductase	167	171
Ca^{2+}-activated ATPase	398	308

that the D-lactate-fumarate oxidoreductase system could energize lactose transport under anaerobic conditions in wild-type cells.

Effect of inhibitors on amino acid transport energized by glycerol in the presence of fumarate in cytochrome-deficient cells

The involvement of ATP, formed by glycolysis from glycerol in the presence of fumarate, in the transport of amino acids under anaerobic conditions by cyto-

TABLE III

EFFECT OF INHIBITORS ON UPTAKE OF PROLINE AND GLUTAMINE UNDER ANAEROBIC CONDITIONS BY CYTOCHROME-DEFICIENT CELLS

E. coli SASX76 was grown anaerobically in the absence of 5-aminolevulinic acid on a glycerol-fumarate medium. Uptake of amino acids was measured at 22 °C at a protein concentration of 0.23 mg protein/ml in the presence of 50 mM glycerol and 30 mM fumarate as described in Materials and Methods. Control rate of uptake: proline, 0.8 nmol/min/mg protein; glutamine, 1.2 nmol/min/mg protein.

Inhibitor	Concentration (mM)	% Inhibition of uptake	
		Proline	Glutamine
Sodium Pyrophosphate	5	52	72
Sodium Arsenate	2	60	61
Sodium Azide	10	96	97
DCCD	0.5	96	96
2,4-dinitrophenol	2	95	98
CCCP	0.05	84	96
Piericidin A	0.05	76	80
Dicoumarol	0.1	68	60
HOQNO	0.025	70	71
Bathophenanthroline	0.25	50	56

chrome-deficient cells was suggested by the effect of inhibitors (Table III). Incubation of cells for 10 min with 2 mM arsenate, which is known to lower the intracellular level of ATP in *E. coli* [6], inhibited the uptake of proline and glutamine by 60 %. The ATPase inhibitors [24] sodium azide (10 mM) and DCCD (0.5 mM) reduced by 96 % the anaerobic uptake of proline and glutamine energized by glycerol with fumarate. Sodium pyrophosphate, also an ATPase inhibitor, inhibited the uptake of proline and glutamine by 52 and 72 %, respectively. The uncouplers, 2,4-dinitrophenol and CCCP effectively blocked transport of both amino acids.

Even in the absence of cytochromes, the anaerobic uptake of proline and glutamine energized by glycerol with fumarate was markedly inhibited by the respiratory chain inhibitors bathophenanthroline, HOQNO, dicumarol, and piericidin A. We have previously shown that these inhibitors prevent the transfer of reducing equivalent from glycerol to fumarate in cytochrome-deficient cells of *E. coli* [9].

Transport of proline by membrane vesicles energized by glycerol in the presence of fumarate

In contrast to whole cells, membrane vesicles prepared from cytochrome-deficient cells, which had been grown anaerobically on glycerol with fumarate, did not transport proline under anaerobic or aerobic conditions when glycerol 3-phosphate

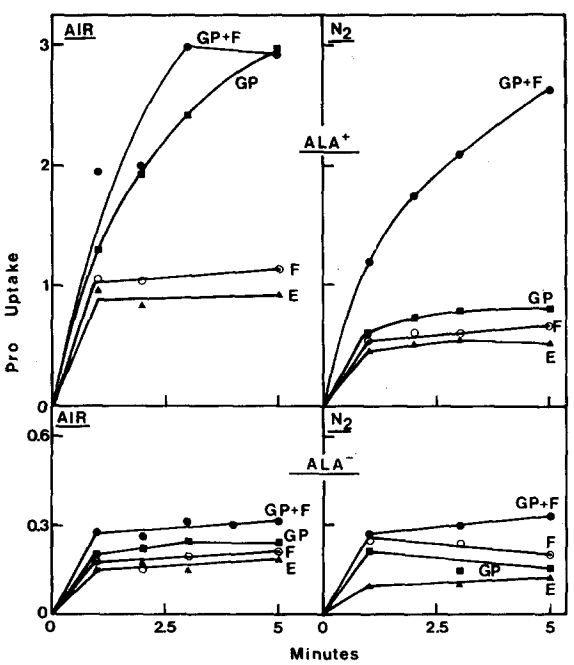

Fig. 3. Uptake of proline under aerobic (AIR) or anaerobic (N_2) conditions by membrane vesicles prepared from cytochrome-containing (ALA^+) or cytochrome-deficient (ALA^-) cells in the absence of an energy source (E), or in the presence of 20 mM glycerol 3-phosphate (GP), 20 mM fumarate (F), or both (GP+F). *E. coli* SASX76 was grown anaerobically at 37 °C to the late exponential phase on a glycerol-fumarate medium in the presence or absence of 5-aminolevulinic acid (ALA). Uptake was measured at 22 °C and is expressed as nmol/mg protein.

with fumarate was used as the energy source (Fig. 3). The inability of this system to carry out transport was not due to the lack of glycerol 3-phosphate dehydrogenase or of fumarate reductase, and that these two enzymes could couple together was shown by the presence of glycerol 3-phosphate-fumarate oxidoreductase activity (Table II). Since Ca^{2+}, Mg^{2+}-activated ATPase activity was also present, it was concluded that the inability of the membrane vesicles to generate ATP by glycolysis from dihydroxyacetone phosphate, the product of dehydrogenation of glycerol 3-phosphate, was responsible for the lack of transport. Kaback [26] has shown that membrane vesicles retain only minute quantities of the cytoplasmic constituents.

In contrast to the results with cytochrome-deficient vesicles, vesicles prepared from cytochrome-containing cells grown under the above conditions accumulated proline under anaerobic conditions in the presence of glycerol 3-phosphate and fumarate. Under aerobic conditions the uptake of proline by vesicles could be energized by glycerol 3-phosphate alone (Fig. 3). These results are consistent with those of Konings and Kaback for lactose transport [10] which suggest that electron transfer from glycerol 3-phosphate to fumarate in the presence of cytochrome can support transport.

Effect of potassium ferricyanide on phenylalanine uptake

Sprott et al. [27] have shown that under anaerobic conditions glycerol in the presence of 7 mM potassium ferricyanide can energize the uptake of phenylalanine by wild-type cells of *E. coli*. Ferricyanide is probably acting as a hydrogen acceptor in this system but its site of interaction is not known.

The uptake of phenylalanine by cytochrome-containing cells of *E. coli* SASX76

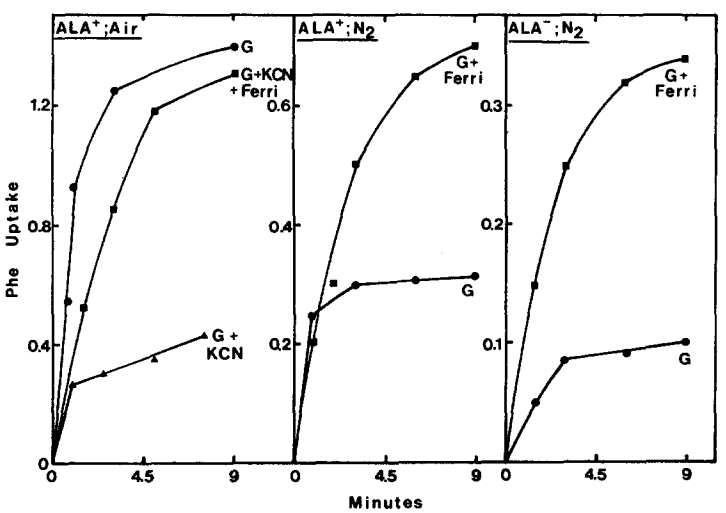

Fig. 4. Uptake of phenylalanine under aerobic (Air) or anaerobic (N_2) conditions by cytochrome-containing (ALA^+) or cytochrome-deficient (ALA^-) cells in the presence of glycerol (G) with or without the addition of 10 mM KCN or 7 mM potassium ferricyanide (Ferri). *E. coli* SASX76 was grown aerobically at 37 °C to the mid exponential phase on a minimal salts-glycerol medium with 5-aminolevulinic acid (ALA) or anaerobically without ALA on glycerol-fumarate medium. Uptake was measured at 22 °C and is expressed as nmol/mg protein.

was stimulated by glycerol under aerobic conditions (Fig. 4). This uptake was blocked by 10 mM potassium cyanide which inhibits the respiratory chain at the level of the cytochrome oxidase [28]. Anaerobiosis also prevented the stimulation of phenylalanine uptake produced by glycerol. Addition of potassium ferricyanide overcame the inhibitory effects of cyanide and anaerobiosis on uptake by providing an alternative electron acceptor to oxygen for the reducing equivalents from glycerol. In cytochrome-deficient cells ferricyanide could also mediate the uptake of phenylalanine under anaerobic conditions in the presence of glycerol indicating that it must accept reducing equivalents from the system prior to the level of the cytochromes. In this respect it closely resembles fumarate and perhaps interacts with the same component of the system as does fumarate reductase.

DISCUSSION

The results presented in this paper extend our previous results with phenylalanine [13] and show that the uptake of proline, glutamine, and succinate by cytochrome-deficient cells in the presence of glucose was dependent on ATP. Devor et al. [29] have obtained similar results for lactose transport using another cytochrome-deficient mutant of *E. coli*. The involvement of the Ca^{2+}, Mg^{2+}-activated ATPase in the coupling of ATP to transport was strongly suggested by the inhibitory effects of ATPase inhibitors on the transport of proline and glutamine.

That ATP is also involved in proline and glutamine transport energized by the glycerol-fumarate system under anaerobic conditions in cytochrome-deficient cells was indicated by the inhibitory effects on transport of the ATPase inhibitors DCCD, pyrophosphate, and azide [24]. Arsenate, which is known to lower the intracellular concentration of ATP [6] was also inhibitory. Further support for the role of ATP in this system was shown by experiments with membrane vesicles prepared from cytochrome-deficient cells. The level of transport activity for proline in the absence of substrate was low and was not markedly increased by glycerol 3-phosphate and fumarate, alone or in combination, under aerobic or anaerobic conditions. The low level of transport activity was not due to the absence of glycerol 3-phosphate-fumarate oxidoreductase activity in these vesicles. Since membrane vesicles are almost completely devoid of cytoplasmic components [26], these results are consistent with a requirement for ATP for transport in the cytochrome-deficient cells using glycerol and fumarate, and with electron transfer alone being unable to support transport. The latter conclusion is in contrast to that of Konings and Kaback [10] and Rosenberg et al. [11] who demonstrated that electron transfer to fumarate could under anaerobic conditions energize the transport of lactose, phosphate and serine. These workers used cytochrome-containing cells or vesicles in their experiments. Vesicles prepared from cells of *E. coli* SASX76 grown with 5-aminolevulinic acid in order to contain cytochromes behaved differently from those without cytochrome. Proline uptake was energized by glycerol 3-phosphate under aerobic but not under anaerobic conditions unless fumarate was present. This confirms the results of Konings and Kaback [10], and Rosenberg et al. [11] and suggests that although the glycerol 3-phosphate-fumarate oxidoreductase system in cytochrome-deficient cells cannot energize transport by electron transfer under anaerobic conditions it can do so if cytochrome is present.

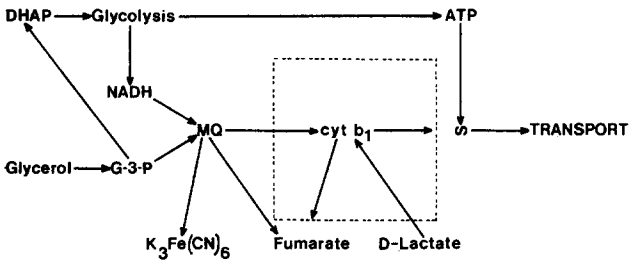

Fig. 5. Interrelationships between the metabolic pathways involved in the metabolism of glycerol under anaerobic conditions which yield ATP and the energized state of the membrane ($\tilde{\omega}$) coupled to transport. Glycerol 3-phosphate (G-3-P) is oxidized by G-3-P dehydrogenase to yield dihydroxyacetone phosphate (DHAP). The reducing equivalents pass via menaquinone (MQ) to fumarate in cytochrome-deficient cells. In cytochrome-containing cells (blocked-in area), the reducing equivalents pass via cytochrome b_1 to fumarate with consequent membrane energization. DHAP is metabolized through the glyolytic pathway with the production of ATP, which is coupled to transport in the cytochrome-deficient cells through the Ca^{2+}, Mg^{2+}-activated ATPase, and of NADH. NADH oxidation in cytochrome-deficient cells under anaerobic conditions can be coupled to the reduction of fumarate.

The existence of cytochrome-dependent and -independent routes in the glycerol 3-phosphate-fumarate oxidoreductase system, with the former pathway only having an energy-coupling site (Fig. 5), explains some previous observations. Thus, Miki and Lin [30] using cytochrome-containing cells had suggested that there was an energy-coupling site in this system although we had not been able to show this in cytochrome-deficient cells [9]. Moreover, we had previously found that the growth rate and growth yield on glycerol with fumarate under anaerobic conditions were higher in the presence of cytochromes than in their absence [9].

In cytochrome-deficient cells growing anaerobically on glycerol with fumarate the ATP required for transport and other cell processes must be generated by glycolysis. Fumarate under these circumstances allows glycolysis to continue with the production of ATP by acting as an electron acceptor for the reoxidation of NADH (Fig. 5). Ferricyanide can replace fumarate in this role. Thus, if glycerol oxidation in cytochrome-containing cells is blocked by cyanide or by anaerobiosis amino acid transport ceases unless ferricyanide is added as an electron acceptor. Ferricyanide will also replace fumarate in the energization of phenylalanine transport in cytochrome-deficient cells indicating that ferricyanide interacts with respiratory chain prior to the level of the cytochromes (Fig. 5).

Although our previous work [9] suggested that the NADH-fumarate oxidoreductase could energize the membrane in cytochrome-deficient cells as measured by the quenching of atebrin fluorescence, the experiments described in the present paper indicate that the NADH-fumarate oxidoreductase cannot energize transport. Thus, in the presence of inhibitors of the ATPase, transport is completely abolished under conditions where the NADH-fumarate oxidoreductase is undoubtedly active. This suggests either that the membrane energization measured by fluorescence quenching is not coupled to transport or that it is of insufficient magnitude to drive the transport of the amino acids.

Berger and Heppel [4, 7] have suggested that the role of ATP in the transport of proline and of glutamine is different in that the energization of the membrane by the

Ca^{2+}, Mg^{2+}-activated ATPase is involved only in the transport of the former amino acid. We have found that the transport of glutamine as well as of proline in cytochrome-deficient cells with glucose or glycerol and fumarate can be almost completely inhibited by inhibitors of the ATPase. Moreover, transport of both amino acids was inhibited by the uncouplers 2,4-dinitrophenol and CCCP suggesting that the energized state of the membrane [1, 25] was involved in transport of both amino acids. Berger and Heppel had found that glutamine transport was relatively resistant to these uncouplers. We conclude that in cytochrome-deficient cells the role of ATP in the transport of glutamine and proline is similar. Plate et al. [31] have arrived at the same conclusion from studies of the effect of colicin K on glutamine and proline transport in a wild-type and an ATPase-deficient mutant of *E. coli*.

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