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REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE DEPENDENT REDUCTION OF FUMARATE COUPLED TO MEMBRANE ENERGIZATION IN A CYTOCHROME DEFICIENT MUTANT OF *ESCHERICHIA COLI* K12

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SUMMARY

Escherichia coli SASX76 does not form cytochromes unless supplemented with 5-aminolevulinic acid. It can grow anaerobically on glycerol and DL-glycerol 3-phosphate in the absence of 5-aminolevulinic acid with fumarate but not with nitrate as the terminal electron acceptor. Cytochrome-independent NADH oxidase, glycerol 3-phosphate- and NADH-fumarate oxidoreductase activities are induced by anaerobic growth on a glycerol-fumarate medium. The pathway of electrons from substrate to fumarate involves menaquinone. The NADH-fumarate oxidoreductase and cytochrome-independent NADH oxidase systems are inhibited by piericidin A, 2-heptyl-4-hydroxyquinoline *N*-oxide, and iron chelating agents. Both systems can energize the membrane particles as indicated by quenching of atebrin fluorescence.

INTRODUCTION

Facultative anaerobes such as *Escherichia coli* can grow on glycerol under anaerobic conditions when either fumarate or nitrate are present as terminal electron acceptors [1, 2]. Anaerobic growth on glycerol in the presence of fumarate induces the formation of an additional glycerol 3-phosphate dehydrogenase as well as fumarate reductase [3]. A particulate system, catalyzing the reduction of fumarate at the expense of either NADH, glycerol-3-phosphate or molecular hydrogen has also been reported for other organisms such as *Streptococcus faecalis* [4], *Bacillus megaterium* [5] *Desulfovibrio gigas* [6], *Propionibacterium arabinosum* [7] and *Propionibacterium freudenreichii* [8]. The fumarate reductase systems of these organisms (except *S. faecalis*) require the participation of quinones (menaquinone or ubiquinone) and cytochrome b_1 in addition to the respective dehydrogenases.

The role of quinones and cytochrome b_1 in the pathway leading to fumarate reduction by either glycerol-3-phosphate or NADH in *E. coli* is not known. Further-

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; DCIP, 2,6 dichlorophenolindophenol, sodium salt.

more, although it has been observed [4] that NADH-dependent fumarate reduction in the cytochromeless bacterium *S. faecalis* is coupled to the generation of ATP, no such reactions have been reported in *E. coli*.

By use of a cytochrome-deficient (*hem A*$^-$) mutant of *E. coli*, we report here that the glycerol-3-phosphate or NADH-dependent reduction of fumarate in *E. coli* involves menaquinone but does not require the participation of cytochromes. Furthermore the reduction of fumarate by NADH is coupled to membrane energization as measured by the quenching of atebrin fluorescence.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade purity. Dicumarol, ATP (disodium salt), D-lactate (lithium salt) and NADH (disodium salt) were purchased from Calbiochem. Glycerol-3-phosphate dehydrogenase and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) were obtained from Sigma Chemical Co. Quinacrine (atebrin) hydrochloride (Nutritional Biochemical Corp.), *o*-phenanthroline (Fisher Scientific Co.), 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) (G. Frederick Smith Chemical Co.), 4,5-dihydroxy-*m*-benzenedisulfonic acid, disodium salt (tiron) (J. T. Baker Chemical Co.), and 2,6-dichlorophenolindophenol, sodium salt (DCIP) (Eastman Organic Chemicals) were obtained as indicated.

Organism and growth

E. coli SASX76 (previously SHSP18), a *hem A*$^-$ mutant of *E. coli* K12, was a generous gift of Dr A. Sasarman (University of Montreal, Canada). Cells were grown anaerobically at 22 °C, unless indicated otherwise, in 4-l flasks (filled to the top) in a minimal salts-glycerol medium containing L-methionine, L-tryptophan, 5-aminolevulinic acid when present, all at a concentration of 50 mg/l [9]. Vitamin-free casamino acids (2 g/l) and fumarate (2.3 g/l) were also present. The culture was harvested in the late exponential phase of growth and washed once with 50 mM Tris·HCl buffer, pH 7.5, containing 5 mM $MgCl_2$. The cells were stored at −20 °C. For testing anaerobic growth on different carbohydrates with or without 5-aminolevulinic acid, fumarate and nitrate, cells were grown in screw cap tubes, completely filled to the top with minimal medium containing the various growth supplements indicated above. No attempt was made to remove dissolved oxygen prior to inoculation.

Preparation of membrane particles

Membrane particles were prepared from washed cells as previously described [9, 10].

Enzyme assays

D-lactate dehydrogenase, DL-glycerol-3-phosphate dehydrogenase, and NADH dehydrogenase activities of membrane particles were measured at 37 °C by following the reduction of DCIP at 600 nm by the corresponding substrate with a Coleman 124 recording spectrophotometer. The assay mixture in a total volume of 1.0 ml, contained 0.1M phosphate buffer, pH 7.0, 0.1 mM DCIP, 0.2–0.4 mg membrane particle protein and either NADH (0.15 mM), DL-glycerol-3-phosphate (20 mM) or D-lactate

(10 mM). The reaction was started by the addition of the particles. Succinate dehydrogenase was determined as before [11].

NADH-fumarate oxidoreductase activity was determined spectrophotometrically under either aerobic or anaerobic conditions at 22 °C by following the decrease in absorbance of NADH at 340 nm. The reaction mixture in a total volume of 1.5 ml contained, 50 mM Tris · HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 0.2 mM NADH, 0.2 to 2.0 mM fumarate, and 0.5 to 2.0 mg membrane particle protein. NADH-fumarate oxidoreductase activity was corrected for NADH oxidase activity when the assay was performed under aerobic conditions. For testing the effect of various inhibitors on this system the water insoluble inhibitors were added to both the control and the assay mixtures as methanolic solutions.

DL-glycerol-3-phosphate-fumarate oxidoreductase activity was measured by following the formation of dihydroxyacetone phosphate under anaerobic conditions by the method of Miki and Lin [12].

All assays were carried out in cuvettes of 1 cm light path.

Ultraviolet irradiation

Membrane particles (12.4 mg protein/ml) were irradiated with a General Electric Mercury Black Light (H 100-FL4) lamp as described by Bragg [13]. The maximum emission from this lamp is light at a wavelength of 360 nm.

Estimation of menaquinone and ubiquinone

The menaquinone and ubiquinone content of membrane particles prepared from cells grown anaerobically in the presence and absence of 5-aminolevulinic acid was determined by the method of Bragg and Polglase [14].

Menaquinone reduction

Menaquinone reduction was measured at 22 °C by the method of Newton et al. [15] using a Turner model 420 spectrofluorometer connected to a Varian strip chart redorder (Model G-14A-1). Menaquinol fluorescence was excited by light at 340 nm and measured at 440 nm. The reaction mixture contained in a total volume of 2.0 ml 10 mM 2-(*N*-2-hydroxyethylpiperazin-N^1-yl) ethane sulfonic acid (HEPES)/KOH buffer, pH 7.5, 300 mM KCl, 5 mM $MgCl_2$ and 3–8 mg membrane particle protein. D-lactate (10 mM), DL-glycerol 3-phosphate (10 mM), NADH (0.2 mM) and L-dihydroorotic acid (5 mM) were used as electron donors and fumarate (0.125–5 mM) was used as an electron acceptor. Cuvettes of 1 cm light path were used.

Atebrin fluorescence

Atebrin (Quinacrine) fluorescence was measured at 22 °C with a Turner model 420 spectrofluorometer connected to a Varian strip chart recorder. The reaction mixture in a final volume of 3.0 ml contained 300 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES /KOH buffer, pH 7.5, 5 μM atebrin and 1–3 mg membrane particle protein. D-Lactate (4 mM), DL-glycerol-3-phosphate (4 mM), ATP (1 mM) and NADH (0,5–0.8 mM) were used as energy-sources and fumarate (2 mM) was added as a terminal electron acceptor. Atebrin fluorescence was excited by light at 430 nm and emmission was measured at 505 nm [16]. Cuvettes of 1 cm light path were used.

Miscellaneous procedures

Protein was determined by the Folin method [17]. Dithionite reduced minus hydrogen peroxide oxidized difference spectra were obtained with a Perkin-Elmer 356 spectrophotometer.

RESULTS

Anaerobic growth of E. coli SASX76 on different carbohydrates in the presence of fumarate or nitrate as terminal electron acceptor

The effect of 5-aminolevulinic acid on the anaerobic growth of *E. coli* SASX76 on different carbon sources in the presence of fumarate or nitrate as the terminal electron acceptor is summarized in Table I. Since cytochromes were not formed without 5-aminolevulinic acid growth on glycerol and glycerol-3-phosphate with nitrate as terminal electron acceptor, and on D-lactate with either fumarate or nitrate as electron acceptors, required the presence of cytochrome. However, the fumarate reductase system could function without cytochrome when glycerol or glycerol-3-phosphate were used as carbon sources.

Cytochrome difference spectra

The cytochrome difference spectra of *E. coli* SASX76 grown under different conditions are shown in Fig. 1. When cells were grown either aerobically or anaerobically on glucose in the absence of 5-aminolevulinic acid no cytochromes could be detected in reduced minus oxidized difference spectra of either whole cells or membrane particles [9]. Similar results were obtained when this organism was grown anaerobically on glycerol in the presence of fumarate but in the absence of 5-aminolevulinic acid (Fig. 1, trace B). A normal cytochrome pattern was exhibited by cells grown with 5-aminolevulinic acid either aerobically or anaerobically on glucose alone or on glycerol plus fumarate (Fig. 1, traces D and E). Membrane-bound cytochromes b_1 (559 nm), a_1 (590 nm) and d (630 nm) were formed. The cytoplasmic fraction showed small amounts of *c*-type (552 nm) and *b*-type cytochromes (Fig. 1, trace C).

TABLE I

ANAEROBIC GROWTH OF *E. COLI* SASX76 ON VARIOUS SUBSTRATES IN THE PRESENCE AND ABSENCE OF 5-AMINOLEVULINIC ACID (ALA) WITH FUMARATE AND NITRATE AS THE TERMINAL ELECTRON ACCEPTORS

+, Growth; −, no growth.

Substrate	Growth with			
	Fumarate		Nitrate	
	−Ala	+Ala	−Ala	+Ala
Glycerol	+	+	−	+
DL-Glycerol 3-phosphate	+	+	−	+
D-Lactate	−	+	−	+
Pyruvate	+	+	+	+

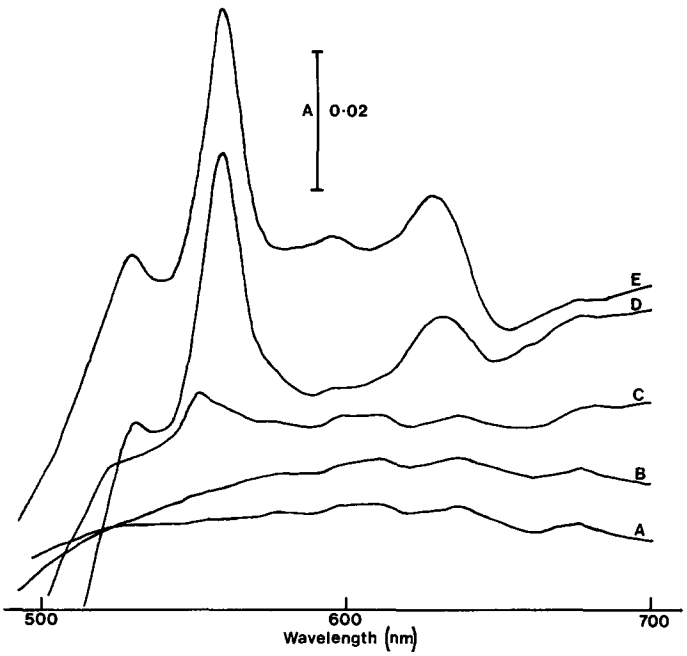

Fig. 1. Dithionite reduced minus oxidized difference spectra of cell fractions from *E. coli* SASX76 grown with or without 5-aminolevulinic acid on glucose or on glycerol in the presence of fumarate. A, base-line; B, membrane particles from cells grown anaerobically on glycerol-fumarate without 5-amino-levulinic acid; C, soluble fraction from cells grown anaerobically on glycerol-fumarate with 5-aminolevulinic acid; D, membrane particles from cells grown anaerobically on glycerol-fumarate with 5-aminolevulinic acid; E, membrane particles from cells grown aerobically on glucose with 5-aminolevulinic acid. Protein concentration: B, D, 10 mg/ml; E, 6 mg/ml.

Content of menaquinone and ubiquinone

It has been reported that menaquinone is involved in fumarate reductase systems of various bacteria when the cells are growing under anaerobic conditions [4–8]. The amount of menaquinone and ubiquinone present in the membrane particles of *E. coli* SASX76 grown anaerobically on glycerol and fumarate in the absence of 5-aminolevulinic acid was 0.385 and 0.073 μmoles per g dry weight of cells, respectively.

NADH-fumarate oxidoreductase

Fumarate-dependent oxidation of NADH by membrane particles from cells grown without 5-aminolevulinic acid was measured aerobically and a typical recorder trace is shown in Fig. 2. This method was used for most of the experiments reported in this paper. In the presence of oxygen NADH was oxidized by the membrane particles prepared from cytochrome-deficient cells, and its oxidation was not stimulated by the addition of 2 mM KNO_3 but was increased four-fold when 0.2 mM fumarate was added to the reaction mixture (Fig. 2). The rate of NADH oxidation was not increased to an appreciable extent when the fumarate concentration was increased from 0.2 mM to 12 mM. It is concluded from these results that the NADH-nitrate oxidoreductase system is not present in cytochrome-deficient cells and that the NADH-fumarate oxidoreductase system does not require the participation of cytochromes.

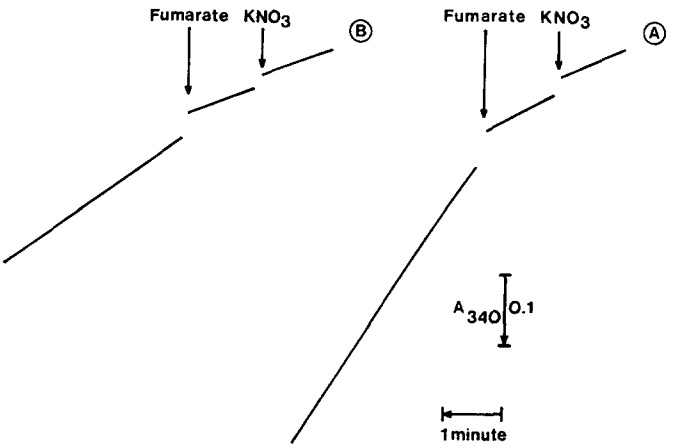

Fig. 2. Assay of NADH oxidase and NADH-fumarate oxidoreductase activity in membrane particles from cytochrome-deficient cells of *E. coli* SASX76. The assay was carried out as described in Materials and Methods. At the arrows 1 mM KNO_3 (final concentration) or 0.2 mM fumarate was added to the cuvette. Membrane particle protein, 0.6 mg/ml. A, normal assay at 22 °C. B, assay mixture containing membrane particles was preincubated at 37 °C for 5 min, cooled to 22 °C, and then NADH added to initiate the reaction.

TABLE II

SPECIFIC ACTIVITY OF NADH- AND GLYCEROL 3-PHOSPHATE ELECTRON ACCEPTOR OXIDOREDUCTASE OF MEMBRANE PARTICLES OF *E. COLI* SASX76

Cells were grown anaerobically without 5-aminolevulinic acid with glycerol as carbon source and fumarate as the terminal electron acceptor.

Electron donor	Electron acceptor	Spec. act. (nmoles/min/mg protein)
NADH	N_2	0
NADH	Fumarate	36.5
NADH	O_2	9.3
NADH	KNO_3	0.7
DL-glycerol 3-phosphate	N_2	1.0
DL-glycerol 3-phosphate	Fumarate	12.5

Oxidation of NADH in the absence of fumarate was abolished when the assay was carried out under nitrogen indicating that oxygen was the terminal electron acceptor for this reaction (Table II). Anaerobiosis had no effect on the rate of fumarate-dependent oxidation of NADH. The traces in Fig. 2 also show the extreme sensitivity of the NADH-fumarate oxidoreductase to heating. The assays were usually carried out at 22 °C since there was considerable loss of activity after only 5 min at 37 °C.

DL-*glycerol-3-phosphate-fumarate oxidoreductase*

In the absence of cytochrome glycerol-3-phosphate-fumarate oxidoreductase activity was present in membrane particles of *E. coli* SASX76 (Table II). The presence

of this enzyme enabled the cells to grow anaerobically on glycerol in the presence of fumarate as electron acceptor.

Effect of inhibitors on NADH-fumarate oxidoreductase and cytochrome-independent NADH oxidase activities

The effect of various inhibitors and metal chelators on fumarate-independent (NADH oxidase) and fumarate-dependent oxidation of NADH (NADH-fumarate oxidoreductase) by membrane particles of cells grown without 5-aminolevulinic acid is shown in Table III. Piericidin A, dicumarol and HOQNO were most effective and inhibited both NADH oxidase and NADH-fumarate oxidoreductase activities with the latter activity being the most sensitive. Among the ferrous iron chelators, bathophenanthroline was more effective in inhibiting these enzyme activities than either *o*-

TABLE III

EFFECT OF INHIBITORS AND CHELATORS ON CYTOCHROME-INDEPENDENT NADH OXIDASE AND NADH-FUMARATE OXIDOREDUCTASE ACTIVITIES OF MEMBRANE PARTICLES

E. coli SASX76 was grown anaerobically without 5-amino-levulinic acid with glycerol as carbon source and fumarate as terminal electron acceptor. The specific activities of NADH oxidase and NADH-fumarate oxidoreductase in the absence of inhibitors were 6.8 and 27 nmoles/min/mg protein. Membrane particles: 3.1 mg protein (2.7 mg for experiment with HOQNO).

Inhibitor	Concentration	% of control activity	
		NADH oxidase	NADH-fumarate oxidoreductase
	(μM)		
Piericidin A	1.2	57	60
	2.4	57	35
	9.6	28	15
	24	14	7
Dicumarol	6	100	83
	15	100	66
	30	60	44
HOQNO	4.0	61	15
	19.5	61	13
Bathophenanthroline	125	100	65
	250	52	19
	530	28	5
	(mM)		
o-Phenanthroline	2	95	70
	4	95	48
	6	68	27
Tiron	2	54	54
	4	54	40
	6	47	33
Salicylaldoxime	2	78	45
	4	35	9
KCN	2	78	100
	10	65	95
Anaerobiosis	—	0	100

phenanthroline or salicylaldoxime. The ferric iron chelator tiron was also inhibitory. As with the other inhibitors NADH oxidase activity was less sensitive than the NADH-fumarate oxidoreductase. KCN, even at a concentration of 10 mM, had only a small inhibitory effect on these enzyme activities.

Involvement of menaquinone

The membrane particles prepared from cells grown without 5-aminolevulinic acid were irradiated with near-ultraviolet light. As shown in Fig. 3, NADH-fumarate oxidoreductase and cytochrome-independent NADH oxidase were very sensitive to light. NAD^+-independent glycerol-3-phosphate dehydrogenase activity was also rapidly inactivated, whereas D-lactate, succinate and NADH dehydrogenase activities were more slowly affected by irradiation. Since quinones are known to be the most light-sensitive components of the respiratory chain of *E. coli* [13, 18] the effect of adding menaquinone and ubiquinone to the irradiated system was examined. Menaquinone-0 or 8 (50–160 μM) or ubiquinone-0, 2,6 or 8 (15–40 μM) added as ethanolic solutions failed to restore the activity of either NADH-fumarate oxidoreductase or glycerol-3-phosphate dehydrogenase.

The involvement of menaquinone in the NADH- and glycerol-3-phosphate-fumarate oxidoreductase pathways was shown by measuring the reduction and oxidation of menaquinone by the fluorimetric technique of Newton et al. [15]. Under the conditions of the experiment there was negligible interference in the assay by ubiqui-

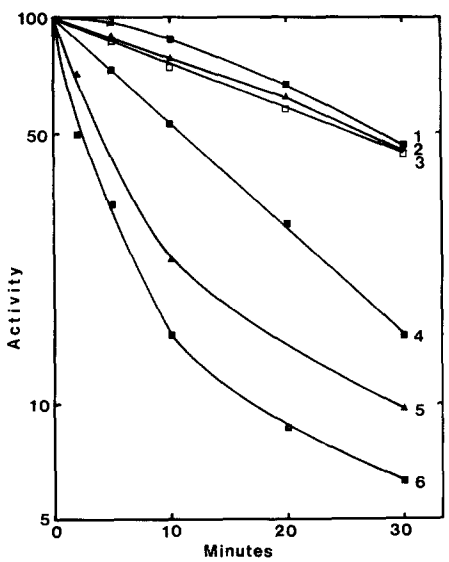

Fig. 3. Effect of near ultraviolet irradiation on NADH-fumarate oxidoreductase and related enzyme activities of membrane particles from cytochrome-deficient cells of *E. coli* SASX76. The irradiation was carried out as described in Materials and Methods. Activity is expressed as % initial spec. act. (nmoles/min/mg protein). Initial specific activities: curve 1, succinate dehydrogenase (169); curve 2, D-lactate dehydrogenase (190); curve 3, NADH dehydrogenase (269); curve 4, DL-glycerol 3-phosphate dehydrogenase (50); curve 5, cytochrome-independent NADH oxidase (6.8); curve 6, NADH-fumarate oxidoreductase (15.5).

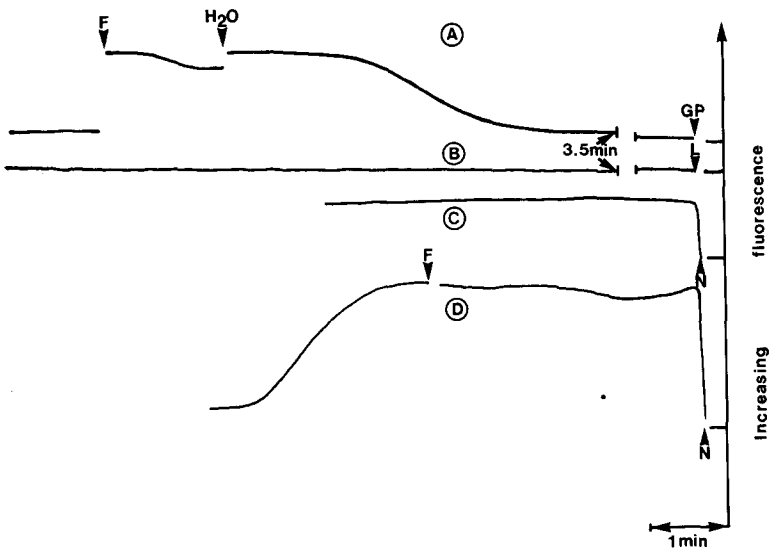

Fig. 4. Reduction of menaquinone by NADH and by glycerol 3-phosphate and reoxidation by fumarate in membrane particles from cytochrome-deficient cells of *E. coli* SASX76. The assay was carried out as described in Materials and Methods. Fluorescence is expressed in arbitrary units. In curve D the light emission was measured with a scale expansion 3-fold greater than in curves A–C. Membrane particle protein: curves A–C, 7.6 mg; curve D, 3.3 mg. At the arrows the following additions were made: GP, 10 mM glycerol 3-phosphate; H_2O, 20 μl water; F (curve A), 5 mM fumarate in 20 μl H_2O; F (curve D), 0.125 mM fumarate in 20 μl H_2O; L, 10 mM D-lactate; N, 0.2 mM NADH. Assay temperatue, 22 °C.

none, NADH, or flavoprotein [28]. Glycerol-3-phosphate, NADH or dihydroorotate (result not shown), but not of D-lactate, caused an increase in fluorescence due to reduction of menaquinone (Fig. 4). Addition of fumarate then caused reoxidation of the menaquinol. The addition of an equivalent volume of water instead of fumarate to correct for the effect of dissolved oxygen produced a transient, partial reoxidation of the menaquinol. When low amounts of NADH were used, menaquinone was reduced to the same steady state level as before but it was subsequently reoxidized when all the NADH had been consumed presumably by way of the cytochrome-independent NADH oxidase pathway.

Atebrin fluorescence

The quenching of fluorescence of acridine dyes has been shown to alter with changes in the energized state of phosphorylating membrane preparations from various sources [19] including *E. coli* [20, 21]. We have therefore used quenching of atebrin fluorescence by membrane particles prepared from cytochrome-less cells of *E. coli* SASX76 to see if the membrane could be energized by fumarate-dependent oxidation of NADH. As shown in Fig. 5A, atebrin fluorescence was partially quenched to a steady state level by oxidation of NADH presumably through the cytochrome-independent NADH oxidase activity. Quenching was still further increased upon the addition of fumarate. Fumarate alone was ineffective, but when fumarate was added first followed by NADH, the extent of quenching of atebrin fluorescence was found to be

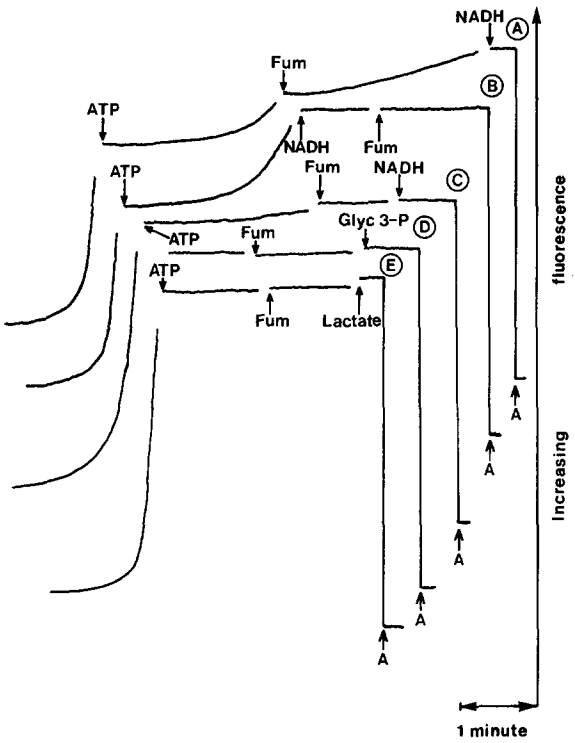

Fig. 5. Atebrin fluorescence in membrane particles from cytochrome-deficient cells of *E. coli* SASX76. The assay was carried out as described in Materials and Methods. Fluorescence is expressed in arbitrary units. Membrane particle protein, 0.96 mg/ml. In experiment C 4.6 μM piericidin A was present. At the arrows the following additions were made: A, 4 μM atebrin (final concentration); 1 mM ATP; 0.75 mM NADH; Fum, 2 mM fumarate; Lactate, 10 mM D-lactate; Glyc 3-P, 10 mM glycerol 3-phosphate. Assay temperature, 22 °C.

equivalent to the sum of quenching produced by NADH oxidase and NADH-fumarate oxidoreductase (Fig. 5B). When ATP was added to this system, the fluorescence of the dye was almost completely quenched. This effect was mediated by the Ca^{2+}, Mg^{2+}-activated ATPase since it was inhibited completely by the ATPase inhibitor DCCD. That the quenching of atebrin fluorescence by fumarate-dependent oxidation of NADH was due to the presence of the NADH-fumarate oxidoreductase was supported by the inhibitory effect of piericidin A on the quenching (Fig. 5C). Piericidin A inhibited the oxygen and fumarate-dependent oxidation of NADH by membrane particles (Table III). It is interesting to note that there was no quenching of atebrin fluorescence upon addition of NADH to membrane particles prepared from cells of another cytochrome-deficient mutant of *E. coli* which had been grown aerobically on glucose [21]. This may have been due to the absence of the cytochrome-independent NADH oxidase activity which is found in our preparation. This system probably shares the NADH dehydrogenase site I region of the respiratory chain with the NADH-fumarate oxidoreductase system.

Miki and Lin [22] in preliminary studies have shown that fumarate-dependent

oxidation of glycerol-3-phosphate was coupled to the formation of ATP with a very low efficiency of 0.1 mole ATP formed per mole glycerol-3-phosphate oxidized. Although glycerol 3-phosphate-fumarate oxidoreductase activity was present in the membrane particles of cytochrome-deficient *E. coli* SASX76 no quenching of atebrin fluorescence was observed with this system (Fig. 5D). Similarly D-lactate in the presence of fumarate did not quench atebrin fluorescence (Fig. 5E).

DISCUSSION

In the absence of cytochrome *E. coli* was able to grow on glycerol and glycerol 3-phosphate, but not on lactate, in the presence of fumarate as a terminal electron acceptor. In contrast, growth on these substrates under anaerobic conditions when nitrate was used as the terminal electron acceptor required the formation of cytochromes. Thus, in the presence of fumarate, *E. coli* can generate enough energy from these substrates in the absence of cytochromes to support growth. Since these growth conditions induce the formation of NADH- and glycerol 3-phosphate-fumarate oxidoreductase activities, it was of interest to determine the components of these pathways and to see if an energy-coupling site was present.

Lin and his coworkers have made extensive studies of the glycerol 3-phosphate dehydrogenase-fumarate oxidoreductase system in *E. coli* [3, 12]. They have shown that under anaerobic conditions an NAD^+-independent L-glycerol 3-phosphate dehydrogenase and fumarate reductase, a different enzyme from succinate dehydrogenase, are induced. These enzymes appear to be coupled together in the membrane particle fraction. Although the dehydrogenase may be a flavoprotein there is no evidence on the nature of the electron carriers between glycerol 3-phosphate and fumarate in this system or in the NADH-fumarate oxidoreductase system.

The NADH-, malate- and glycerol 3-phosphate-fumarate oxidoreductases of *Bacillus megaterium* [5], the glycerol 3-phosphate-fumarate oxidoreductase of *Propionibacterium freudenreichii* [8], the NADH,- glycerol 3-phosphate-, and D-lactate-fumarate oxidoreductases of *P. arabinosum* [7], and the hydrogen-fumarate oxidoreductase of *Desulfovibrio gigas* [6] all contain functional menaquinone and b-type cytochrome. The NADH-fumarate oxidoreductase of *Proteus rettgeri* contains menaquinone but it is not clear if a *b*-type cytochrome is involved [23]. Thus, a *b*-type cytochrome and menaquinone might be expected to act as electron carriers in the NADH- and glycerol 3-phosphate-fumarate oxidoreductase systems of *E. coli*. The results presented in this paper show that cytochrome is not an obligatory component of these systems in *E. coli* since they are active under conditions where cytochrome is absent.

However, the specific activity of the NADH-fumarate oxidoreductase in *E. coli* SASX76 grown in the absence of 5-amino-levulinic acid was 36.5 nmoles/min/mg protein. This value is close to that (44 nmoles/min/mg protein) found in the cytochrome-lacking organism *Streptococcus faecalis* but somewhat less than the values of 320 and 120 nmoles/min/mg protein determined for *P. rettgeri* [23] and *P. arabinosum* [7]. Since the NADH-fumarate oxidoreductase of the last two organisms may involve cytochrome it is possible that cytochrome may enhance but not be absolutely required for the activity of the oxidoreductase. This view is supported by the finding that *E. coli* SASX76 grown anaerobically on glycerol with fumarate in the absence of 5-aminolevulinic acid required 30 h to reach the stationary phase of growth compared to

12 h for cells grown with 5-aminolevulinic acid. The growth yield in the former case was 82 % of that of the supplemented cells.

The higher content of menaquinone than of ubiquinone present in our preparation suggested that it might play a role in the NADH- and glycerol 3-phosphate oxidoreductase systems.

Irradiation with ultraviolet light, which is known to inactivate menaquinones [18] but may also have effects on other components of the respiratory chain [13], inactivated the oxidoreductase systems. However, we have not successfully restored activity by addition of menaquinone or ubiquinone possibly because the inactivated quinone remained in situ. Attempts to remove the inactivated quinone by extraction with solvent [24] have been defeated by the extreme lability of the system. Newton et al. [15], using a mutant of *E. coli* unable to form menaquinone, showed that the dihydroorotate-fumarate oxidoreductase system required this quinone. They suggested that the NADH-fumarate oxidoreductase system also might involve menaquinone since the oxidation of NADH by membranes from anaerobically grown cells of the mutant was markedly stimulated by menaquinone. Using a fluorimetric method we have shown that menaquinone is reduced by NADH and glycerol 3-phosphate, but not by D-lactate, and that the reduced menaquinone can be reoxidized by fumarate. Therefore, menaquinone is probably a component of the glycerol 3-phosphate- and NADH-fumarate oxidoreductase systems.

The sensitivity of the NADH-fumarate oxidoreductase to iron chelators suggests that nonheme iron is a component of this system as has been suggested also for *S. faecalis* [4]. It is also of interest that HOQNO inhibits the NADH-fumarate oxidoreductase since this clearly demonstrates that cytochrome is not the site of action of this compound. This agrees with the previous suggestions [5, 6] that the site of action of HOQNO is close to the site of quinone in the respiratory chain.

The cytochrome-independent NADH oxidase activity which is present in our preparation is inhibited by the same compounds as inhibit the NADH-fumarate oxidoreductase but it is less sensitive than the latter system. This suggests that these systems could have some components in common. Of interest in this regard is the finding that both systems can energize the membrane as measured by the quenching of atebrin fluorescence. Although their effects are additive this may be due to a higher steady state level of energization being generated by the same site when an additional electron acceptor is provided. This would occur if the rate-limiting step in the oxidation of NADH occurred between the site of energization and the electron acceptors. Addition of ATP can raise the level of energization of the membrane still further.

The presence of an energy-coupling site in the NADH-fumarate oxidoreductase system of *E. coli* is a further point of similarity of this system with that of *S. faecalis* [4]. Miki and Lin [22] have obtained some evidence for an energy-coupling site in the glycerol 3-phosphate oxidoreductase system of *E. coli* but we were not able to demonstrate energization of the membrane by this system. We have no explanation for this observation. Growth yield studies with *P. freudenreichii* [8] and *P. rettgeri* [27] have also indicated the presence of an energy coupling site in the glycerol 3-phosphate- and NADH-fumarate oxidoreductase systems, respectively.

In our previous studies [9] we have shown that transport of phenylalanine was stimulated by glucose in cells of *E. coli* SASX75 grown anaerobically on glucose in the absence of 5-aminolevulinic acid. Under similar conditions, D-lactate with or without

fumarate was ineffective in energizing transport. We concluded that phenylalanine transport was energized by ATP in these cells. The present results do not contradict this conclusion. Fumarate reductase is not induced by growth of *E. coli* SASX76 under the above conditions. Moreover, the lack of quenching of atebrin fluorescence by D-lactate under conditions when fumarate reductase is formed suggests that membrane energization by this substrate requires the formation of cytochrome. This hypothesis is also supported by the inability of *E. coli* SASX76 to grow anaerobically on lactate with fumarate in the absence of 5-aminolevulinic acid (Table I).

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