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ENERGY-LINKED NICOTINAMIDE ADENINE DINUCLEOTIDE TRANSHYDROGENASE IN MEMBRANE PARTICLES FROM *ESCHERCHIA COLI*

ROBERT J. FISHER AND D. RAO SANADI

Boston Biomedical Research Institute, Department of Cell Physiology, 20 Staniford Street, Boston, Mass. 02114 (U.S.A.)

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SUMMARY

The energy-linked nicotinamide adenine dinucleotide transhydrogenase from *Escherichia coli* can be driven by energy obtained from ATP or from respiration. *E. coli* grown anaerobically has the ATP-driven transhydrogenase but no respiration-driven transhydrogenase activity. The ATP-driven activity is inhibited by 50 μM *N,N'*-dicyclohexylcarbodiimide (DCCD) in membrane particles prepared from both aerobically and anaerobically grown cells, but the respiration-driven transhydrogenase is not inhibited.

Low levels of DCCD enhance the respiration-driven transhydrogenase activity in membrane particles prepared from aerobically grown cells. The oxidation of NADH is one-fifth as low in particles prepared from anaerobically grown cells and is less sensitive to KCN than in particles from aerobically grown cells.

The results suggest that anaerobically grown *E. coli* can produce and utilize the nonphosphorylated high-energy intermediate from ATP. However, this membrane appears to be depleted in its ability to transduce oxidative energy to form the high-energy intermediate.

INTRODUCTION

In mitochondria, the energy-linked nicotinamide nucleotide transhydrogenase reaction can utilize energy either from ATP or from the respiratory chain by the oxidation of succinate or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. When the energy is supplied by oxidation, the reaction is insensitive to oligomycin and is not affected by treating the submitochondrial particles with 2 M urea to drastically reduce the ATPase activity[1].

In bacteria this reaction has not been studied much and only the ATP-driven nicotinamide nucleotide transhydrogenase reaction has been demonstrated in

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimadazole; oxidative transhydrogenase, the transhydrogenase driven by energy derived from aerobic oxidation; ATP-driven transhydrogenase, the transhydrogenase driven even under anaerobic conditions by ATP energy.

Escherichia coli[2,3,*]. The first part of this paper deals with oxidatively driven transhydrogenase in membrane particles prepared from *E. coli*.

There are many reports in the literature that anaerobically grown cells still have energy-linked functions which are senstive to uncouplers[4,5]. Anaerobically grown *E. coli* show little uncoupler-sensitive esterification of P_i, and when these cells are exposed to oxygen for 2 h, the incorporation increases 10-fold to a level comparable to that found in aerobically grown cells[6]. This means that anaerobically grown *E. coli* does not carry out oxidative phosphorylation. However, it an electron acceptor such as nitrate is used, oxidative phosphorylation becomes apparent[7,8]. These cells are similar to aerobic cells with respect to oxidative phosphorylation except that nitrate replaces O_2 as the terminal electron acceptor. In cells that are grown anaerobically there is no evidence for oxidative phosphorylation in the absence of nitrate. However, the induction of β-galactosidase in anaerobically grown *E. coli* is still sensitive to uncouplers[4]. These results are best explained by the existence of an uncoupler-sensitive, non-phosphorylated, high-energy intermediate in anaerobically grown cells which is in equilibrium with ATP[9]. The second part of this paper presents evidence for a non-phosphorylated, high-energy intermediate in aerobically and anaerobically grown *E. coli*.

METHODS

Growth of organism

E. coli K-12 was grown aerobically on glucose as described previously[10]. Anaerobic cultures were grown in 10-l batches in glucose medium supplemented with 0.5 % yeast extract (Difco). The 10-l cultures were flushed with high-purity N_2 gas (less than 0.001 % O_2) to obtain anaerobiosis. When the cells reached a density of 1.5 g wet wt./l, they were quickly chilled and harvested in less than 15 min in a Sharples-Super centrifuge. This procedure minimized the possibility of induction of the oxidative phosphorylation system by exposure to O_2. It has been shown that under growth conditions, it takes 90 min to induce aerobic levels of oxidative phosphorylation[6].

The preparation and storage of membrane particles is described elsewhere[10].

Assays

Oxidative transhydrogenase will refer to the reaction driven by oxidative energy. It was measured in a medium containing 50 μmoles Tris sulfate at pH 7.8, 700 μmoles sucrose, 0.3 μmole NAD, 5 μmoles dithiothreitol, 2 mg bovine serum albumin, 100 μg crystalline yeast alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1.), 90 μmoles ethanol and membrane particles (0.5–1.0 mg protein) in 2.9 ml. The medium was incubated for 2 min at 38° and the reaction was initiated by the addition of 1.5 μmoles NADP. The increase in absorbance at 340 nm was followed in a Gilford multiple sample absorbance recorder.

Anaerobic or ATP transhydrogenase refers to the ATP driven activity. The aerobic *plus* anaerobic transhydrogenase was assayed in the same manner except that

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the medium contained, in addition to the above components, 6 μmoles ATP and 10 μmoles $MgCl_2 \cdot 6H_2O$.

The non-energy-linked transhydrogenase was measured in a medium containing 100 μmoles phosphate at pH 6.5, 1.5 μmoles 3-acetylpyridine-NAD, 1 μM KCN and 250 μg membrane protein in a total volume of 2.95 ml (ref. 11). The assay medium was incubated for 2 min and the reaction was initiated by the addition of 1.5 μmoles of NADPH. The increase in absorbance was followed at 375 nm in a Gilford multiple sample absorbance recorder.

ATPase (ATP phosphohydrolase, EC 3.6.1.4) activity was measured in a medium containing 25 μmoles Tris chloride at pH 7.5, 5 μmoles phosphoenolpyruvate, 5 units pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40), 0.5 mg membrane protein in a total volume of 0.96 ml. The mixture was incubated at 30° and the reaction was initiated by the addition of 2 μmoles $MgCl_2 \cdot 7H_2O$ and 0.1 μmole of ATP in 0.04 ml. The mixture was incubated for 5 min at 30° and the reaction was terminated by addition of 0.1 ml of 50% trichloroacetic acid. The precipitate was removed by centrifugation and inorganic phosphate was determined in the supernatant liquid[12]. All enzymes and reagents were purchased from Sigma Chemical Company, Inc., and were of the highest purity available.

Protein was determined by the biuret procedure[15].

RESULTS

The oxidation of NADH in mitochondrial particles is inhibited by low concentrations of rotenone, which allows convenient measurement of the aerobic transhydrogenase by using an NADH generating system and utilizing the oxidation of succinate to provide energy for the reduction of NADP by NADH. The NADH oxidase from *E. coli*, however, is not inhibited by rotenone so that the energy for the aerobic transhydrogenase is derived at least in part from the oxidation of NADH. The use of an NADH generating system ensures that the steady-state level of NADH is maintained during its oxidation by the electron transport chain and concomitant transhydrogenation of NADP. We have previously shown the dependence of the aerobic transhydrogenase on O_2 concentration[10]. When the oxygen in the medium is consumed, the reaction stops. If ATP is also present, the reaction continues independently of the respiratory chain. Fig. 1 also demonstrates the oxidative and ATP-driven transhydrogenases. If the oxidative reaction is allowed to proceed until all oxygen is consumed, no further increase in absorbance is observed unless ATP is added. The addition of 60 μM 4,5,6,7-tetrachloro-2-trifluoromethylbenzimadazole (TTFB) uncouples the reaction and there is no further increase in absorbance. The sudden increase in absorbance on the addition of TTFB is due to the absorbance of the uncoupler.

In order to demonstrate that the increase in absorbance was due to NADP reduction, the experiment shown in Fig. 2 was performed. The complete reaction mixture *minus* alcohol dehydrogenase and NADP was introduced into the cuvette at time 0. Alcohol dehydrogenase was added at 1 min, which produces an immediate increase of absorbance due to NADH formation. Addition of NADP at 2.5 min initiates the oxidative transhydrogenase reaction with the concomitant absorbance increase. At 10 min, oxidized glutathione *plus* excess glutathione reductase (NADPH: glutathione oxidoreductase, EC 1.6.4.2) was added, which immediately decreased the

absorbance approximately to the level which was attained before the addition of NADP. This decrease was due to the reoxidation of NADPH. Subsequently, pyruvate *plus* lactate dehydrogenase (L-lactate: oxidoreductase, EC 1.1.1.27) was added at 11 min which decreased the absorbance further to the original base due to the oxida-

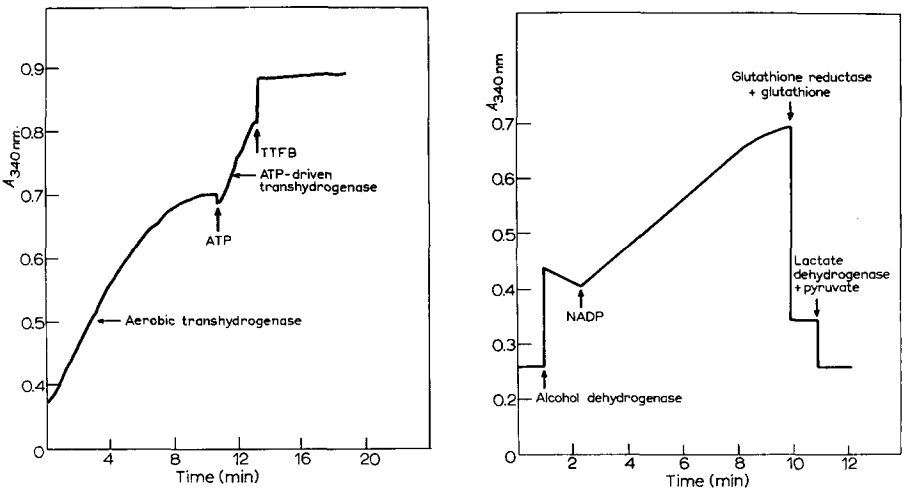

Fig. 1. Aerobic and anaerobic transhydrogenase in a membrane preparation from *E. coli*. 1.3 mg protein of *E. coli* membrane particles (glucose-grown) in 2.9 ml of medium containing 50 μmoles Tris sulfate, pH 7.8, 700 μmoles sucrose, 0.3 μmole NAD; 2 mg bovine serum albumin, 50 μg crystalline yeast alcohol dehydrogenase, 90 μmoles ethanol, 10 μmoles of $MgCl_2 \cdot 7H_2O$; the reaction was initiated by the addition of 1.5 μmoles of NADP. After the aerobic reaction had stopped, 6 μmoles of ATP were added in order to demonstrate the anaerobic transhydrogenase. The uncoupler 4,5,6,7-tetrachloro-2-trifluoromethylbenzimadazole (TTFB) was added at a concentration of 60 μM at the point indicated.

Fig. 2. Demonstration of NADPH formation during aerobic transhydrogenase reaction. 1.2 mg of *E. coli* membrane as described in the legend to Fig. 1 *minus* alcohol dehydrogenase, $MgCl_2 \cdot 7H_2O$, ATP and NADP. At 1 min, 50 μg alcohol dehydrogenase was added, and after the maximum NADH was formed 1.5 μmoles of NADP was added. Near the completion of the reaction 2 μg of yeast glutathione reductase (NADPH:glutathione oxidoreductase, EC 1.6.4.2) and 0.10 μmole of oxidized glutathione were added at the point indicated and the decrease of absorbance was followed until its maximum. At this point, 100 μg of lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) and 0.1 μmole of pyruvate were added at the point indicated.

TABLE I

REQUIREMENTS FOR THE ENERGY-LINKED TRANSHYDROGENASE BY MEMBRANES ISOLATED FROM AEROBICALLY GROWN *E. coli*

Details of the experiment as described in the legend to Fig. 1.

Medium	*NADPH (nmoles/min per mg protein)*
Complete	35.0
—alcohol dehydrogenase	0.9
—ATP	16.8
—NADP	0.0
$-Mg^{2+}$	31.8
—dithiothreitol	31.8
Complete + 60 μM TTFB	1.5

tion of NADH. Under the assay conditions described here, the increase in absorbance at 340 nm after the addition of NADP is entirely due to the formation of NADPH.

Table I demonstrates the requirements for the energy-driven transhydrogenase system. There was no reaction in the absence of alcohol dehydrogenase or NADP. When ATP was omitted from the system, the reaction proceeded at the aerobic rate. TTFB at a concentration of 60 μM inhibited both the aerobic and anaerobic reactions demonstrating energy requirement for both reactions. Thus, the *E. coli* transhydrogenase responds to the components and energy source just as the mitochondrial system does.

Facultatively anaerobic bacteria offer a unique tool in studying oxidative phosphorylation in that they may be manipulated physiologically to deplete the organism of components necessary for oxidative phosphorylation[6,13]. It has been shown that *E. coli* grown anaerobically lack cytochromes and oxidative phosphorylation activity; these may be induced by oxygen[6]. Table II compares the activities of the membrane preparations from aerobically and anaerobically grown *E. coli*. Major differences in the NADH oxidation and respiration-driven transhydrogenase reaction are evident. The rate of NADH oxidation is only 20% of that in the aerobic membranes. This activity is inhibited 40% by 1 mM KCN, while it is inhibited 80% in the aerobic membranes. The aerobic energy-linked transhydrogenase activity is virtually absent in the anaerobically grown *E. coli* membrane, but the energy-independent reaction is quite active. The ATP-driven transhydrogenase is still functional in these membranes

TABLE II

ACTIVITIES OF MEMBRANE PARTICLES FROM *E. coli*

1. ATPase activity: Complete reaction mixture contained 5 units pyruvate kinase, 50 μmoles Tris chloride, 5 μmoles phosphoenol pyruvate, 2 μmoles $MgSO_4$, 1 μmole ATP and 250 μg membrane protein in 1.0 ml at pH 7.5. The mixture was incubated at 30° for 5 min and the reaction terminated by the addition of 0.1 ml of 50% trichloroacetic acid. The P_i was measured by the method of FISKE AND SUBBAROW[12]. Activity is expressed as nmoles P_i/min per mg protein. 2. The NADH oxidase reaction was measured polarographically at 37°. The medium contained 50 μmoles Tris sulfate, pH 7.8, 700 μmoles sucrose, 2 mg bovine serum albumin and 0.5 mg membrane protein in a total volume of 1.4 ml. The reaction was initiated by the addition of 6 μmoles NADH. The activity is expressed as natoms O_2/min per mg protein. 3. Energy-linked transhydrogenase activities were measured in a medium containing 50 μmoles Tris sulfate, pH 7.8, 2.95 ml H_2O, 700 μmoles sucrose, 0.3 μmole NAD, 2 mg bovine serum albumin, 50 μg crystalline yeast alcohol dehydrogenase, 90 μmoles ethanol, and 0.5 mg membrane protein at 38°; total volume, 2.95 ml. The reaction was initiated by the addition of 1.5 μmoles NADP. The aerobic + anaerobic transhydrogenase was measured by inclusion of 6 μmoles ATP and 10 μmoles $MgCl_2$. Activity is expressed in nmoles NADPH/min per mg protein. 4. The energy-independent transhydrogenase was measured in a medium consisting of 500 μmoles phosphate, pH 6.5; 3 μmoles KCN, 1.5 μmoles NADPH, 1.5 μmoles acetylpyridine NAD and 0.5 mg membrane protein in 3 ml. The reaction was initiated by addition of the acetylpyridine NAD. Activity is expressed in nmoles acetylpyridine NADH/min per mg protein.

Growth conditions	*ATPase*	*NADH Oxidation*		*Transhydrogenase*		
		−CN⁻	*+1 mM CN⁻*	*Aerobic*	*Aerobic + anaerobic*	*Energy-independent*
Glucose, aerobic	211	389	75	24	51	178
Glucose + yeast extract, anaerobic	256	78	46	1.5	18.5	80

at a rate comparable to that found in the aerobic membrane. This activity is obtained by subtracting the aerobic acitivity from the ATP-driven *plus* aerobic reaction rate. The high ATPase activity of the anaerobic membrane is consistent with the presence of high anaerobic (ATP-driven) transhydrogenase.

Table III shows the requirements of the energy-linked transhydrogenase in anaerobic membranes. The source of energy is clearly ATP and the level of activity in the absence of ATP cannot be reduced further by the presence of uncouplers.

In mitochondria *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibits energy-linked reactions driven by ATP and appears to act much like oligomycin[14]. The inhibition of the ATP-dependent transhydrogenase by DCCD in both aerobic and anaerobic membranes indicates that the route for utilization of the ATP energy is probably the same

TABLE III

REQUIREMENTS FOR ENERGY-LINKED TRANSHYDROGENASE ACTIVITY IN MEMBRANES ISOLATED FROM ANAEROBICALLY GROWN *E. coli*

Details of the experiment are described in the legend to Fig. 1.

Medium	*NADPH (nmoles/min per mg protein)*
Complete	17
—alcohol dehydrogenase	0.3
—ATP	1.5
—NADP	0.0
—Mg^{2+}	14.6
—dithiothreitol	14.6
Complete + 60 μM TTFB	1.5

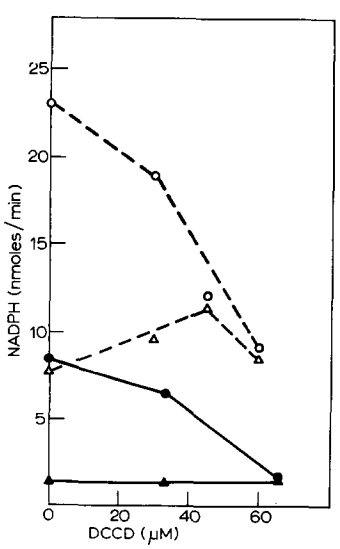

Fig. 3. Inhibition of the anaerobic transhydrogenase by DCCD. The reaction medium was the same as described for Fig. 1, except that the reaction was initiated by the addition of NADP. The aerobic membranes were prepared from glucose-grown *E. coli* and the anaerobic membranes were prepared from glucose-grown *E. coli* and the anaerobic membranes were prepared from glucose and casamino acids-grown *E. coli*. ▲—▲, anaerobic membranes, *minus* ATP; ●—●, anaerobic membranes, *plus* ATP; △---△, aerobic membranes, *minus* ATP; ○---○, aerobic membranes, *plus* ATP. 0.5 mg membrane protein was used.

as in mitochondria (Fig. 3). As expected from the analogy with mitochondrial systems, low levels of DCCD also stimulate the transhydrogenase activity slightly (Table IV). Similarly, the ATPase in both the aerobic and anaerobic membranes is inhibited by 60 % by 50 μM DCCD, a level which completely inhibits the ATP-driven transhydrogenase (Fig. 3).

In our routine assay, the energy for the aerobic transhydrogenase is derived from NADH oxidation. It is possible to increase the activity of the transhydrogenase system by adding succinate and DCCD. The aerobic membrane activity with NADH is 24, with NADH + succinate the activity is 34 and in the presence of DCCD the activity is 48 (Table IV). In the anaerobic membrane, the activity increases from a rate comparable to that of the non-energy-linked reaction of 2.4 to 6.7 in the presence of succinate and DCCD. Thus, under the optimal conditions for highest activity, the anaerobic membranes have roughly one-seventh the activity of the aerobic membrane.

TABLE IV

STIMULATION OF THE ENERGY-LINKED TRANSHYDROGENASE IN *E. coli* MEMBRANE PARTICLES BY DCCD

Details of the experiment are described in the legend to Fig. 1.

	NADPH (nmoles/min per mg protein)	
	Anaerobic membrane	*Aerobic membrane*
NADH	2.4	24
NADH + 60 μM TTFB	2.4	1.7
NADH + 16 μM DCCD	3.6	36.2
NADH + 20 mM succinate	5.4	34
NADH + 20 mM succinate + 16 μM DCCD	6.7	48.2
NADH + 20 mM succinate + 50 μg 2-heptyl-4-hydroxyquinoline-*N*-oxide	3.6	not determined

DISCUSSION

The energy-dependent transhydrogenase reaction in membrane particles from *E. coli* is similar to that found in mammalian mitochondria. Previous work had documented the ATP-driven reaction, and the data presented here establish the utilization of energy from respiration. Both reactions are uncoupled by TTFB, enhanced by low levels of DCCD and when ATP is the energy source, inhibited by high levels of DCCD. The reaction described here provides the first convenient kinetic assay for measuring the utilization of energy from respiration in membrane particles. The results are consistent with the view of SLATER[9] and KOVAC AND KUZELA[4] that a non-phosphorylated high-energy intermediate (X ~C) drives the transhydrogenase and other energy-requiring reactions in *E. coli*. These workers noted that enzyme adaption in anaerobic yeast, glutamate uptake by *Staphylococcus aureus*, and induction of β-galactosidase in anaerobic *E. coli* were sensitive to uncouplers. When the energy for the transhydrogenase is derived from the respiratory chain, the reaction is inhibited by TTFB but not by DCCD. On the other hand, if the energized state is produced from ATP, the reaction is sensitive to both types of inhibitors. KLEIN *et al.*[16] have reported that proline transport is probably energized by a non-phosphorylated high-energy intermediate. It has not been possible to drive this reaction with ATP energy

in isolated membrane vesicles, possibly due to the inability of ATP to penetrate the membrane vesicle or to the depletion of ATP-forming enzymes. By analogy with the work of KOVAC AND KUZELA[4] on the induction of β-galactosidase in anaerobically grown *E. coli*, ATP should be capable of energizing proline uptake in anaerobically grown cells.

It is known that when *E. coli* is growing anaerobically, it does not make ATP oxidatively, and oxidative phosphorylation can be induced by exposure to oxygen in the presence of added carbon source[6]. However, the anaerobically grown *E. coli* does have those features of the oxidative phosphorylation system which are necessary for anaerobic growth, namely a method of producing an energized state from ATP for the purposes of transport of ions and metabolites, and producing reducing equivalents (NADPH) for biosynthesis. The low cyanide-sensitive respiration in anaerobically grown *E. coli* appears to be related to the low concentrations of cytochromes and not to low concentrations of NADH and succinate dehydrogenases[6]. In addition, the cyanide-sensitive respiration could be due to the induction of the oxidase during the harvesting of the organism. When the anaerobic cells are exposed to oxygen energy transduction is induced due to the formation of cytochromes and/or possibly energy transfer factors between the respiratory chain and the common non-phosphorylated high-energy intermediate. The presence of high ATP-driven transhydrogenase and low respiratory activity make the membrane of the anaerobically grown *E. coli* highly suitable for studies on the *in vitro* reconstitution of oxidative phosphorylation using purified respiratory enzymes, cytochromes and coupling and energy transfer factors[17].

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