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H^+/e^- Stoichiometry for NADH Dehydrogenase I and Dimethyl Sulfoxide Reductase in Anaerobically Grown *Escherichia coli* Cells

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Anaerobically grown *Escherichia coli* cells were shown to acidify the reaction medium in response to oxygen or dimethyl sulfoxide (DMSO) pulses, with the H^+/e^- stoichiometry being close to 2.5 and 1.5, respectively. In the presence of the NADH dehydrogenase I (NDH-I) inhibitor 8-methyl-*N*-vanillyl-6-nonenamide (capsaicin) or in mutants lacking NDH-I, this ratio decreased to 1 for O_2 and to 0 for DMSO. These data suggest that (i) the H^+/e^- stoichiometry for *E. coli* NDH-I is at least 1.5 and (ii) the DMSO reductase does not generate a proton motive force.

NADH oxidation by the respiratory chain of *Escherichia coli* is catalyzed via at least two distinct NADH:quinone oxidoreductases, i.e., NADH dehydrogenase I (NDH-I) and NADH dehydrogenase II (NDH-II) (5, 15). NDH-II comprises a single polypeptide containing flavin adenine dinucleotide, and this enzyme cannot form $\Delta\bar{\mu}_{H^+}$ (31). NDH-I consists of 14 individual subunits containing flavin mononucleotide and five to eight FeS clusters (12, 21, 24); its NADH-oxidizing activity is coupled to $\Delta\bar{\mu}_{H^+}$ formation. The operon encoding NDH-I was cloned and sequenced (24), and the protein was purified and characterized (12). The sequence and the set of prosthetic groups of this enzyme closely resemble the mitochondrial complex I. In fact, *E. coli* NDH-I is considered to be a simplified version of this type of enzyme or a so-called minimal form of complex I.

The H^+/e^- stoichiometry of the mitochondrial complex I is usually assumed to be 2 (see, e.g., references 17 and 27), although values of 1.5 (23) and 2.5 (13) have also been discussed. The H^+/e^- stoichiometry for NDH-I from *E. coli* still awaits elucidation, mainly because of the wide diversity of NDH-I-independent respiratory chain-linked dehydrogenases present in this bacterium. These enzymes reduce quinone without $\Delta\bar{\mu}_{H^+}$ generation, which can result in a decrease in the measured H^+/e^- ratio for NDH-I. To overcome this difficulty, one can use *E. coli* cells growing anaerobically. In such cells, synthesis of all the noncoupled dehydrogenases is strongly repressed by the *Arc* and *Fnr* regulatory systems (14), while the expression of NDH-I is decreased only slightly (4). Anaerobic growth of *E. coli* can also be accompanied by the appearance of a number of additional membrane-bound dehydrogenases (anaerobic glycerophosphate dehydrogenase, formate dehydrogenase, and hydrogenase) and a wide variety of reductases such as fumarate reductase, trimethylamine *N*-oxide (TMAO) reductase, dimethyl sulfoxide (DMSO) reductase, nitrate reductase, and nitrite reductase (8).

The DMSO reduction in anaerobic cultures of *E. coli* is catalyzed by an enzyme (DMSO reductase) which is composed of three different subunits containing four FeS clusters and a molybdopterin cofactor (1, 19). DMSO reduction by anaerobically grown *E. coli* is accompanied by H^+ extrusion from the cells with H^+/e^- stoichiometry close to 1.5 (3). It was unknown whether any respiratory chain enzymes that might precede DMSO reductase (first of all, NDH-I) are involved in $\Delta\bar{\mu}_{H^+}$ formation. If NDH-I is involved in this process, DMSO could be used as an electron acceptor to measure the H^+/e^- ratio for NDH-I, because the quinol-DMSO reaction in solution is not accompanied by any pH change, and the K_m value of DMSO reductase for DMSO is low, i.e., ~170 μM (2). In the work described in this paper, we studied the H^+/e^- stoichiometry of NDH-I and DMSO reductase. Ratios of 1.5 and 0, respectively, were found.

MATERIALS AND METHODS

Strains. The strains used in this study are listed in Table 1. *E. coli* GR70N, GO104, MWC215, and MWC217 were kindly provided by R. B. Gennis; AN387 and ANNO91 were provided by H. Weiss; and ZK1141 and ZK1362 were provided by R. Colter.

Bacterial growth. In anaerobic cultures, *E. coli* cells were grown in M9 growth medium containing 0.4% (vol/vol) glycerol, 60 mM DMSO, 1 μM ammonium molybdate and 0.1% tryptone. Strains GR70N, GO104, MWC215, MWC217, AN387, and ANNO91 were grown in this medium supplemented with thiamine (1 μg/ml). When indicated, the growth medium was supplied with 0.4% glucose instead of glycerol. Cells were grown for 16 h at 37°C. In aerobic cultures, *E. coli* cells were grown for 5 to 6 h to the midlogarithmic phase in M9 medium without DMSO.

H^+/e^- ratio measurements. Cells were harvested from the growth medium by centrifugation for 10 min at 7,000 × *g*, washed twice with medium containing 150 mM KCl and 0.5 mM dithiothreitol, and resuspended in reaction medium A (100 mM KCl, 25 mM KSCN, 10 mM glycerol, 0.5 mM morpholineethanesulfonic acid [MES]-KOH [pH 6.5]) or B (medium A supplemented with 7 mM NaF); the final protein concentration was 5 mg/ml. This suspension was immediately injected into a 2-ml vessel equipped with a pH electrode. After being bubbled with argon, the vessel was sealed and the pH was adjusted to 6.5 with KOH. During O_2 or DMSO pulses, 5 nmol of O_2 or 25 nmol of DMSO was injected into the vessel. pH shifts were measured with a combination pH electrode. Calibration was carried out with 50-nmol additions of HCl from a stock solution saturated with argon. All measurements were performed within the pH range 6.2 to 6.5. When using benzyl viologen as the electron donor, 1 mM benzyl viologen and 0.4 mM sodium dithionite were injected into the sealed vessel containing the argon-saturated cell suspension. To estimate H^+/e^- ratios, a linear extrapolation method was used (27).

Enzyme assays. NADH- and deamino-NADH-oxidase activities were evaluated by means of a Hitachi-557 spectrophotometer at 30°C. For NADH-oxidase activity measurements, the *E. coli* cells grown under different growth conditions were washed twice with medium containing 100 mM NaCl and 10 mM K_2HPO_4 (pH 6.5), resuspended in reaction medium C (50 mM K_2HPO_4, 0.5 mM EDTA [pH 6.5]), and disrupted by a single pass through a French press at 16,000 lb/in^2. Intact cells and cell debris were precipitated by centrifuging for 15 min at

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TABLE 1. Bacterial strains used in this study

Strain	Phenotype	Genotype	Reference or source
GR70N	Wild type	F$^-$ *thi rpsL gal*	16
GO104	Cyo$^-$	GR70N Δ(*cyoABCDE*)*456*::Kan^r	16
MWC215	NDH-II$^-$	GR70N *ndh*::Cm^r	5
MWC217	Cyo$^-$, NDH-II$^-$	GO104 *ndh*::Cm^r	5
AN387	Wild type	F$^-$ *thi* Str^r	H. Weiss
ANNO91	NDH-I$^-$	AN387 *nuoI*	H. Weiss
ZK1141	Wild type	ZK126 *rpoS819 sga rpsL*	32
ZK1362	NDH-I$^-$	ZK1141 *nuoA*::mini-Tn*10*Cm^r	32

14,000 × *g*. The supernatant was diluted with reaction medium C and immediately used for the NADH-oxidase activity measurements.

Benzyl viologen:DMSO oxidoreductase activity in *E. coli* cells was measured by a method described previously (19) or by alkalinization of the reaction medium following the benzyl viologen:DMSO reductase reaction ($H^+/e^- = 1$) under conditions described in the previous section.

Protein determination. The protein concentration was estimated by a biuret method with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

As demonstrated by Bilous and Weiner (3), *E. coli* cells grown anaerobically on minimal medium in the presence of glycerol and DMSO acidify the reaction medium on DMSO addition with an H^+/e^- stoichiometry of about 1.5. We found the same protonophore-sensitive H^+/e^- ratio during DMSO pulses in *E. coli* GR70N cells ($H^+/e^- = 1.38 \pm 0.06$ [six experiments]; Fig. 1). Addition of the NDH-I inhibitor 8-methyl-*N*-vanillyl-6-nonenamide (capsaicin) (30) resulted in almost complete inhibition of acidification (the H^+/e^- ratio was less than 0.2). A possible explanation of this fact is that all the H^+ pumping is coupled to NDH-I whereas DMSO reductase is a noncoupled enzyme.

To test this hypothesis, we measured the H^+/e^- ratio in anaerobically grown *E. coli* cells with oxygen as the electron acceptor. This ratio was initially rather low and decreased even more during anaerobic incubation of the cells. This effect could be explained by the fact that during anaerobic incubation of anaerobically grown *E. coli* cells, formate, which is an inhibitor of terminal oxidases, is produced. Introduction of the glycolysis inhibitor fluoride (when switching to reaction medium B) resulted in an increase in the H^+/e^- ratio which proved to be incubation time independent. The only disadvantage of fluoride is that the decay of the proton gradient after the O_2 or DMSO pulse became faster as a result of increased proton permeability of the membrane. Figure 2 shows that in the presence of 7 mM NaF, the *E. coli* GO104 cells acidify the reaction medium with a stoichiometry of about 2.5 upon O_2 addition. This mutant strain contains only cytochrome *bd* as the terminal oxidase. It is known that the *E. coli* cytochrome *bd* forms $\Delta\bar{\mu}_{H^+}$ solely as a result of production of $2H_2O$ from O_2 when four electrons and four H^+ ions come from two opposite sides of the membrane ($H^+/e^- = 1$) (18). The above data, indicating that the H^+/e^- ratio is 2.5 when O_2 is added, mean that the stoichiometry for NDH-I is around 1.5, just as with DMSO as the electron acceptor. The addition of 300 μM capsaicin decreased the H^+/e^- ratio to 1 in the oxygen pulse experiments. This value was expected, assuming that $\Delta\bar{\mu}_{H^+}$ is generated solely by cytochrome *bd* under these conditions. The very fact that DMSO pulses (in contrast to oxygen pulses) failed to induce acidification in the presence of capsaicin is in line with the inability of DMSO reductase to function as a $\Delta\bar{\mu}_{H^+}$ generator.

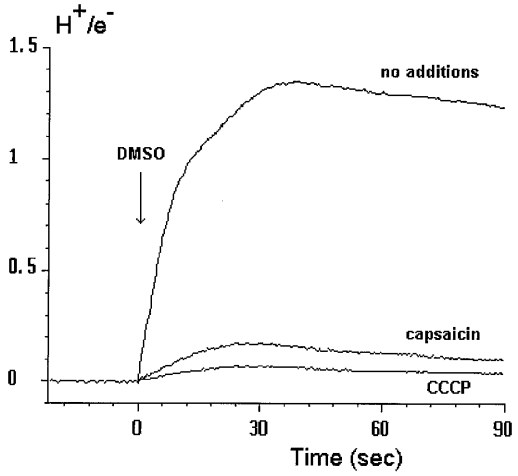

FIG. 1. H^+/e^- ratios measured in medium A for anaerobically grown *E. coli* GR70N (wild type) with DMSO as the electron acceptor. Additions were 300 μM capsaicin (the cells were preincubated with the inhibitor for 10 min) and 30 μM carbonyl cyanide M-chlorophenylhydrazone (CCCP).

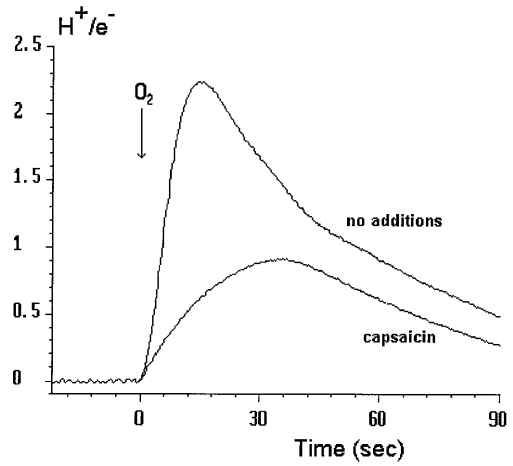

FIG. 2. H^+/e^- ratios measured in medium B for anaerobically grown *E. coli* GO104 (Δ*cyo*) with oxygen as the electron acceptor. Where indicated, the cells were preincubated with 300 μM capsaicin for 10 min.

In the next series of experiments, *E. coli* mutant strains lacking NDH-I were studied. Figure 3 shows that during O_2 and DMSO pulses, the H^+/e^- stoichiometry in the wild-type *E. coli* AN387 was 2.5 and 1.5, respectively. Although this strain is capable of synthesizing both the *bd*- and *bo*-type terminal oxidases, it demonstrated the same H^+/e^- ratio as did *E. coli* GO104 (Δ*cyo*). This is obviously due to a strong repression of cytochrome *o* biosynthesis under anaerobic growth conditions (6, 9). In contrast to the parental strain, *E. coli* ANNO91 (*nuoI*) lacking NDH-I acidified the reaction medium with the addition of oxygen but not DMSO (Fig. 4), exhibiting an H^+/e^- stoichiometry of 1 during the O_2 pulse, i.e., the ratio characteristic of cytochrome *bd*. The DMSO injection did not lead to any significant acidification of the reaction medium. It should be emphasized that the activity of the DMSO reductase in ANNO91 remained high. The latter statement is confirmed by the rapid alkalinization of the reaction medium when reduced benzyl viologen and DMSO were added to *E. coli* ANNO91 cells. These observations are also consistent with the

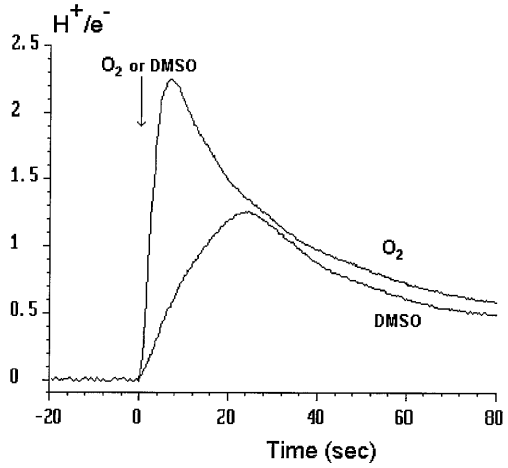

FIG. 3. H^+/e^- ratios measured in medium B for anaerobically grown wild-type *E. coli* AN387.

H^+/e^- ratio for DMSO reductase being 0. A mild acidification ($H^+/e^- < 0.1$) at DMSO pulses could possibly be explained by contribution of formate reductase or hydrogenase, since both these enzymes are competent in $\Delta\bar{\mu}_{H^+}$ generation (10, 11).

Another *E. coli* mutant lacking NDH-I (ZK1362) also showed H^+/e^- stoichiometries of 1 and 0 during oxygen and DMSO pulses, respectively (data not shown).

Thus, the data obtained indicate that the H^+/e^- stoichiometry for DMSO reductase and NDH-I of the *E. coli* respiratory chain is 0 and 1.5, respectively. It should be pointed out that although the ΔG of the menaquinol:DMSO oxidoreduction (about 230 mV) is high enough to achieve a translocation of at least 1 proton per electron, the DMSO reductase is not capable of generating $\Delta\bar{\mu}_{H^+}$ when DMSO is used as the electron acceptor. However, since DMSO reductase can use as electron acceptors various *N*-oxides (for example, trimethylamine *N*-oxide) as well as *S*-oxides (25), this enzyme should form the respective amines. If one accepts the topographic model of DMSO reductase of Weiner and coworkers (3, 19) and assumes the *N*-oxide reduction site to be located on the cytoplasmic side of the membrane, reduction of *N*-oxides by

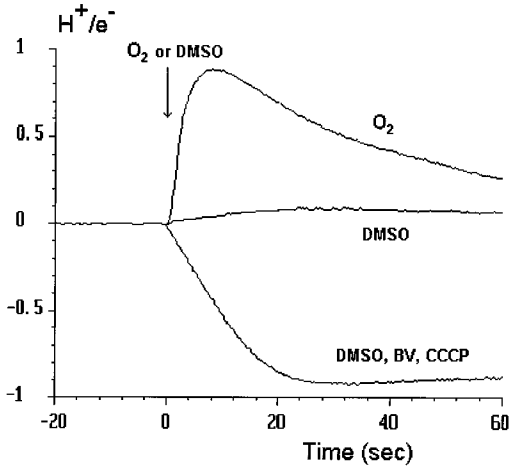

FIG. 4. H^+/e^- ratios measured in medium B for anaerobically grown *E. coli* ANNO91 (*nuoI*). Additions were reduced benzyl viologen (BV) and 30 μM CCCP.

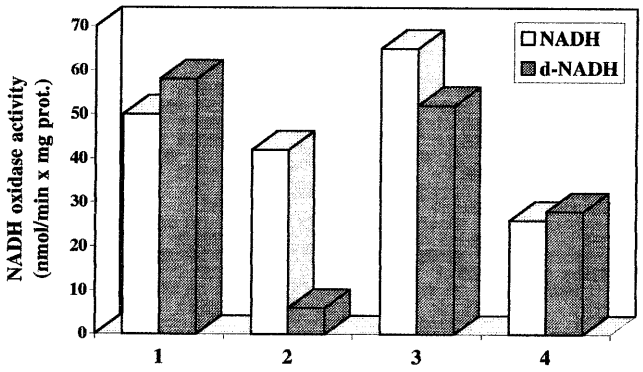

FIG. 5. NADH and deamino-NADH (d-NADH) oxidase cell extract activity of *E. coli* strains. 1, MWC215 (Δ*ndh*) aerobically grown with glycerol; 2, ANNO91 (*nuoI*) aerobically grown with glycerol; 3, AN387 (wild type) aerobically grown with glycerol; 4, AN387 anaerobically grown with glycerol plus DMSO.

DMSO reductase will lead to alkalinization of the cytoplasm with an H^+/e^- ratio of 0.5. This process can lead to the formation of ΔpH of the right direction (alkaline inside) and hence can contribute to the $\Delta\bar{\mu}_{H^+}$ generation.

Usually, the H^+/e^- ratio for mitochondrial complex I is assumed to be 2, i.e., larger than 1.5, the value we observed for the *E. coli* analog of the enzyme. Some underestimation of the H^+/e^- ratio for bacterial NDH-I could be due to the participation of some noncoupled dehydrogenases. However, we obtained the same stoichiometry of 1.5 for NDH-I when growing *E. coli* GO104 anaerobically in the presence of glucose or DMSO plus glucose (instead of DMSO plus glycerol), i.e., under conditions favoring maximal repression of the aerobic respiratory chain dehydrogenases as well as the anaerobic glycerophosphate dehydrogenase (data not shown). On NDH-I-depleted strains grown under the same conditions, we observed a total lack of acidification after the oxygen pulse, which is a consequence of a greatly decreased respiration rate. A possible explanation of these effects is that the *E. coli* cells grown in the presence of glucose do not possess any noncoupled dehydrogenase activity; as with *E. coli* cells grown in the presence of glycerol, the contribution of these noncoupled dehydrogenases to the quinol pool reduction is very moderate against the background of a high NDH-I activity.

The lack of significant medium acidification after a DMSO pulse with NDH-I mutants suggests that the hydrogenase and formate hydrogenase contributions to quinone pool reduction are also minimal under these conditions, possibly because of low concentrations of the respective substrates.

We also assume that NDH-II does not significantly contribute to the anaerobic respiratory chain function, because it is known that the expression of this enzyme is strongly repressed during growth under oxygen-depleted conditions (22). This statement can be substantiated by the fact that like GO104, *E. coli* MWC217 (Δ*ndh* Δ*cyo*), lacking NDH-II, demonstrated the same H^+/e^- ratio (1.5) for the NDH-I span of the respiratory chain when either DMSO or O_2 was used as the electron acceptor (data not shown). At the same time, the expression of NDH-I under anaerobic growth conditions was decreased only slightly (4). This assertion is confirmed by data on the NADH- and deamino-NADH-oxidase activities (Fig. 5).

As for a partial DMSO reduction via some cytoplasmic oxidoreductases which could also lead to a decreased H^+/e^- ratio, it is unlikely, because the use of oxygen as the electron acceptor gave the same ratio for NDH-I as that obtained with

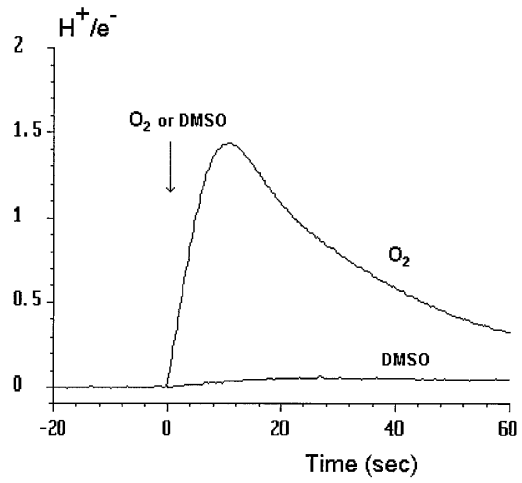

FIG. 6. H^+/e^- ratios measured in medium B for aerobically grown *E. coli* GO104 (Δ*cyo*).

DMSO. Thus, although we can assert only that the H^+/e^- ratio for NDH-I is not less than 1.5, we have no reason to assume a larger ratio.

The finding that NADH oxidation by DMSO is coupled with transport of less than 2 H^+ can be somehow related to the fact that DMSO reductase preferentially uses menaquinol as the electron donor (28, 29). Thus, one can assume that in anaerobically grown *E. coli* cells with DMSO as the sole electron acceptor, the NDH-I is also forced to use menaquinone. Menaquinol, as a compound with a more negative redox potential than ubiquinol, seems to be a more convenient reductant for anaerobic electron acceptors such as fumarate. For the same reason, ubiquinone should be a better oxidant for, e.g., succinate, an aerobic substrate. Moreover, ubiquinol is more convenient to use under aerobiosis, since the parasitic reaction of one-electron O_2 reduction by menasemiquinone should be much faster than by the more positive ubisemiquinone (20).

In any case, the energy produced by the anaerobic NADH: menaquinone reductase reaction may be insufficient to support translocation of more than 1.5 H^+ per electron (4). It is not excluded that in aerobically grown *E. coli* cells the NADH: ubiquinone reductase reaction can have a higher H^+/e^- ratio. However, it is practically impossible to test this suggestion by using the whole cells, since aerobiosis is known to induce succinate dehydrogenase and numerous other substrate dehydrogenases which reduce ubiquinone in an NDH-I-independent fashion. This decreases the measured H^+/e^- ratio. In fact, the H^+/e^- stoichiometry, when measured in the aerobically grown *E. coli* cells, proved to have a lower value than in the anaerobically grown cells (Fig. 6). It is also impossible to use DMSO as electron acceptor in aerobically grown *E. coli* cells, since the DMSO reductase synthesis is repressed under such growth conditions (2, 7).

On the other hand, an H^+/e^- ratio of 1.5 may be, according to Vinogradov (23), a property of any H^+-translocating NADH:quinone oxidoreductase, since it is a direct consequence of the electron transfer mechanism in the initial span of the respiratory chain.

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