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Engineering *Escherichia coli* for Fermentative Dihydrogen Production: Potential Role of NADH-Ferredoxin Oxidoreductase from the Hydrogenosome of Anaerobic Protozoa

Phi Minh Do · Alexander Angerhofer · Ivan Hrdy · Lucie Bardonova · Lonnie O. Ingram · K. T. Shanmugam

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Abstract *Trichomonas vaginalis* generates reduced ferredoxin within a unique subcellular organelle, hydrogenosome that is used as a reductant for H_2 production. Pyruvate ferredoxin oxidoreductase and NADH dehydrogenase (NADH-DH) are the two enzymes catalyzing the production of reduced ferredoxin. The genes encoding the two subunits of NADH-DH were cloned and expressed in *Escherichia coli*. Kinetic properties of the recombinant heterodimer were similar to that of the native enzyme from the hydrogenosome. The recombinant holoenzyme contained 2.15 non-heme iron and 1.95 acid-labile sulfur atoms per heterodimer. The EPR spectrum of the dithionite-reduced protein revealed a [2Fe–2S] cluster with a rhombic symmetry of g_{xyz}=1.917, 1.951, and 2.009 corresponding to cluster N1a of the respiratory complex I. Based on the Fe content, absorption spectrum, and the EPR spectrum of the purified small subunit, the [2Fe–2S] cluster was located in the small subunit of the holoenzyme. This recombinant NADH-DH oxidized NADH and reduced low redox potential electron carriers, such as viologen dyes as well as *Clostridium* ferredoxin that can couple to hydrogenase for H_2 production from NADH. These results show that this unique hydrogenosome NADH dehydrogenase with a critical role in H_2 evolution in the hydrogenosome can be produced with near-native properties in *E. coli* for metabolic engineering of the bacterium towards developing a dark fermentation process for conversion of biomass-derived sugars to H_2 as an energy source.

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P. M. Do · L. O. Ingram · K. T. Shanmugam (✉)
Department of Microbiology and Cell Science, University of Florida, P.O. Box 110700, Gainesville, FL 32611, USA
e-mail: shan@ufl.edu

A. Angerhofer
Department of Chemistry, University of Florida, Gainesville, FL 32611, USA

I. Hrdy · L. Bardonova
Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic

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Introduction

Nonrenewable nature of fossil fuels combined with an increase in atmospheric CO_2 has prompted a search for alternate sources of energy to support human endeavors. Among the various energy sources that are being explored, H_2 is attractive due to its higher energy density. The product of combustion of dihydrogen is water and does not contribute to environmental pollution and global warming. Dihydrogen is an energy carrier and can be produced by several methods [1]. Biologically, H_2 can be produced by photosynthetic microorganisms from water along with O_2 and also by fermentation of sugars using microbial biocatalysts [2–5].

Photosynthetic microorganisms are known to produce H_2 from water using sunlight as the energy source [6–8]. Gaffron demonstrated H_2 production by green algae over 60 years ago [9], and this process is sensitive to evolved O_2, produced by photosynthesis, that limited the yield and rate of H_2 production from water. This O_2 sensitivity of the algal hydrogenase has been overcome recently by separating O_2 evolving photosynthesis from H_2 production phase by manipulating the S-content of the medium [7]. In the first photosynthetic phase, carbohydrates were synthesized from CO_2 and water using light energy, and the accumulated carbohydrates were fermented to H_2 in the second S-limitation phase that minimizes O_2 evolution. Similar separation of photosynthesis and H_2 evolution was also reported for cyanobacteria [10]. We have previously reported fermentation of sugars by cyanobacteria in a photoheterotrophic mode under N-limitation reaching a H_2 to fructose ratio as high as 15 [11].

Dihydrogen produced by light-dependent microbial biocatalysts may not be cost-competitive with other sources of energy, and strictly from an economic point of view, a dark fermentation process to H_2 could be a viable option [2]. In such a dark fermentation process, sugars derived from photosynthetic processes, such as stored carbohydrates or lignocellulosic biomass, can be fermented by anaerobic and facultative microbial biocatalysts to H_2 and CO_2. Microorganisms are well known for their ability to produce copious amounts of dihydrogen during fermentation of sugars [3]. Although the dark fermentation proceeds at high rate, the net yield of H_2 per glucose is usually less than two (Fig. 1). Either formate or reduced ferredoxin produced by appropriate bacteria is the main electron donor for H_2 production and in most cases the only source of H_2 produced by fermenting bacteria. The NADH produced by the glycolytic pathway is normally not used as a source of H_2 and instead is reoxidized to maintain redox balance by reduction of organic molecules such as pyruvate or acetyl-CoA leading to characteristic end products of fermentation of the organism. There are reports of bacteria that produce H_2 yield of higher than 2.0 but never reaching the expected maximum of 4.0 H_2/glucose [12–14]. For hydrogen to be a viable energy source, the yield needs to reach at least 10 H_2/glucose [15]. To reach this high H_2 to hexose ratio, microbial biocatalysts need to be metabolically engineered to divert all the NADH produced during glycolysis and TCA cycle to H_2. This requires a unique set of enzymes that can couple oxidation of NADH to reduction of electron carriers such as ferredoxin that can transfer the electrons to hydrogenase and ultimately released as H_2 (Fig. 1). In their pioneering work, Greenbaum and his colleagues demonstrated a biochemical pathway using a combination of enzymes that can catalyze the conversion of hexose to H_2 at a yield per hexose that is close to the theoretical maximum [16]. This was further extended to include starch [17].

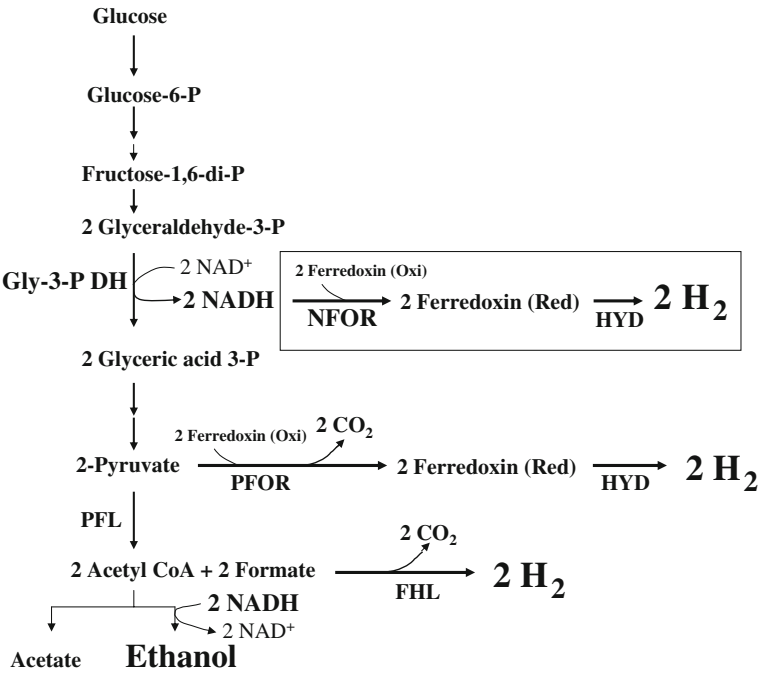

Fig. 1 Fermentative pathways in microorganisms that lead to H_2 production. The reactions within the rectangular box are found in the hydrogenosome of anaerobic protozoa. pyruvate ferredoxin oxidoreductase (*PFOR*) dependent reactions are found in Clostridia and the pyruvate formate-lyase (*PFL*) based reactions are normally seen in facultative bacteria. *FHL*, formate hydrogen-lyase; *HYD*, hydrogenase

The biochemical reaction presented in Fig. 1 (within the box) is an alternate pathway for H_2 production that has been reported in the hydrogenosomes of anaerobic protozoan, such as *Trichomonas vaginalis* [18]. Trichomonads lack mitochondria but contain unique subcellular organelles, the hydrogenosomes [19]. This organelle is critical for anaerobic energy metabolism of the protozoan and likely share evolutionary ancestry with the mitochondrion [19–21]. A NADH-dehydrogenase (NADH-DH) was purified from *T. vaginalis* hydrogenosomes and this enzyme reduced *Trichomonas* ferredoxin in vitro [22]. This ferredoxin is likely the in vivo electron acceptor of NADH-DH [22]. Once reduced, the electrons are transferred from ferredoxin to hydrogenase for H_2 evolution. The *Trichomonas* NADH-DH consists of two subunits (51 and 24 kDa subunits, designated Tvh-47 and Tvh-22 [22]; this nomenclature is maintained here for the proteins) that are similar to the core NADH-oxidizing subunits of the NADH:ubiquinone oxidoreductase of mitochondrial or bacterial complex I (complex I; NDH; EC 1.6.5.3). The ability of the hydrogenosome NADH-DH to reduce electron acceptors at the midpoint redox potential of ferredoxin (−0.36 V; [23]) or to more electronegative electron acceptors such as methyl viologen (E_0' of −0.44 V) has not been reported in any of its homologs that constitute the respiratory complex of aerobic organisms. This raises an interesting possibility that a metabolically engineered *Escherichia coli* that produces the unique hydrogenosome NADH-DH, ferredoxin, and hydrogenase could couple NADH oxidation to H_2 production in *E. coli* also. As a first step towards constructing such a NADH to H_2 pathway, we have cloned and expressed the corresponding genes from *T. vaginalis* in *E. coli* and biochemically characterized this critical component of the pathway, the recombinant NADH-DH. Since

the heterodimer is expected to have cofactors, FMN and Fe/S, it is important that the recombinant protein is endowed with these cofactors for full activity. The results presented in this communication show that the *T. vaginalis* NADH-DH produced by *E. coli* is fully active and catalyzes the reduction of methyl viologen with midpoint redox potential close to that of H_2 and can serve as a starting point for the construction of microbial biocatalysts for fermentative H_2 production at high yield.

Experimental Procedures

Materials

All organic and inorganic chemicals were from Fisher Scientific and were analytical grade. Biochemicals were from Sigma Chemical Co. Reagents, enzymes and supplies for molecular biology experiments were from New England Biolabs unless specified otherwise.

Medium and Culture Condition

E. coli cultures were grown in rich medium (L-broth) as described previously [24]. *E. coli* strains with plasmids were selected and maintained in rich medium with ampicillin (100 mg/L). *E. coli* strain Rosetta (Novagen) carries lambda DE3 with T7 gene *1* encoding RNA polymerase under *lac* promoter at the λ*att* site and a plasmid pRARE (Novagen) encoding a set of less-abundant tRNAs of *E. coli*. *E. coli* strain JM109(DE3) was from Promega.

E. coli Expression Plasmid with *nuoE* and *nuoF* Genes

Plasmids containing *T. vaginalis nuoE* (Tvh-22) and *nuoF* (Tvh-47) genes encoding the two subunits of the NADH-DH were described previously [22, 25]. Using predicted signal peptide cleavage site [22], two sets of primers were synthesized for polymerase chain reaction (PCR) amplification of the two genes and cloning into expression plasmid, pET15b (Novagen; Electronic Supplementary Material).

Plasmid, pPMD40, in which both genes are expressed from independent T7-gene *10* promoters, was constructed to produce approximately equimolar quantities of both subunits in the same cell towards production of an active NADH-DH. Plasmid pPMD40 was constructed after PCR amplification of the entire *nuoF* gene along with the phage T7-promoter (forward primer: CGGCAAGCTTCCACGATGCGTCCGGCGTAG; reverse primer: GCCGAAGCTTTTGGTTATGCCGGTACTGCC) and inserting this DNA into a HindIII site in plasmid pPMD38 (Electronic Supplementary Material). Plasmid pPMD40 contains tandem T7-gene *10* promoters each expressing *nuoE* or *nuoF* gene.

Expression of *nuoE* and *nuoF* Genes in *E. coli*

NADH dehydrogenase was produced in *E. coli* strain JM109(λDE3, pRARE) after inducing the *nuo* genes with arabinose since the protein produced after IPTG induction was inactive. The level of expression from T7 gene *10* promoter found in T7 expression plasmids was lower with arabinose as the inducer compared to IPTG, the traditional *lac* inducer. For these experiments, a freshly transformed *E. coli* JM109(λDE3, pRARE) with plasmid pPMD40 (or plasmid pPMD38 or pPMD39; Electronic Supplementary Material)

was cultured in 1 L LB medium in a shaker at 200 RPM. When the OD420nm of the culture reached about 0.6 (Beckman DU640 spectrophotometer), arabinose (1.5% *w*/*v*) was added to the medium and incubation continued at 28 °C for additional 4 h. Ferrous sulfate was also added at the same time ($FeSO_4$ $7H_2O$, 20 mg/L) to support production and incorporation of Fe/S clusters into the amplified proteins. Cells were harvested by centrifugation (10,000×*g*, 10 min, 4 °C), washed once with ice-cold Buffer A (K-phosphate, 50 mM, pH 7.5; NaCl, 0.1 M) and stored at −20 °C until use.

Purification of NADH-DH

Cells from a 500-mL (or 1 L) culture were suspended in 10 mL (or 20 mL) of Buffer A and broken by passage through a French pressure cell (20,000 psi). The extract was clarified by centrifugation (30,000×*g*; 45 min) and the supernatant was filtered through a 0.22-μm filter. The filtered extract was loaded on a Hi Trap chelating column (5 mL; General Electric) in Buffer A that was prewashed with $NiCl_2$ (0.1 M) in Buffer A. Unadsorbed and loosely bound proteins were washed off the column with five volumes of Buffer A followed by five volumes of Buffer A with imidazole (50 mM). Proteins were eluted with a linear imidazole gradient of 50 to 300 mM imidazole in Buffer A. The NADH-DH eluted at about 80 mM imidazole as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and activity. Fractions with highest purity of the protein, as determined by SDS-PAGE, were pooled and the N-terminal His-tags from both proteins (Tvh-22 and Tvh-47) were removed by thrombin (75 units; General Electric) treatment for 16 h at 4 °C during dialysis of the protein against Buffer A with DTT (0.5 mM). The protein was further purified by gel filtration on a Hi Prep Sephacryl S-200 HR column (2.6/60 cm; General Electric) in Buffer A with DTT (0.5 mM). Fractions with NADH-DH activity were pooled and dialyzed overnight against 4 L of K-phosphate buffer (50 mM, pH 7.5) with DTT (0.5 mM). To one half of the total protein, glycerol was added to a final concentration of 20% and stored at −75 °C. This protein was stable for several months at −75 °C. The other half of the protein was maintained on ice and used for biochemical assays.

Individual subunits of NADH dehydrogenase were also purified essentially as described for the holoenzyme. Since the subunits lack enzyme activity, purification was followed by SDS-PAGE.

Enzyme Activity

NADH-DH activity was determined spectrophotometrically using NADH as the electron donor and various artificial electron acceptors. Standard reaction mixture consisted of K-phosphate buffer (50 mM, pH 7.5), NADH (1 mM), and benzyl viologen (BV) (1 mM). Reaction mixture in a 13×100-mm tube was sealed with a serum stopper, and the gas phase was replaced by evacuation and refill with N_2. Enough sodium dithionite was added to the reaction mixture to titrate out the residual O_2. Reaction was initiated by the addition of enzyme, and BV reduction was monitored continuously at 600 nm at room temperature. Although the small amount of added dithionite will reduce both the Fe–S cluster and flavin in the protein, the presence of excess BV in the reaction mixture is expected to reoxidize these cofactors and not interfere with the assay. Under these conditions, the small amount of added dithionite did not affect the kinetics of BV reduction. With ferricyanide as electron acceptor, assays were under aerobic condition. Concentrations of BV, methyl viologen (MV), and K-ferricyanide were 1.00 mM in the experiments leading to determination of *K*m of NADH. Molar extinction coefficients used for BV and MV are 7,800 and 6,300,

respectively, at 600 nm with 1.00 cm path length. Ferricyanide-dependent NADH-DH activity was determined in the same buffer as the BV assay (410 nm; extinction coefficient of 1,020 M^{-1} cm^{-1}). Ferredoxin-dependent NADH-DH activity was determined in the same buffer with 0.10 mM NADH and 40 μM *Clostridium acetobutylicum* ferredoxin by following the oxidation of reduced NADH at 340 nm. One unit of enzyme activity is defined as 1 μmol substrate reduced min^{-1} mg $protein^{-1}$.

Clostridium Ferredoxin Purification

C. acetobutylicum strain 824 (NRRL B-23491) obtained from USDA-ARS (Peoria, IL, USA) was cultured in Reinforced Clostridial Medium (Oxoid) without the agar. Ferredoxin was isolated from the cells as described previously [26, 27]. Protein concentration was determined from the molar extinction coefficient of 30,600 cm^{-1} at 390 nm.

Non-heme iron and sulfur determination—Non-heme iron was determined as described by Harvey et al. [28] and sulfur was determined using the method described by Cline [29].

EPR Measurements

Cw-EPR spectra of the NADH-DH complex at cryogenic temperatures were determined using a commercial EPR spectrometer (Bruker Elexsys E580) equipped with an Oxford Instruments ESR900 helium-flow cryostat and the standard TE_{102} mode rectangular cavity (Bruker ER4102ST). EPR samples were placed in a Wilmad 3×4 (ID×OD)-mm quartz tubes (CFQ), prefrozen in liquid nitrogen, before insertion into the precooled cryostat.

Analytical Methods

Protein concentration was determined using Coomassie blue (Bradford reagent) or BCA assay [30, 31] with bovine serum albumin as standard. SDS-PAGE utilized 12.5% gel as per Laemmli [32]. The protein standards used in SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA) were aprotinin (6,500 Da), lysozyme (14,400 Da), trypsin inhibitor (21,500 Da), carbonic anhydrase (31,000 Da), ovalbumin (45,000 Da), serum albumin (66,200 Da), phosphorylase b (97,400 Da), β-galactosidase (116,250 Da), and myosin (200,000 Da). Protein standards used to calibrate Hi Prep Sephacryl S-200 HR column used for gel filtration (Sigma Chemical Co., St. Louis, MO, USA) were bovine carbonic anhydrase (29,000 Da), ovalbumin (45,000 Da), bovine serum albumin (66,000 Da), and yeast alcohol dehydrogenase (150,000 Da).

Results

Purification of the Recombinant NADH-DH

T. vaginalis hydrogenosome NADH dehydrogenase is a heterodimer comprising two subunits with anhydrous molecular weights of 20,706 and 45,723, respectively [22, 25]. Optimal expression of the two proteins in *E. coli* required plasmid pRARE (Novagen) expressing *E. coli* less-abundant tRNAs. The two subunits of the protein were expressed separately in *E. coli* host BL21(DE3)(pRARE) and attempts to reconstitute an active enzyme either from crude extracts or from purified subunit components were unsuccessful. Similar results were also obtained when the two genes were expressed from the same

promoter in the cytoplasm. An active protein was only detected when both proteins were expressed from tandem promoters in plasmid pPMD40 in the same cell. Although induction of gene expression with IPTG (as low as 50 μM) supported significant amount of NADH-DH protein synthesis, the activity of the recombinant protein was very low. In order to alleviate this, arabinose was used as an inducer for production of T7-polymerase from the *lac* promoter in λDE3. Arabinose is not a known inducer of *lac* promoter; however, a metabolic product of arabinose appears to induce the *lac* promoter at a lower level [33]. The specific activity of NADH-DH after arabinose induction and purification increased to about 600 units with ferricyanide as electron acceptor (Table 1), an activity that is similar to that of the native enzyme [22].

This protein migrated through the gel filtration column as a heterodimer with a molecular mass of 69,100 and SDS-PAGE revealed the two subunits corresponding to Tvh-47 and Tvh-22. The specific activity of the recombinant enzyme was highest with ferricyanide as electron acceptor (Table 1) as seen with the native enzyme. The specific activity of the enzyme with benzyl viologen as electron acceptor was comparable to that of the values with ferricyanide. The recombinant protein also reduced methyl viologen (about 65% of the ferricyanide value). The activity of the recombinant protein with methyl viologen as electron acceptor was significantly higher than that of the enzyme purified directly from the hydrogenosome. In addition, the enzyme also reduced Clostridial ferredoxin. These results show that the recombinant NADH-DH reduces low-potential electron acceptors such as methyl viologen (E_0' of −0.44 V) readily.

Apparent *K*m for NADH for the recombinant enzyme was dependent on the electron acceptor and varied between 0.10 mM with BV and 0.31 mM with ferricyanide (Table 2). These values were significantly higher than the 0.021 mM reported for the native enzyme with ferricyanide as the electron acceptor [22]. The affinity of the recombinant protein to ferricyanide was significantly higher than that of the native protein purified from the hydrogenosome (*K*m of 0.06 vs 0.29 mM). The *K*m for BV was about threefold higher than that of ferricyanide and the electron acceptor with the highest *K*m was methyl viologen. The reaction rate was also highest with ferricyanide as the electron acceptor although the Vmax with the other two acceptors was not that dissimilar. These results show that the *T. vaginalis* hydrogenosome NADH-DH produced in *E. coli* is capable of reducing electron acceptors

Table 1 Specific activities of recombinant *T. vaginalis* hydrogenosome NADH-DH produced in *E. coli* with arabinose as inducer.

Electron acceptor	Specific activity (μmol min^{-1} mg $protein^{-1}$)	
	Arabinose-induced[a]	Native[b]
Ferricyanide	604±24	690
Benzyl viologen	582±44	ND
Methyl viologen	392±19	262
Ferredoxin	24±0.2[c]	48[d]

[a] Values are the mean of 3–5 replicates with standard deviation

[b] These values for the native enzyme isolated from *Trichomonas vaginalis* hydrogenosomes were from Hrdy et al. [22]

[c] *Clostridium acetobutylicum* ferredoxin

[d] *T. vaginalis* ferredoxin

ND not determined

Table 2 Kinetic properties of recombinant *T. vaginalis* hydrogenosome NADH-DH (arabinose-induced) purified from *E. coli*.

Acceptor	*K*m (NADH) (mM)	*K*m (acceptor) (mM)	Vmax[a]	*K*cat (s^{-1})
Ferricyanide	0.31	0.06	870	1,170
Benzyl viologen	0.10	0.17	645	725
Methyl viologen	0.22	0.44	570	650

*K*m for NADH was determined in an assay mixture containing 50 mM phosphate buffer, pH 7.5 with 5 mM benzyl viologen, 20 mM methyl viologen, or 1 mM potassium ferricyanide. *K*m for electron acceptors was determined in a reaction mixture containing 50 mM phosphate buffer, pH 7.5, 1 mM NADH and the electron acceptor at various concentrations. The reaction was followed by the reduction of electron acceptor (ε_{600nm} BV=7,800 M^{-1} cm^{-1} ; ε_{600nm} MV=6,300 M^{-1} cm^{-1} ; ε_{410nm} ferricyanide=1,020 M^{-1} cm^{-1}). All assays with viologen dyes were preformed under anaerobic conditions at room temperature using $S_2O_4^{-2}$ to titrate out residual oxygen in the assay mixture

[a] Vmax—μmoles min^{-1} (mg protein)$^{-1}$

with standard redox potentials that are significantly lower than that of NADH such as methyl viologen.

Absorbance Spectrum and Iron/Sulfur Content

The holoenzyme had a spectrum typical of Fe–S proteins with absorbance peaks at 326, 420, 463, and 551 nm (Fig. 2). Upon reduction with dithionite, the peaks at 420 and 463 nm disappeared, and the absorbance at 551 nm was lower than the protein as purified. Oxidation of the reduced protein by air restored the original spectrum. A spectrum similar to that of the holoenzyme was also obtained with the small subunit (Tvh-22) alone that was purified separately (Fig. 2). These spectra resemble the spectra of the corresponding flavoprotein subcomplex of respiratory NADH dehydrogenase complex I and the 25 KDa subunit of the same complex from *Paracoccus denitrificans* [34, 35] and also a [2Fe–2S] clostridial hydrogenase N-terminal domain [36].

The active NADH-DH had 2.15 non-heme iron and 1.95 acid-labile sulfur atoms per heterodimer. The Tvh-22 protein expressed and purified separately also had Fe and acid-labile sulfur at the same level. These results show that the holoenzyme only contains a [2Fe–2S] cluster that is located in the small subunit.

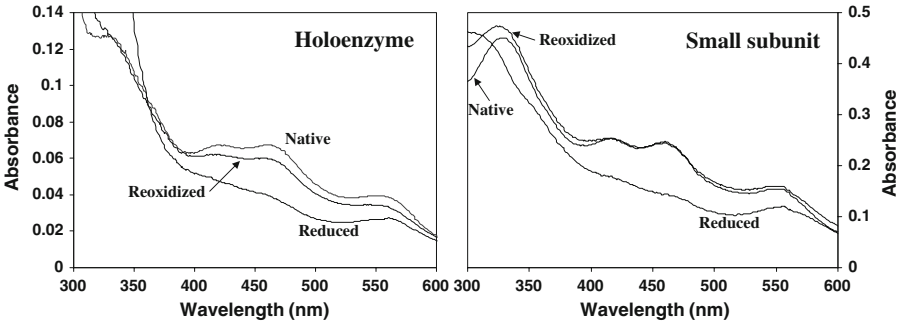

Fig. 2 Absorption spectrum of recombinant *T. vaginalis* hydrogenosome NADH-DH. Native spectrum represents the protein as purified. Reduced spectrum was obtained after titrating the protein with sodium dithionite. Reoxidized spectrum was obtained after gently mixing the reduced protein with air

The absence of Fe/S in the large subunit of NADH-DH is unique since the Tvh-47 homologs from respiratory chain complex I contain a [4Fe–4S] cluster [34, 37] and the cysteines implicated in liganding the tetranuclear Fe–S cluster in these proteins are conserved in the *Trichomonas* protein. The presence of only [2Fe–2S] cluster in the recombinant holoenzyme indicates that the Tvh-47 component lacks the anticipated tetranuclear N3 cluster. In agreement with this, the Tvh-47 protein expressed and purified separately also did not have any detectable Fe and labile S.

EPR Measurements of the Recombinant NADH-DH

In order to confirm the presence of only the binuclear Fe–S cluster in the purified NADH-DH, EPR spectra of the protein were obtained. The purified protein did not show an EPR spectrum but reduction with dithionite generated a spectrum that was rhombic in symmetry with g_{xyz} values corresponding to 1.917, 1.951, and 2.009 (Fig. 3). Although NADH was a substrate for the enzyme, NADH failed to reduce it to an EPR-active form. An EPR signal corresponding to a tetranuclear Fe–S cluster N3 (g_{xyz}=1.87, 1.94, and 2.04) found in homologous 54 KDa proteins of the respiratory complex I [34] was not detected in this *Trichomonas* NADH-DH purified from *E. coli*. The Tvh-22 protein by itself also produced an EPR spectrum that was similar to the holoenzyme upon reduction by dithionite (g_{xyz}= 1.92, 1.953, and 2.008; Fig. 4). These EPR studies support the conclusion that the protein contains only the binuclear Fe–S cluster corresponding to N1a in the small subunit. It is unlikely that an additional Fe–S cluster is present in the holoenzyme that is not reduced by dithionite to a paramagnetic state for detection by EPR since the spectrum of the holoenzyme in the 400–500 nm range was completely bleached by dithionite, indicating complete reduction of the Fe–S clusters in the protein (Fig. 2). In addition, the Fe content of the purified protein was only 2/heterodimer.

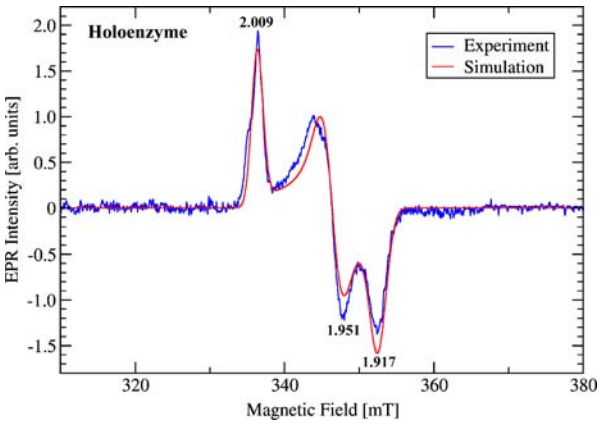

Fig. 3 EPR spectrum of the recombinant *T. vaginalis* hydrogenosome NADH-DH holoenzyme produced in *E. coli*. Spectrum of the holoenzyme (123.3 μM) was recorded at a microwave power of 2.0 mW after reducing the protein with sodium dithionite. EPR conditions: sample temperature, 25°K; microwave frequency, 9.45801 GHz; modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 80 ms; scan rate, 160 ms/data point for 4.9 G/s, 0.78 G/data point, receiver gain 60 dB. Wavy lines represent the experimental data and the smooth line is the simulation of the spectrum

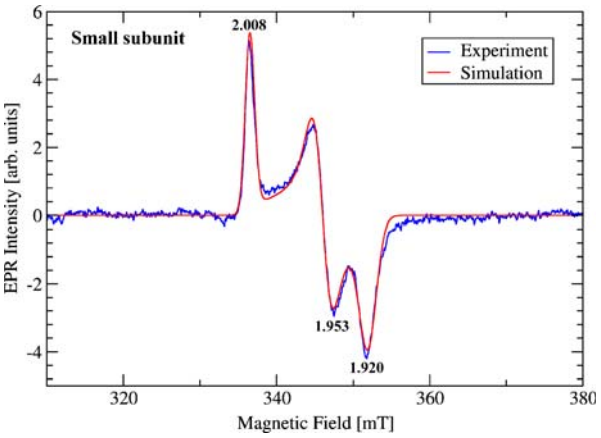

Fig. 4 EPR spectrum of Tvh-22 of the *T. vaginalis* NADH-DH produced in *E. coli*. Spectrum of the small subunit (121.0 μM) was obtained after reducing the protein with sodium dithionite. Other conditions were as listed for Fig. 3

Discussion

The hydrogenosomal NADH dehydrogenase is the only known enzyme with a predicted physiological role of coupling NADH oxidation to H_2 evolution that has been purified and biochemically characterized. As a first step in our attempt to engineer *E. coli* for production of H_2 at a higher yield, we have developed methods for optimum expression of the protein in an active form in *E. coli*. The purified protein reduced ferredoxin, and it is likely that the NADH-DH reduces ferredoxin in vivo also as an intermediate in H_2 evolution pathway (Fig. 1). In metabolic engineering of *E. coli* for H_2 production, the other components of the hydrogenosome pathway, ferredoxin, and hydrogenase need to be cloned and expressed in *E. coli*. In the interim, the ability of the NADH-DH to reduce viologen dyes leads to the possibility of using these intermediate electron carriers to couple the recombinant NADH-DH with the native hydrogenase 3 isoenzyme for H_2 evolution. *E. coli* whole cells as well as isolated membranes are well known for their ability to utilize BV or MV as an intermediate electron carrier in coupling dithionite to HYD-3 for H_2 evolution and this reaction could replace the need for ferredoxin and hydrogenase from the hydrogenosome. The [NADH]/[NAD^+] ratio of anaerobic *E. coli* is also significantly higher than that of an aerobically growing *E. coli* [38] and this could lead to a reduction in the in vivo midpoint redox potential of the NADH/NAD^+ couple (less than the Eo' of −0.32 V) to facilitate reduction of viologen dyes by NADH-DH in vivo. However, this requires complete removal of O_2 from the fermenting *E. coli* since the reduced viologen dyes react with O_2 to generate superoxide radicals that are detrimental to the cell. An alternate possibility is to engineer the bacterium to be less sensitive to superoxide.

The tetranuclear N3 cluster that plays a critical role in electron transport to ubiquinone [37, 39, 40] in all homologs of the respiratory complex I is absent in the recombinant *T. vaginalis* NADH-DH produced in *E. coli*. This is similar to the observed lack of the tetranuclear N3 cluster in the recombinant *Paracoccus denitrificans* NADH-DH produced in *E. coli* [34]. In contrast to the recombinant *T. vaginalis* protein of this study, the recombinant *P. denitrificans* protein also lacked FMN and NADH-dependent enzyme activity. Reconstitution of the recombinant *P. denitrificans* protein with FMN alone (without the N3 cluster but with N1a cluster) only produced about 25% of the NADH-dependent ferricyanide reduction activity of a complex that was reconstituted with FMN, iron, and S_2^-. This raises the possibility that a specific chaperone may be needed for

insertion of the N3 cluster in heterologous NADH-DH produced in *E. coli*. The absence of the N3 cluster in the recombinant NADH-DH is not due to high level of expression of the protein in *E. coli* is shown by the presence of N3 cluster in recombinant NuoF subunit of *E. coli* [41]. However, it should be noted that the *T. vaginalis* protein produced in *E. coli*, although lacking the N3 cluster, did reduce several electron acceptors at rates that are comparable to the native enzyme isolated from the hydrogenosome (Table 1).

The reported midpoint redox potential of FMN (−340 mV) in the respiratory complex I [37] may not suggest efficient electron flow from $FMNH_2$ to the N1a cluster of the recombinant *T. vaginalis* NADH-DH. However, the higher $[NADH]/[NAD]^+$ ratio of the anaerobic cell coupled with an increase in the ratio of $[FMNH_2]/[FMN]$ may lower the redox potential sufficiently to facilitate transfer of electrons to the [2Fe–2S] cluster at a midpoint potential of about −0.37 V. With the anticipated physiological role in NADH oxidation to H_2 production in a microbial biocatalyst such as recombinant *E. coli*, the tetranuclear cluster N3, if present in the flavoprotein of the NADH-DH, would drain electrons from NADH to a more oxidized form (−0.25 V) that is not energetically favorable to H_2 production.

In summary, we have taken the first step towards constructing a recombinant *E. coli* that can potentially oxidize glucose to at least 10 H_2 by cloning and expressing an active NADH-DH that can readily reduce low-potential electron carriers such as viologen dyes and ferredoxin. Further metabolic engineering of the bacterium with appropriate ferredoxin and hydrogenase genes could introduce an active pathway for release of the reductant in NADH as H_2 towards developing a microbial biocatalyst for production of H_2 as an energy source by dark fermentation of biomass derived sugars.

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