Novel FMN-Containing Rotenone-Insensitive NADH Dehydrogenase from *Trypanosoma brucei* Mitochondria: Isolation and Characterization†

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ABSTRACT: A rotenone-insensitive NADH dehydrogenase has been isolated from the mitochondria of the procyclic form of African parasite, *Trypanosoma brucei*. The active form of the purified enzyme appears to be a dimer consisting of two 33-kDa subunits with noncovalently bound FMN as a cofactor. Hypotonic treatment of intact mitochondria revealed that the NADH dehydrogenase is located in the inner membrane/matrix fraction facing the matrix. The treatment of mitochondria with increasing concentrations of digitonin suggested that the NADH dehydrogenase is loosely bound to the inner mitochondrial membrane. The NADH:ubiquinone reductase activity is insensitive to rotenone, flavone, or dicumarol; however, it was inhibited by diphenyl iodonium in a time- and concentration-dependent manner. Maximum inhibition by diphenyl iodonium required preincubation with NADH to reduce the flavin. More complete inhibition was obtained with the more hydrophobic electron acceptors, such as Q_1 or Q_2, as compared to the more hydrophilic ones, such as Q_0 or dichloroindophenol. Kinetic analysis of the enzyme indicated that the enzyme followed a ping-pong mechanism. The enzyme conducts a one-electron transfer and can reduce molecular oxygen forming superoxide radical.

The presence of rotenone-insensitive NADH dehydrogenase, the so-called alternative NADH dehydrogenase (or type II NADH dehydrogenase), has recently been reported in the mitochondria of plants and fungi as well as in bacterial membranes (*1*, *2*). These enzymes, generally a single polypeptide containing FAD as a cofactor, provide an alternative pathway to the proton-pumping NADH:ubiquinone oxidoreductase (complex I)[1] in the transfer of reducing equivalents to the electron transport chain (*1*, *2*). The first identification of an alternative NADH dehydrogenase was in plants, which often contain, in addition to complex I, four different NADH dehydrogenases located both on the external and internal faces of the inner mitochondrial membrane (*1*, *2*). Subsequently, three different rotenone-insensitive NADH dehydrogenases were discovered in the yeast *Saccharomyces cerevisiae* that does not contain complex I (*3*, *4*). The NADH dehydrogenase facing the mitochondrial matrix has been purified from *S. cerevisiae* mitochondria as a single polypeptide of 53 kDa (*3*). Two alternative NADH dehydrogenases located on the exterior face of the inner mitochondria have been identified in *S. cerevisiae* with the suggestion that their role is to transfer electrons from the NADH generated in the cytoplasm into the mitochondria (*4*). More recently, it was reported that the obligate aerobic yeast *Yarrowia lipolytica* contains, in addition to complex I, a single alternative NADH dehydrogenase, located on the exterior face of the inner mitochondria membrane (*5*). By contrast, *Neurospora crassa* mitochondria are reported to contain an alternative NADH dehydrogenase, which faces the mitochondrial matrix (*6*). *Escherichia coli* has an alternative NADH dehydrogenase (NDH-2 encoded by the *ndh* gene) in addition to complex I, NDH-1 encoded by the *nuo* operon (*1*, *7*).

Trypanosoma brucei, the African trypanosome, has a dual life cycle in the bloodstream of the mammalian host and the insect vector. In the mammalian bloodstream, the trypanosomes exist as dividing long slender forms that lack well-developed mitochondria, cytochromes, and cyanide-sensitive electron transport (*8*, *9*). The energy requirements of these long slender forms are fulfilled by glycolysis, using the abundant glucose present in the bloodstream of the mammalian host (*10*). The procyclic form, present in the midgut of the insect host, has a single large mitochondrion that contains a cyanide-sensitive, cytochrome-containing electron-transport chain similar to that of eukaryotes (*8*). In recent studies in our laboratory, a rotenone-sensitive NADH dehydrogenase activity was observed in procyclic *T. brucei* mitochondria, suggesting the presence of complex I in this organism (*11*, *12*). A large molecular weight complex having rotenone-sensitive NADH:ubiquinone oxidoreductase activity was isolated from procyclic *T. brucei* mitochondria by sucrose gradient centrifugation and blue native PAGE (BN−PAGE) (*13*). The isolated complex has a molecular weight of approximately 600 kDa and contains at least 11 subunits.

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[1] Abbreviations: Complex I, proton-pumping NADH:ubiquinone oxidoreductase; IDP, diphenyl iodonium; DM, dodecyl maltoside; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_1, ubiquinone-5; Q_2, ubiquinone-10; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; Q_6, ubiquinone-30; DCPIP, dichlorophenol−indophenol; BN−PAGE, blue native−PAGE; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

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During the sucrose gradient centrifugation of detergent-solubilized mitochondria from *T. brucei*, a fraction containing rotenone-insensitive NADH:ubiquinone reductase activity was observed. Immunoblotting studies with specific antibodies of the proteins in this fraction revealed the absence of the 51 kDa, TYKY, and PSST subunits of complex I, suggesting the presence of an alternative NADH dehydrogenase in the procyclic form of *T. brucei* (*13*). In the current study, we first report the isolation and characterization of a protein with rotenone-insensitive NADH:ubiquinone oxidoreductase activity from the mitochondrial membranes of the procyclic form of *T. brucei*. This alternative NADH dehydrogenase contains noncovalently bound FMN as a cofactor and appears to be a one-electron donor to ubiquinone or oxygen. The active enzyme appears to be a dimer, consisting of 2 subunits of 33 kDa, and is located on the inner mitochondrial membrane facing the matrix. To our knowledge, this is the first report of an alternative NADH dehydrogenase in a parasitic protozoan.

MATERIALS AND METHODS

Strain and Chemicals. *T. brucei* Mit 1.2 (strain 427) obtained from Dr. Paul Englund, Johns Hopkins University, Baltimore, MD, was used in this work. Diphenyl iodonium (IDP) and dodecyl maltoside (DM) were obtained from Fluka and Anatrace, respectively. The derivatives of ubiquinone, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0), ubiquinone-5 (Q_1), ubiquinone-10 (Q_2), ubiquinone-30 (Q_6), and 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB), plus other chemicals were purchased from Sigma Co.

Preparation of Mitochondria. Growth of the procyclic form of *T. brucei* was as described previously (*13*). To prepare mitochondrial membranes, cells were harvested by centrifugation at 2000*g* at 4 °C for 15 min and washed twice with a 20 mM sodium phosphate buffer containing 150 mM NaCl and 20 mM glucose (pH 7.9). The cells were then resuspended at a density of 10^{8-9} cells/mL in buffer A (20 mM Tris-HCl, 250 mM sucrose, 2 mM EDTA, and appropriate cocktail proteases inhibitor (pH 7.8)) and washed once. The washed cells were broken with glass beads (425−600 μm) using 8 cycles of 15 s duration stroke at 2 min intervals in a Braun mixer at 0−4 °C. The broken cells were centrifuged for 15 min at 1500*g* and 4 °C. The sediments were discarded, and the supernatant was centrifuged at 38 000*g* and 4 °C for 30 min. The pellets (containing mitochondrial membranes) were washed once in buffer A and maintained at −80 °C.

To prepare intact mitochondria, the washed cells suspended in buffer A were forced three times through a 26 G$^1/_2$ needle at high pressure at 0−4 °C. The suspension was then centrifuged at 1500*g* and 4 °C for 15 min to remove the unbroken cells and cell fragments. The supernatant was centrifuged at 16 000*g* and 4 °C for 20 min. The pellets containing mitochondria were washed once carefully in buffer A, maintained on ice, and used within 2 h. The integrity of the isolated mitochondria was determined by measuring the citrate synthase activity.

Hypotonic swelling of mitochondria was performed according to Söllner (*14*). Separation of the mitoplasts from the broken mitochondrial outermembranes and interspace proteins were carried out by centrifugation at 16 000*g* and 4 °C for 20 min.

Permeabilization of intact mitochondria by digitonin was carried out by incubating mitochondria with different amounts of digitonin (in buffer A) on ice for 25 min. After incubation, the suspension was centrifuged at 120 000*g* and 4 °C for 20 min to separate the released matrix proteins from the permeabilized mitochondria. The activities of released NADH dehydrogenase and citrate synthase in the supernatant were determined.

Purification of Rotenone-Insensitive NADH Dehydrogenase. The isolated mitochondrial membranes were solubilized with DM, in a proportion of 1.5 mg of DM/mg of membrane protein, for 30 min on ice, and the resulting suspension was subjected to centrifugation for 30 min at 38 000*g* and 4 °C. The pellets were discarded and the supernatant was dialyzed against buffer B (50 mM Tris-HCl, 1 mM EDTA, 5% glycerol, and 0.025% DM (pH 7.8)) containing cocktail protease inhibitors. All chromatographic procedures were performed on a Pharmacia FPLC system (LCC-500) at 4 °C. The dialyzed membrane proteins were first applied to an anion exchange column (HiTrap Q (Pharmacia) equilibrated with buffer B) and eluted with a NaCl gradient (0−0.8 M, in buffer B). The fractions containing NADH:Q_1 reductase activity were pooled, concentrated, and dialyzed against buffer C (50 mM Tris-HCl, 1 mM EDTA, 5% glycerol, and 0.025% DM (pH 8.5)). The enzyme was then loaded on the second HiTrap Q column (equilibrated with buffer C) and eluted with a NaCl gradient (0−0.4 M, in buffer C). The fractions containing NADH:Q_1 reductase activity were pooled, concentrated, and applied to a gel filtration column (Superdex 200, HR 10/30; Pharmacia) that was equilibrated with buffer B containing 150 mM NaCl. The proteins were eluted at a flow rate of 0.15 mL/min. The purified NADH dehydrogenase was store at −80 °C.

Enzyme Assays. NADH dehydrogenase activity was measured in 50 mM sodium phosphate, 1 mM EDTA, 2 mM KCN, 200 μM NADH, and 100 μM Q_1 (pH 6.5). The oxidation of NADH (NADPH and deamino-NADH) was monitored at 340 nm (extinction coefficient at 340 nm = 6.22 mM^{-1} cm^{-1}). Reduction of dichlorophenol−indophenol (DCPIP) was monitored at 600 nm (extinction coefficient at 600 nm = 16.26 mM^{-1} cm^{-1} (pH 6.5)), and NADH:$K_3[Fe(CN)_6]$ reductase activity was assayed at 420 nm (extinction coefficient at 420 nm = 1.05 mM^{-1} cm^{-1}). All activities were determined in a Cary 50 Bio UV-spectrophotometer at room temperature. The citrate synthase activity was determined as described previously (*15*, *16*).

Electrophoresis. SDS−PAGE was carried out as described by Laemmli (*17*) with 10% polyacrylamide resolving gels. Blue native PAGE (BN−PAGE) was performed according to Schägger's method (*18*, *19*) with a small modification. The proteins eluted from the second ion exchange column were concentrated and added to Bistris buffer (50 mM Bistris, 30% glycerol, and 0.1% Serve Blue G (pH 7.0)) in a 1:1 mixture. The sample was separated on a 5−20% linear gradient gel with a 4% stacking gel (1.5 mm thick). The cathode buffer was 50 mM Tricine, 15 mM Bistris, and 0.001% Serve Blue G (pH 7.0). The NADH dehydrogenase activity in the native gel was visualized with nitro blue tetrazolium as electron acceptor in the presence of NADH (*20*). The band containing NADH dehydrogenase activity was excised, immersed in 1% mercaptoethanol, and 1% SDS solution at room temperature for 2 h, washed with deionized

water to remove the mercaptoethanol for a few seconds, and then subjected to SDS−PAGE. A 0.5-cm lane of the gel was placed on a glass plate at the usual position for the stacking gel. After positioning the spacers (0.75 mm thick) and covering with the second glass plate, the separating gel (10%) was poured in through the gap between the gel strip and spacers. After polymerization, the stacking gel (4%) was poured in to fill the gap. The comb was positioned until the bottom of it reached the strip gel. After polymerization of the stacking gel, the purified enzyme obtained from the Superdex column and the molecular weight markers were loaded, and the electrophoresis was started immediately.

Flavin Analysis. The flavin present as a cofactor of the enzyme was determined fluorometerically (*21*). The purified enzyme was boiled for 3−4 min followed by centrifugation, and the presence of noncovalently bound FMN was determined fluorometerically using an excitation wavelength of 450 nm and an emission of 525 nm.

The released flavin was analyzed by HPLC using a Waters Alliance System consisting of the 2690 separation module, the 2487 dual λ absorbance detector, and the 474 scanning fluorescence detector controlled by Millennium^{32} software. A 150 × 4.6 mm i.d. Supelcosil LC-DP column (Supelco) was used. The composition of the mobile phase was 80% of 0.1% TFA in water and 20% of 0.1% TFA in 40% acetonitrile. The flow rate through the column at ambient temperature was 1 mL/min. The excitation and emission wavelengths of the fluorescence detector were set at 450 and 525 nm, respectively.

LC/ESI-MS studies of the flavin were performed using a Micromass ZMD 4000 quadrupole mass sepectrometer coupled to a Waters Alliance HPLC System controlled by a computer running Windows NT based Micromass MasslynxNT V3.5 software. The source temperature was 100 °C with the cone voltage set to 40 kV. Solvent flow through the LC-DP column was 1.0 mL/min, with 35% of the flow being diverted to the mass spectrometer. Scan data were collected over a range to *m*/*z*: 50−500 Da in the full scanning mode and *m*/*z*: 348 and 457 Da in the SIR mode.

EPR Studies. Determination of the semiquinone radical of Q_0 was performed in 40 mM sodium phosphate, 200 μM NADH, 100 μM Q_0, and 1 mM EDTA (pH 6.5) in a total volume of 200 μL. The reaction was started by addition of the rotenone-insensitive NADH dehydrogenase and subjected to EPR detection immediately. The center field is 3475 G with a sweep width of 100 G. Microwave frequency and microwave power were 9.740 G and 20.120 mW. Sweep time was 83.886 s.

Determination of $O_2^{\bullet -}$ produced by the rotenone-insensitive NADH dehydrogenase was performed in 40 mM phosphate, 1 mM EDTA, 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 200 μM NADH, and purified rotenone-insensitive NADH dehydrogenase (pH 6.5) in a total volume of 200 μL. The reaction was initiated by addition of NADH and subjected to immediate detection by EPR in an EMX EPR spectrometer (Bruker) at room temperature. EPR spectra were recorded at a center field of 3480 G with a sweep width of 100 G. Microwave frequency and microwave power were 9.752 G and 20.070 mW. Sweep time was 167.772 s.

Other Methods. Protein concentration was determined by the method of Lowry et al. (*22*). Molecular mass determination of the active form of the enzyme was performed in the previously described gel filtration column, using mass protein standards range from 12.4 to 240 kDa.

Table 1: Purification of Rotenone-Insensitive NADH Dehydrogenase from *T. brucei* Mitochondria[a]

purification step	protein (mg)	specific activity (μmol/min/mg)	yield (100%)	purification fold
DM extract	587.3	0.18	100	1.0
HiTrap Q (pH 7.8)	60.1	0.81	46	4.5
HiTrap Q (pH 8.5)	20.0	1.70	32	9.4
Superdex 200	0.8	4.80	3.6	26.7

[a] The NADH dehydrogenase activity was measured in 50 mM sodium phosphate, 1 mM EDTA, 2 mM KCN, 200 μM NADH, and 100 μM Q_1 (pH 6.5) at room temperature. The oxidation of NADH was monitored at 340 nm with an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The yield is calculated as total activity as a percentage of the total activity in the DM extract.

RESULTS

Purification of a 33 kDa Rotenone-Insensitive NADH Dehydrogenase. In our previous attempts to isolate complex I from detergent solubilized mitochondria of *T. brucei*, we observed rotenone-insensitive NADH:Q_1 reductase activity in a fraction obtained by sucrose gradient centrifugation. Immunoblotting with specific antibodies revealed the absence of three known subunit proteins of complex I, suggesting the presence of an alternative rotenone-insensitive NADH dehydrogenase in *T. brucei* (*13*). To confirm this suggestion, we attempted to isolate and purify such a protein. Mitochondrial membranes isolated from the procyclic form of *T. brucei* were solubilized with DM prior to anion exchange chromatography. Using a three-step chromatographic procedure, the rotenone-insensitive NADH dehydrogenase was purified to apparent homogeneity with a yield of about 3.6% (Table 1). In the final step, the NADH:Q_1 reductase activity was eluted from the Superdex column with an apparent molecular weight of 65 kDa. The purified protein was light yellow and catalyzed NADH:Q_1 reductase activity that was insensitive to rotenone.

Analysis of the purified enzyme by SDS−PAGE revealed a single major band with a molecular weight of 33 kDa. The purified enzyme, however, was eluted as a peak with an apparent molecular weight of 65 kDa through exclusion chromatography. To confirm that the 33 kDa protein observed on SDS−PAGE corresponds to the alternative NADH dehydrogenase with a molecular weight of 65 kDa obtained by filtration chromatography, the fraction containing activity from the second anion exchange column was subjected to BN−PAGE. To visualize the protein and reduce the background of the native gel, the sample was treated with 0.05% dye before electrophoresis, and the cathode buffer contained only 0.001% Serve blue G. After BN−PAGE, a few light blue bands were observed in the gel. To establish which band contained activity, the gel was treated with nitro blue tetrazolium, which acts as an electron acceptor for NADH dehydrogenase (*20*). The single band on the gel that gradually turned purple and dark, because of the reduction of the electron acceptor, contains NADH dehydrogenase activity (Figure 1A). This band was excised and treated with SDS plus mercaptoethanol prior to SDS−PAGE in parallel with the enzyme purified by gel filtration. The proteins isolated by these two different procedures migrated to the same position in SDS−PAGE with an apparent molecular

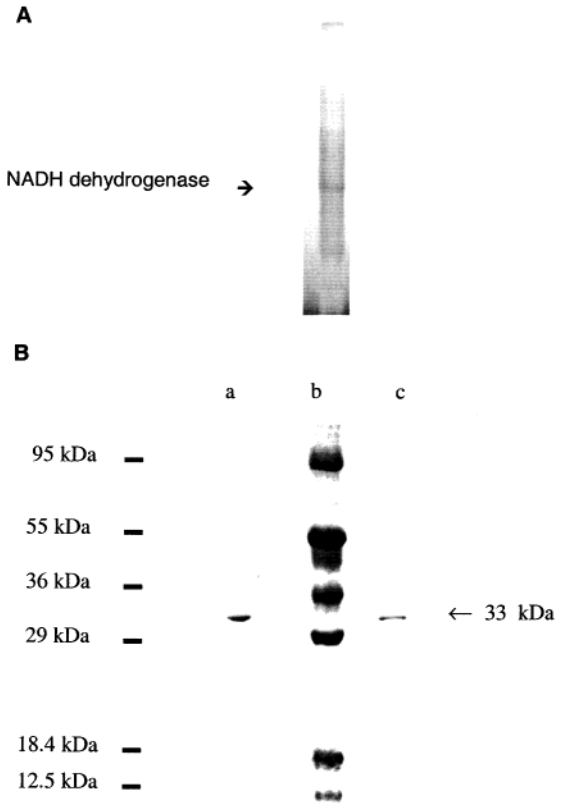

FIGURE 1: (A) BN−PAGE of the partially purified NADH dehydrogenase isolated from the second ion exchange column. The proteins were stained with serve blue G and loaded on a blue native gradient gel (5−20%). After electrophoresis, the NADH dehydrogenase was visualized with nitro blue tetrazolium (*20*). The arrow indicates the protein having NADH dehydrogenase activity. (B) SDS−PAGE of purified NADH dehydrogenase obtained by gel filtration and BN−PAGE. Electrophoresis was performed in a 10% acrylamide gel: lane a, purified protein obtained by through gel filtration; lane b, molecular weight markers; lane c, NADH dehydrogenase purified by BN−PAGE.

weight of 33 kDa (Figure 1B, lanes a and c). These results suggest that the rotenone-insensitive NADH dehydrogenase of *T. brucei* exists as a native dimer containing two 33 kDa subunits.

Rotenone-Insensitive NADH Dehydrogenase Contains FMN as Cofactor. The supernatant obtained after boiling the purified enzyme followed by centrifugation fluoresced with a strong emission maximum at 525 nm when excited at 450 nm, indicating the presence of noncovalently bound flavin (Figure 2A). Separation of standard flavins by HPLC as specified in Materials and Methods revealed retention times for FAD and FMN of 7.54 and 8.75 min, respectively (Figure 2B). The released flavin from the purified NADH dehydrogenase separated as a single peak at 8.74 min (Figure 2B). (Authentic FAD boiled under these conditions was stable and generated little FMN). These results suggest that FMN is the cofactor present in the rotenone-insensitive NADH dehydrogenase of *T. brucei.*

Further studies using LC/ESI-MS analysis provided additional evidence for the presence of FMN as cofactor in the rotenone-insensitive NADH dehydrogenase. In the full scanning mode, the sample from the enzyme displayed the same retention time (6.64 min), molecular weight (apparent MH^+ ion at *m*/*z*: 457), and fragmentation pattern as authentic FMN (data not shown). In the single ion monitoring mode,

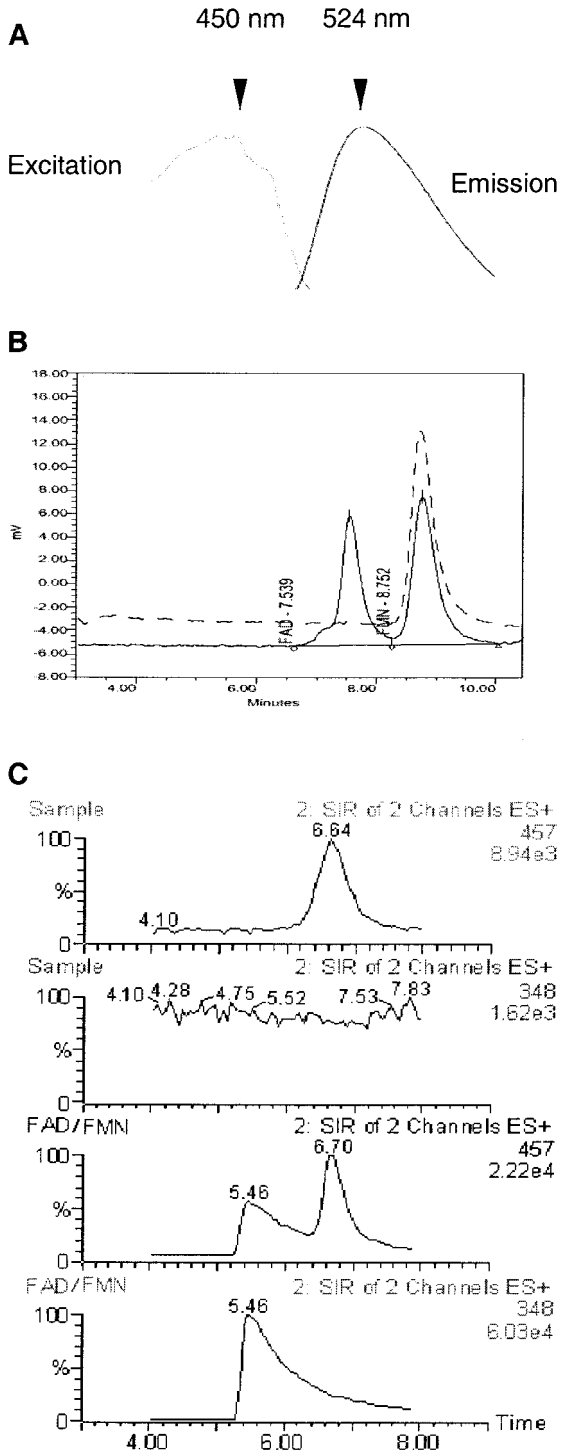

FIGURE 2: Analysis of the flavin cofactor of rotenone-insensitive NADH dehydrogenase of *T. brucei*. (A) Fluoresence excitation (left) and emission (right) spectra of the supernatant released from the enzyme by boiling. (B) HPLC analysis: FAD and FMN standards (—), and sample (− −). The retention time for FAD was 7.54 min, for FMN was 8.75, and for the sample was 8.74 min. (C) MS detection of authentic FAD and FMN standards and the sample using single ion monitoring at *m*/*z*: 348 and 457, respectively: top panel, *m*/*z*: 457 of the sample; 2nd panel, *m*/*z*: 348 of the sample; 3rd panel, *m*/*z*: 457 of FAD and FMN standards, bottom panel, *m*/*z*: 348 of FAD and FMN standards.

the sample separated as an apparent ion at *m*/*z*: 457 and a retention time of 6.64 min, identical to the FMN standard (Figure 2C, top panel). Moreover, the sample did not show an apparent fragmentary ion at *m*/*z:* 348 or 457 at the

Table 2: Kinetic Parameters of the Rotenone-Insensitive NADH Dehydrogenase of *T. brucei* Mitochondria

substrates	apparent K_m (μM)	V_{max} (μmol/min/mg)
NADH[a]	120	12.0
deamino-NADH	160	3.1
NADPH	400	1.4
Q_0[b]	33	12.8
Q_1	71	9.6
DCPIP	100	16.8
ferricyanide	190	23.8

[a] For determination of the kinetics for NADH, deamino-NADH, and NADPH, 150 μM Q_0 was used as the electron acceptor. [b] In measurements of the kinetic parameters of different acceptors, 250 μM NADH was the electron donor.

retention time of 5.46 min observed for authentic FAD in the single ion monitoring mode (Figure 2C, second panel). These results indicate that the cofactor of the rotenone-insensitive NADH dehydrogenase is FMN.

Catalytic Characterization of the Alternative NADH Dehydrogenase. The kinetic parameters of the purified rotenone-insensitive NADH dehydrogenase of *T. brucei* were examined using different electron donors and acceptors (Table 2). The purified NADH dehydrogenase isolated from *T. brucei* mitochondria can reduce several ubiquinone analogues, DCPIP, and ferricyanide. The purified enzyme can also reduce more hydrophobic ubiquinone analogues such as Q_2, DB, and Q_6; however, their high hydrophobicity and low solubility in water prevented the accurate determination of K_m values. In addition, the enzyme can oxidize NADPH and deamino-NADH but at much lower rates, 10% and 25% of that observed with NADH as substrate. Many rotenone-insensitive NADH dehydrogenases show a similar preference for NADH over deamino-NADH, as substrate and do not oxidize NADPH (*23*); however, the alternative NADH dehydrogenases isolated from plants are capable of oxidizing NADH and deamino-NADH but show no preference for NADH or NADPH (*24*, *25*).

To determine the mechanism of the reaction catalyzed by the purified alternative dehydrogenase of *T. brucei*, the initial velocity was determined using DCPIP as an electron acceptor. DCPIP, an artificial electron acceptor, was selected because of its high solubility in aqueous solution and the large molar extinction coefficient for the reduced form. The double reciprocal plots showing the dependence of the reaction rate for both substrates appear parallel at concentrations of the substrate that varied (data not shown). Similarly, replotting the reciprocal of V_{max} against the reciprocal of the fixed substrate concentrations displayed a linear relationship suggesting that catalysis by the alternative NADH dehydrogenase occurs by a ping-pong mechanism.

The alternative NADH dehydrogenase of *T. brucei* was stable within the pH range from 4.5 to 9.5, with a pH optimum between pH 6.0−6.5.

Inhibition of the Enzyme by Diphenyl Iodonium. Several well-known inhibitors of complex I and other NADH dehydrogenases were tested for their effects on the alternative NADH dehydrogenase of *T. brucei*. NADH:Q_1 reductase activity was not affected by rotenone, the well-known inhibitor of complex I, by flavone, an inhibitor of NDI1 of *S. cerevisiae* (*1*), or by dicumarol, an inhibitor of cytosolic NAD(P)H:quinone oxidoreductase (*26*, *27*); however, activity

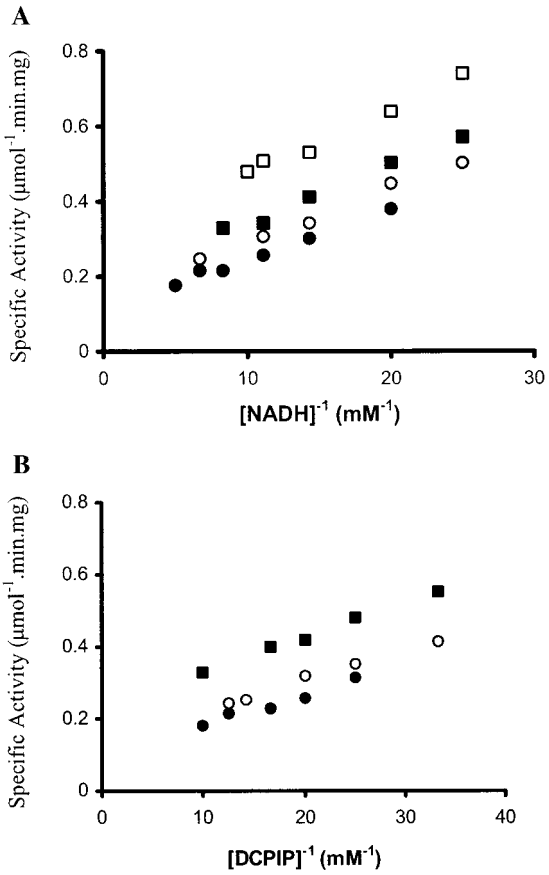

FIGURE 3: Initial velocity pattern for rotenone-insensitive NADH dehydrogenase with both substrates. (A) Initial velocity pattern of the enzyme with [NADH] varied at fixed levels of DCPIP. (Concentrations of DCPIP: (●) 100 μM; (○) 70 μM; (■) 50 μM; (□) 25 mM.) (B) Initial velocity pattern for the enzyme with [DCPIP] varied at fixed levels of NADH. (Concentrations of NADH: (●) 200 μM; (○) 100 μM; (■) 50 μM.)

of the purified enzyme was blocked by addition of diphenyl iodonium (IDP), a well-characterized inhibitor of flavoproteins (*28*−*30*), in a time and concentration-dependent manner (Figure 4A). A loss of 90% of the NADH:Q_1 reductase activity was observed after a 1 min incubation of the enzyme with 200 μM IDP in the presence of NADH. Incubation of the enzyme with 50 μM IDP under the same conditions resulted in the loss of 40% of NADH:Q_1 reductase activity; however, if the incubation time was 6 min, 95% of the activity was lost. The effectiveness with which IDP inhibited the purified alternate NADH dehydrogenase depended on the electron acceptor used in the assay (Figure 4B). A 98% inhibition was observed with Q_2 as acceptor, 90% with Q_1, 68% with Q_0, and 54% with DCPIP after incubation of the enzyme with IDP for 1 min. An increase in the incubation time of the purified enzyme with NADH and IDP to 20 min increased the inhibitory rate just to 66% with DCPIP as electron acceptor. Similar low inhibitory effects of IDP were observed with ferricyanide as electron acceptor (data not shown).

One-Electron Transfer by the Enzyme. An earlier report indicating that IDP is an inhibitor of flavoproteins conducting one-electron transfer (*31*) suggested that the alternative NADH dehydrogenase of *T. brucei* might catalyze a one-electron transfer. To establish the ability of the NADH dehydrogenase to catalyze the transfer of one electron to ubiquinone, we used EPR to assay directly the formation of

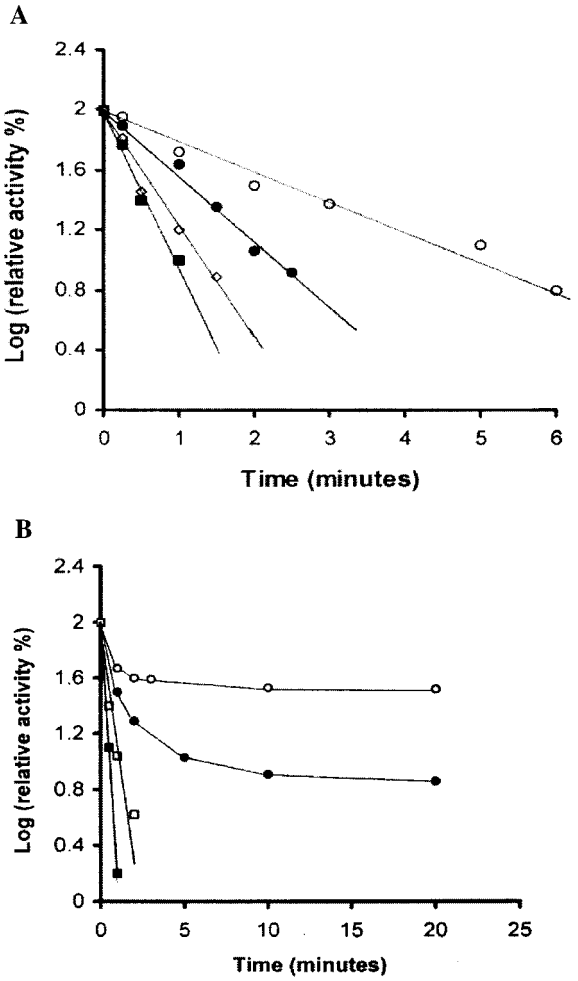

FIGURE 4: IDP inhibited rotenone-insensitive NADH dehydrogenase of *T. brucei*. (A) Inhibition of rotenone-insensitive NADH:Q_1 reductase by IDP. Rotenone-insensitive NADH dehydrogenase was incubated with IDP in the presence of NADH (200 μM) in buffer containing 50 mM sodium phosphate (pH 6.5) at room temperature. At the times indicated, enzyme assays were started immediately by addition of 100 μM Q_1 in a volume of 10 μL. The relative activity was determined by calculating the ratio of the residual NADH:Q_1 reductase activity observed in the presence of IDP to that obtained in the absence of IDP (100% activity was 105 nmol/min). (Concentrations of IDP used: (■) 200 μM; (□) 150 μM; (●) 100 μM; (○) 50 μM.) (B) Inhibition of the rotenone-insensitive NADH dehydrogenase activity by IDP with different acceptors. (Acceptors used: (■) 20 μM Q_2; (□) 100 μM Q_1; (●) 100 μM Q_0; (○) 80 μM DCPIP.)

the ubisemiquinone radical (Figure 5). Incubating the enzyme with NADH and Q_0 produced a partially resolved (five lines) characteristic signal of a Q_0 semiquinone radical with hyperfine structure due to splittings from the methyl protons (*32*). This result indicates that the enzyme reduces ubiquinone through one-electron transfer.

Production of Superoxide Radicals. To determine if the rotenone-insensitive NADH dehydrogenase from procyclic *T. brucei* mitochondria can reduce O_2 directly with the production of $O_2^{\bullet-}$, EPR was used to detect directly the superoxide radical signal. Because the $O_2^{\bullet-}$ radical is unstable with a short lifetime, DMPO was used as a spin trap. When the purified enzyme was incubated with NADH aerobically, strong EPR signals that are typical of the DMPO−OOH formed by $O_2^{\bullet-}$ were observed (Figure 6A). If superoxide dismutase (200 units/mL) were added prior to starting the

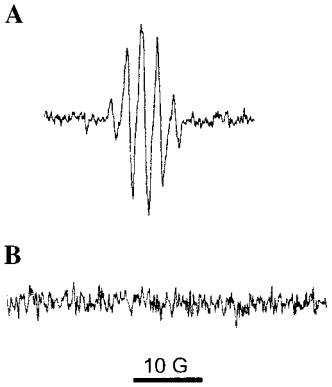

FIGURE 5: Formation of semiquinone radical by rotenone-insensitive NADH dehydrogenase. NADH (200 μM) was incubated in 40 mM sodium phosphate with 100 μM Q_0 and 1 mM EDTA (pH 6.5) in a total volume of 200 μL. The reaction was started by addition of purified NADH dehydrogenase and subjected to immediate analysis by EPR. The EPR spectrum was recorded at room temperature in a Bruker EMX EPR spectrometer and measured at a center field of 3475 G with a sweep width of 100 G. Microwave frequency and microwave power were 9.740 G and 20.120 mW. The sweep time was 83.886 s: (A) Q_0 semiquinone radical production by rotenone-insensitive NADH dehydrogenase; (B) control (in the absence of rotenone-insensitive NADH dehydrogenase).

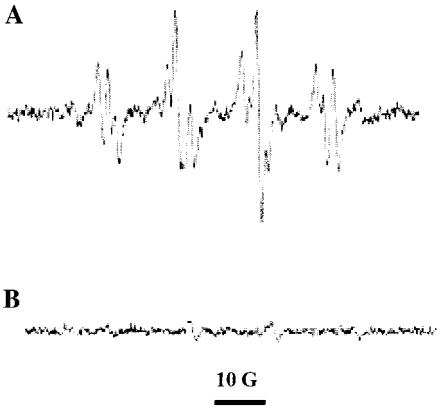

FIGURE 6: Formation of superoxide radical by alternative NADH dehydrogenase. The determination was performed in 40 mM phosphate, 1 mM EDTA, 100 mM DMPO, and 200 μM NADH (pH 6.5) in a total volume 200 μL. The reaction was started by the addition of NADH and subjected to immediate analysis by EPR in a Bruker EMX EPR spectrometer. The EPR spectrum was measured at a center field of 3480 G with a sweep width of 100 G. Microwave frequency and microwave power were 9.752 G and 20.070 mW. Sweep time was 167.772 s: (A) generation of superoxide in the presence of rotenone-insensitive NADH dehydrogenase; (B) inhibition of superoxide formation by addition of superoxide dismutase.

reaction, little signals were generated (Figure 6B). These results suggest that, under aerobic conditions, the alternative NADH dehydrogenase of *T. brucei* can generate superoxide radicals in the presence of NADH.

Intramitochondrial Location of the Alternative NADH Dehydrogenase. To test for the intactness of the mitochondria isolated from the procyclic form of *T. brucei*, the activity of rotenone-insensitive NADH:Q_1 reductase was compared to that of the mitochondrial matrix enzyme, citrate synthase, which is inaccessible to exogenous substrates if mitochondria remain intact. The activity of citrate synthase of the isolated *T. brucei* mitochondria was 9 nmol/min/mg (Table 3). Treatment of the mitochondria with 1% DM prior to assay resulted in a 9−10-fold increase in citrate synthase activity

Table 3: Determination of Rotenone-Insensitive NADH:Q_1 Reductase and Citrate Synthase Activities of *T. brucei* Mitochondria

	enzyme activity (nmol/min/mg)			
			hypotonic-treated mitochondria	
enzyme	intact mitochondria	1% DM-treated mitochondria	supernatant	mitoplast
rotenone-insensitive NADH:Q_1 reductase	21	220	0	24
citrate synthase	9	87	nd[a]	nd

[a] nd = not determined.

to 87 nmol/min/mg, suggesting that mitochondria isolated from *T. brucei* are intact with an integrity of approximately 90%. Similarly, the rotenone-insensitive NADH:Q_1 reductase activity of the *T. brucei* mitochondria was increased from 21 to 220 nmol/min/mg after DM treatment (Table 3). The low activity of NADH:Q_1 reductase activity in the isolated mitochondria may result from broken mitochondria, because NADH cannot permeate the inner mitochondrial membrane (*33*).

Hypotonic swelling of intact mitochondria under controlled conditions results in the destruction of the outer mitochondrial membrane while maintaining the inner membrane/matrix fraction (mitoplast) intact. The NADH:Q_1 reductase activity of the mitoplasts was 24 nmol/min/mg, a value similar to that observed with intact mitochondria. No NADH:Q_1 reductase activity was observed in the supernatant obtained after centrifugation of the treated mitochondria, suggesting that the alternative NADH dehydrogenase is located in the inner membrane/matrix fraction (Table 3).

To obtain a more precise location of the alternative NADH dehydrogenase, the isolated mitochondria were treated with increasing concentrations of digitonin in order to achieve a sequential opening of the matrix compartment. The release of NADH:Q_1 reductase activity was compared with that of the marker enzyme citrate synthase, a soluble protein localized in the mitochondrial matrix, which is released with increasing concentrations of digitonin. At a 0.1% digitonin concentration, 12% of the total rotenone-insensitive NADH dehydrogenase activity was released to the supernatant compared to 46% of the citrate synthase activity (Figure 7). Raising the concentration of digitonin to 1.6% resulted in the release of 40% of the NADH dehydrogenase activity as compared to the release of 80% of the citrate synthase activity. The results of the hypotonic swelling and digitonin treatment suggest that, unlike citrate synthase, the rotenone-insensitive NADH dehydrogenase is bound to the inner mitochondrial membrane facing the mitochondrial matrix.

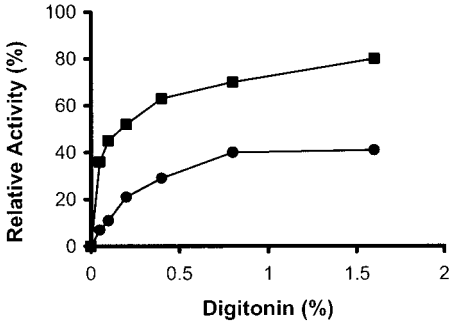

FIGURE 7: Treatment of *T. brucei* mitochondria with increasing concentrations of digitonin. Aliquots of intact mitochondria suspended in buffer A were incubated with increasing concentrations of digitonin on ice for 25 min and centrifuged at 120 000*g* for 20 min at 4 °C. The activities of NADH:Q_1 reductase and citrate synthase were determined in the supernatant. The enzymatic activity of the mitochondria in each aliquot was defined as 100%. (-●-) Released NADH dehydrogenase; (-■-) Released citrate synthase.

DISCUSSION

In the current study, we have identified and characterized a rotenone-insensitive NADH dehydrogenase in mitochondria of the procyclic form of *T. brucei*. The active form of the enzyme elutes from Superdex chromatography as a dimer of 65 kDa, which separates after SDS−PAGE as a 33 kDa subunit, and contains FMN as cofactor. In general, alternative NADH dehydrogenases present in mitochondria from other organisms are single polypeptides with molecular weights ranging from 40 to 60 kDa containing FAD as cofactor (*1*, *2*). The presence of noncovalently bound FMN as cofactor in the *T. brucei* alternative NADH dehydrogenase raises the possibility that FMN as cofactor may confer the enzyme with properties or functions that differ from other FAD-containing alternative NADH dehydrogenases. The rotenone-insensitive NADH dehydrogenase in *T. brucei* mitochondria is located on the inner membrane facing the matrix similar to the NADH dehydrogenase of *N. crassa* (*6*) and to one or more of the alternative dehydrogenases of *S. cerevisiae* (*3*) or plants (*2*).

Inhibitors for alternative rotenone-insensitive NADH dehydrogenases are rare and nonspecific. For example, the alternative NADH dehydrogenase isolated from red beetroot is sensitive to dicumarol (*25*), while the NDI1 of *S. cerevisiae* is inhibited by flavone (*1*). The enzyme isolated from *T. brucei* is not inhibited by either of these compounds but is sensitive to IDP, a potent inhibitor of flavoproteins such as neutrophil NADPH oxidase (*28*). The inhibition of NADPH oxidase by IDP is proposed to occur though a reductive mechanism in which the reduced flavin transfers one electron to IDP, resulting in the formation of a flavin semiquinone and a neutral diphenyliodonyl radical (*28*−*30*). The diphenyliodonyl radical disproportionates to iodobenzene and a reactive phenyl radical, which is proposed to combine with the flavin semiquinone to form a phenylated adduct. The observation that incubation of the rotenone-insensitive NADH dehydrogenase of *T. brucei* with IDP and NADH was a prerequisite step for enzyme inhibition indicates that the inhibitory effects of IDP also require a reduced redox center. In addition, greater inhibition by IDP was observed with the more hydrophobic electron acceptors. One explanation for this observation might be that the phenyl radicals reacted with amino acid residues adjacent to the cofactor, resulting in the inaccessibility of the active site to the electron acceptors especially the hydrophobic ubiquinones.

The rotenone-insensitive NADH dehydrogenase of *T. brucei* has the ability to transfer one electron from the FMN cofactor to various electron acceptors, including oxygen and ubiquinone-0. By contrast, other reported alternative NADH dehydrogenases containing FAD as cofactor (*1*, *2*) are reported to conduct two-electron reduction (*3*, *34*). The transfer of one electron would make the NADH dehydrogenase of *T. brucei* particularly prone to produce reactive

oxygen species, because superoxide is generated when molecular oxygen is reduced by one electron transfer (*35*). The possible role of the rotenone-insensitive NADH dehydrogenase in the production of superoxide radicals in *T. brucei* mitochondria is currently under investigation.

The physiological functions of alternative NADH dehydrogenase are still ambiguous. In *S. cerevisiae* mitochondria, the internal NADH dehydrogenase has been suggested to play a role in regulating the redox balance at the level of mitochondrial NADH produced by the citric acid cycle (*36*). The external NADH dehydrogenases of *S. cerevisiae* have been suggested to contribute to the reoxidation of cytosolic NADH (*4*, *37*, *38*). By contrast, the external NADH dehydrogenase isolated from *Yarrowia lipolytica* was not required for cell growth (*5*). A recent report suggested that the type II NADH dehydrogenase present in the cyanobacterium *Synechocystis* sp. strain might be involved in regulation of metabolism rather than respiration (*39*, *40*). The procyclic form of *T. brucei* has a well-developed mitochondrion with cytochromes and cyanide-sensitive respiration. The major reducing equivalents are provided by the oxidation of the abundant amino acids, such as proline, in the midgut of the insects. A rotenone-sensitive NADH dehydrogenase from procyclic *T. brucei* mitochondria has been identified and characterized (*11*−*13*); however, the activity of complex I in *T. brucei* mitochondria was low as compared to that observed in other species and may be rate limiting for NADH oxidation. One possible function of the alternative NADH dehydrogenase described in this study might be a complement of complex I mediating the electron feeding from NADH to the electron-transfer chain. Its location on the inner mitochondrial membrane facing the matrix is consistent with this suggestion.

In this paper, a rotenone-insensitive NADH dehydrogenase in *T. brucei* was identified, isolated, and characterized. As far as we know, this is the first report of an alternative NADH dehydrogenase in protozoan mitochondria. Attempts are currently underway to investigate the physiological function of the alternative NADH dehydrogenase in the intermediary metabolism of *T. brucei* by isolating the gene for the protein and constructing null mutants.

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