Alternative Pathway of Metronidazole Activation in *Trichomonas vaginalis* Hydrogenosomes

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Metronidazole and related 5-nitroimidazoles are the only available drugs in the treatment of human urogenital trichomoniasis caused by the protozoan parasite *Trichomonas vaginalis*. The drugs are activated to cytotoxic anion radicals by their reduction within the hydrogenosomes. It has been established that electrons required for metronidazole activation are released from pyruvate by the activity of pyruvate:ferredoxin oxidoreductase and transferred to the drug by a low-redox-potential carrier, ferredoxin. Here we describe a novel pathway involving the drug activation within the hydrogenosome. The source of electrons is malate, another major hydrogenosomal substrate, which is oxidatively decarboxylated to pyruvate and CO₂ by NAD-dependent malic enzyme. The electrons released during this reaction are transferred from NADH to ferredoxin by NADH dehydrogenase homologous to the catalytic module of mitochondrial complex I, which uses ferredoxin as electron acceptor. *Trichomonads* acquire high-level metronidazole resistance only after both pyruvate- and malate-dependent pathways of metronidazole activation are eliminated from the hydrogenosomes.

For more than 40 years, metronidazole and related derivatives of 5-nitroimidazole have been the drugs of choice in the treatment of infections caused by anaerobic or microaerophilic microbes, both prokaryotic and eukaryotic. The susceptible organisms are characterized by the presence of low-redox-potential electron-transporting systems that are absent in aerobes. These pathways involve ferredoxin-like electron carriers that use the nitroimidazole prodrugs as electron acceptors, generating short-lived reactive anion radicals that inflict multiple types of cellular damage and subsequent cell death (5, 14).

In *Trichomonas vaginalis*, an amitochondriate flagellate causing human urogenital trichomoniasis, metronidazole is activated within hydrogenosome. This double-membrane-bound organelle harbors a catabolic pathway in which pyruvate or malate is oxidatively decarboxylated with concomitant generation of electrons. Pyruvate is converted to acetyl coenzyme A (acetyl-CoA) and CO₂ by a pyruvate:ferredoxin oxidoreductase (PFOR). While acetyl-CoA is utilized in a substrate-level synthesis of ATP, the released electrons are transferred via ferredoxin to hydrogenase that produces molecular hydrogen (15). Malate is converted to pyruvate and CO₂ by malic enzyme. The electrons released during this reaction are transferred from NADH to ferredoxin by NADH dehydrogenase homologous to the catalytic module of mitochondrial complex I, which uses ferredoxin as electron acceptor. Trichomonads acquire high-level metronidazole resistance only after both pyruvate- and malate-dependent pathways of metronidazole activation are eliminated from the hydrogenosomes.

*MATERIALS AND METHODS*

**Organisms.** *Trichomonas vaginalis* strain TV 10-02, which is susceptible to metronidazole (9), and its laboratory-induced derivatives lacking PFOR activity, which express low (TV 10-02 MR 5) or high (TV 10-02 MR 100) levels of metronidazole resistance (8, 17), were used in the experiments monitoring the formation of metronidazole anion radicals by isolated hydrogenosomes. *T. vaginalis* strain T1 (provided by Patricia Johnson, University of California, Los Angeles, Calif.) was used for the purification of NDH. The cells were grown in tryptophan-extract-maltose (TYM) medium (3) supplemented with 10% heat-inactivated horse serum and 0.05% agar (wt/wt) at 37°C. Cultures of the resistant strains were maintained in a TYM medium with metronidazole (5 μg ml⁻¹ for the MR 5 strain and 50 μg ml⁻¹ for the MR 100 strain). Large-volume cultures for isolation of hydrogenosomes were grown without agar and metronidazole.

**Cell fractionation.** The cells (approximately 2 liters of culture) were harvested by centrifugation, washed with ST buffer (250 mM sucrose, 0.5 mM KCl, 10 mM Tris-HCl, pH 7.2) and suspended in ST buffer with 50 μg of N-tosyl-l-lysine chloromethyl ketone (TLCK) per ml and 10 μg of leupeptin per ml. The cells were disrupted by sonication and subjected to differential centrifugation as described previously (4). The resulting hydrogenosome-enriched fraction was further purified by isopycnic centrifugation on 45% Percoll (19). These highly purified hydrogenosomes were used throughout this study.

**Purification of NDH.** NDH was purified from the hydrogenosomes by liquid chromatography on cation exchange and hydroxypatite columns as described in...
an earlier publication (6). Aliquots from purification steps were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were visualized by Coomassie staining.

Preparation of T. vaginalis recombinant ferredoxin. Recombinant T. vaginalis [2Fe-2S] ferredoxin without a hydrogenosomal targeting sequence was expressed in Escherichia coli and isolated by two steps of liquid chromatography as described previously (19).

Enzyme activity determinations. Activity of malic enzyme was determined spectrophotometrically at 340 nm as the rate of malate-dependent reduction of NAD\(^+\) as described previously (4). Activities of PFOR and NDH were determined under anaerobic conditions in 100 mM potassium phosphate buffer, pH 7.4, containing 25 mM mercaptoethanol and 10 mM methyl viologen. The PFOR activity was determined with 6 mM pyruvate and 0.5 mM CoA; 8.5 mM NADH was the substrate for NDH. Activities of both enzymes were determined spec-trophotometrically at 600 nm using a molar extinction coefficient, \(\varepsilon_{600}\), of 6,300 M\(^{-1}\) cm\(^{-1}\). Activity of NDH in the course of purification was monitored using 2,6-dichlorophenol (DCIP) as the electron acceptor. The reaction mixture contained 50 mM KCl, 100 mM Tris-HCl, pH 8 (assay buffer), 3.3 mM NADH, and 100 \(\mu\)M DCIP. The reduction of acceptor was monitored at 600 nm, and the \(\varepsilon_{600}\) of DCIP was taken as 21000 M\(^{-1}\) cm\(^{-1}\). All spectrophotometric determinations were done at 25°C. One unit of enzyme activity was defined as amount of protein catalyzing the consumption of 1 micromole of substrate or the formation of 1 micromole of product per minute. Protein concentrations were determined by the method of Lowry.

The activities of PFOR, malic enzyme, and NDH were determined in isolated hydrogenosomes of all strains prior to the EPR experiments. All the activities were in the same range as those determined previously for the same strains (17), except for NDH (called NADH:ferredoxin oxidoreductase in the reference), for which the activity was found in this work to be about twofold higher in wild-type and low-resistance strains.

EPR spectroscopy. EPR spectroscopy was used to detect the metronidazole anion radical formed by isolated hydrogenosomes and also by purified NDH. Assay mixtures used to detect the metronidazole radical formation catalyzed by PFOR, malic enzyme, and NDH within isolated organelles were similar to those used for spectrophotometric determination of the respective enzymatic activities, except for the omission of the methyl viologen electron acceptor in the PFOR and NDH reactions. The assay mixture (0.8 ml) contained 43 mM metronidazole, approximately 50 \(\mu\)g/ml T. vaginalis ferredoxin, 25 mM mercapto-ethanol, and 1.2 mg/ml Triton X-100, plus either 7.5 mM pyruvate plus 0.27 mM CoA, 14 mM malate plus 1.1 mM NAD\(^+\), or 2.2 mM NADH. The solution was degassed by argon flow for 10 min, and the reaction was started by the addition of approximately 300 \(\mu\)g of hydrogenosomal protein of a wild-type, low-resistance strain. Upon addition of the protein sample, each mixture was immediately drawn into a 150-\(\mu\)l flat detection cell. EPR spectra of the hydrogenosomal preparations were recorded at 25°C on a Bruker ESP 300 spectrometer (Bruker BioSpin). Formulation of metronidazole nitro anion radicals by purified NDH was monitored in the assay buffer containing 2.2 mM NADH and 12 \(\mu\)M metronidazole. The mixture (2 ml) was flushed with oxygen-free nitrogen for 10 min, and then recombinant T. vaginalis ferredoxin (approximately 700 \(\mu\)g) was added and the reaction was started by the addition of approximately 4 \(\mu\)g of purified NDH. Spectra were recorded on a Bruker ELEXSY SES80 spectrometer at 25°C. Hyperfine coupling constants for the metronidazole anion radicals were determined using the SimFonia program.

RESULTS

Formation of metronidazole anion radicals in isolated hydrogenosomes. In order to test whether electrons generated by malic enzyme are used in metronidazole activation, we monitored the formation of metronidazole anion radicals by EPR spectroscopy in the reaction mixture containing hydrogenosomes, T. vaginalis ferredoxin, malate, NAD\(^+\), and metronidazole. The signal corresponding to reduced metronidazole was observable in the reaction with malic enzyme (Fig. 1A). The amplitude of the signal generated by malate-dependent activity was lower than that generated by PFOR, indicating a lower activity of metronidazole reduction catalyzed by the alternative system (Fig. 1).

In the next experiment, NADH only was used as an electron donor for metronidazole reduction by the hydrogenosomes of drug-sensitive trichomonads; this sole substrate should have been sufficient if NDH was involved in metronidazole reduction. Indeed, the metronidazole anion radicals were formed and detected with the same amplitude as that in the case of the malic enzyme-catalyzed reaction (Fig. 1C), indicating that NDH activity is the limiting factor in malate-dependent reduction of metronidazole. Reduction of ferredoxin by NDH is relatively slow (approximately 10 \(\mu\)mol/min per mg of purified protein [unpublished data]), as opposed to the rapid reoxidation of reduced ferredoxin by metronidazole (21). Addition of external ferredoxin in all reactions had only a marginal effect; omitting ferredoxin resulted in somewhat lower amplitudes of the signals, showing that the ferredoxin concentration in the hydrogenosomal preparations was sufficient to support the drug reduction.

Using the same experimental setup as described above, the hydrogenosomes from the PFOR-deficient strain (TV 10-02 MR 5), displaying a low level of resistance, were tested for their capacity to catalyze the reductive activation of metronida-zole. Trace A in Fig. 2 shows that metronidazole was not
reduced when pyruvate and CoA were added into the reaction, consistent with the absence of PFOR activity in this strain. However, metronidazole anion radicals were formed by activities of both malic enzyme and NDH (Fig. 2B and C, respectively). As described above, the amplitudes of the signals were comparable in malic enzyme- and NDH-catalyzed reactions.

Finally, we tested the formation of metronidazole anion radicals using the hydrogenosomes from the T. vaginalis strain displaying a high level of metronidazole resistance (TV 10-02 MR 100). This strain lacks detectable activities of PFOR, malic enzyme, and NDH and does not express ferredoxin (17). Consistent with the absence of all these proteins, metronidazole was reduced neither by the PFOR-dependent reaction nor by the alternative pathway involving malic enzyme and NDH (Fig. 2D; only the trace corresponding to NDH activity is shown).

**Reduction of metronidazole by NDH.** To provide the direct evidence that NDH provides electrons for metronidazole activation, we tested the formation of metronidazole anion radicals using the hydrogenosomes from the T. vaginalis strain displaying a high level of metronidazole resistance (TV 10-02 MR 100). This strain lacks detectable activities of PFOR, malic enzyme, and NDH and does not express ferredoxin (17). Consistent with the absence of all these proteins, metronidazole was reduced neither by the PFOR-dependent reaction nor by the alternative pathway involving malic enzyme and NDH (Fig. 2D; only the trace corresponding to NDH activity is shown).

**DISCUSSION**

In this study, we report on a novel PFOR-independent metronidazole-activating pathway present in T. vaginalis hydrogenosomes that consists of malic enzyme, NDH, and ferredoxin. Until recently, the principal hydrogenosomal activity responsible for the reductive activation of metronidazole had been ascribed to PFOR (2, 12, 16), based on the findings (i) that PFOR catalyzed generation of nitro anion radicals in Tritrichomonas foetus hydrogenosomal fraction when pyruvate, CoA, and metronidazole were present (13) and (ii) that PFOR activity was lost in in vitro-derived strains of T. foetus (1) and T. vaginalis (8) resistant to high concentrations of metronidazole. These strains were able to grow in vitro in the presence of >100 μg/ml metronidazole. However, more-recent studies of metabolic changes accompanying the in vitro induction of a high-level metronidazole resistance in T. vaginalis revealed that PFOR activity is among the first enzymes that disappear

![FIG. 2. EPR spectra demonstrating formation of metronidazole anion radicals by the hydrogenosomes of low-resistance (strain TV 10-02 MR 5; traces A to C) and highly resistant (strain TV 10-02 MR 100; trace D) T. vaginalis strains in the presence of 43 mM metronidazole. (A) Absence of signal in the presence of pyruvate, ferredoxin, and CoA. (B) Signal generated in the presence of malate, ferredoxin, and NADH" (malic enzyme activity). (C) Signal generated in the presence of NADH and ferredoxin (NDH activity). (D) Absence of signal in the presence of hydrogenosomes of highly resistant T. vaginalis, NADH, and ferredoxin (NDH activity). Instrument settings were as described for Fig. 1.](http://aac.asm.org/content/49/19/5035/F2.large.jpg)

![FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Coomassie-stained subcellular fractions of T. vaginalis and isolated NDH. Lanes: 1, total homogenate; 2, cytosol; 3, hydrogenosomes; 4, two subunits of NDH purified from hydrogenosomes.](http://aac.asm.org/content/49/19/5035/F3.large.jpg)

![FIG. 4. EPR spectrum of metronidazole anion radical formed by purified NDH in the presence of NADH and Trichomonas ferredoxin. The spectrum was recorded with microwave power of 0.505 mW, a frequency of 9.7854 GHz, and a modulation amplitude of 0.4 mT.](http://aac.asm.org/content/49/19/5035/F4.large.jpg)
from the hydrogenosomes in the process of resistance development. The early-stage parasites, which were already PFOR deficient, were still quite susceptible to the drug, growing in the presence of no more than 5 μg/ml metronidazole (17). These observations suggested that other hydrogenosomal enzymes participating in redox reactions account for metronidazole activation. Hydrogenosomes contain a great abundance of malic enzyme, which by oxidative decarboxylation of malate provides reducing power in the form of NADH (4), and a NADH:ferredoxin oxidoreductase. This enzyme is highly resistant phenotype is acquired only after malic enzyme and NADH:ferredoxin oxidoreductase activities are markedly reduced or completely disappear from the hydrogenosome to- and flavodoxin genes have subsequently been annotated during replacement machinery (10). Indeed, several other ferredoxin systems awaits experimental verification.

In conclusion, we have demonstrated the presence of a novel pathway of metronidazole reduction in T. vaginalis hydrogenosomes. Unlike the PFOR-dependent activity, where pyruvate is the source of electrons, the alternative pathway uses electrons released from malate in the form of NADH plus H⁺ by the action of malic enzyme. NADH dehydrogenase then recycles NADH by reducing ferredoxin, which provides electrons to metronidazole. Thus, ferredoxin plays a pivotal role in both pyruvate- and malate-dependent activations of metronidazole.

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REFERENCES


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