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Characterization of the NAD(P)H Oxidase and Metronidazole Reductase Activities of the RdxA Nitroreductase of *Helicobacter pylori*

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Abstract

Metronidazole (MTZ) is widely used in combination therapies against the human gastric pathogen *Helicobacter pylori*. Resistance to this drug is common among clinical isolates and results from loss of function mutations in *rdxA*, which encodes an oxygen insensitive nitroreductase (NTR). The RdxA-associated MTZ-reductase activity of *H. pylori* is lost upon cell disruption. Here we provide a mechanistic explanation for this phenomenon. Under aerobic conditions, His₆-tagged RdxA protein (purified from *Escherichia coli*), catalyzed NAD(P)H-dependent reductions of nitroaromatic and quinone substrates including: nitrofurazone, nitrofurantoin, furazolidone, CB1954, and 1,4 benzoquinone, but not MTZ. Unlike other NTRs, His₆-RdxA exhibited potent NAD(P)H oxidase activity ($k_{cat} = 2.8 \text{ s}^{-1}$) which suggested two possible explanations for the role of oxygen in MTZ reduction: 1) NAD(P)H oxidase activity promotes cellular hypoxia (nonspecific reduction of MTZ); and 2) molecular oxygen out-competes MTZ for reducing equivalents. The first hypothesis was eliminated upon finding that *rdxA* expression, while increasing MTZ toxicity in both *E. coli* and *H. pylori* constructs, did not increase paraquat toxicity even though both are of similar redox potential. The second hypothesis was confirmed by demonstrating NAD(P)H-dependent MTZ-reductase activity (apparent $K_m = 122 \pm 58 \text{ }\mu\text{M}$, $k_{cat} = 0.24 \text{ s}^{-1}$) under strictly anaerobic conditions. The MTZ reductase activity of RdxA was 60-times greater than for NfsB (*E. coli* NTR), but 10 times lower than the NADPH-oxidase activity. Whether molecular oxygen directly competes with MTZ or alters the redox state of the FMN cofactors is discussed.

Keywords

Metronidazole; flavoprotein; nitroreductase; NAD(P)H oxidase; *Helicobacter*

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Enzymes: RdxA (EC 1.6.3.1; EC 1.6.5.2)

INTRODUCTION

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] (see Fig. 1) and related 5-nitroimidazoles are redox-active prodrugs commonly used to treat infections caused by anaerobic bacteria and intestinal parasites[1]. In these organisms, MTZ is reduced (nitroreduction) to mutagenic and DNA-damaging hydroxylamine adducts by ferredoxin electron carriers associated with the pyruvate/ ketoacid: ferredoxin oxidoreductases (P/KFOR) (reviewed by[2]). Clinically significant MTZ resistance is rare in anaerobes because P/KFOR enzymes are essential components of core metabolism (reviewed by[3]). In contrast, MTZ resistance is common among clinical isolates of the gastric microaerophile *Helicobacter pylori*, especially those from geographic regions where MTZ usage is high[4]. *H. pylori* establish life long infections of the gastric mucosa of billions of people worldwide[5]. It is the major cause of peptic and duodenal ulcers and is a key risk factor in gastric cancer and MALT lymphoma. Drug resistance can be a major impediment to the success of MTZ-based eradication therapies[4].

Genetic tests revealed that MTZ resistance in *H. pylori* requires loss of function mutations in *rdxA* (HP0954) a nonessential gene encoding a ~26 kDa oxygen-insensitive nitroreductase[6]. Moreover, expression of *rdxA* in *E. coli*, which ordinarily is highly resistant to MTZ (MTZ^r), confers a MTZ^s phenotype. This correlates with dose-dependent increases in both mutation frequency and extent of DNA fragmentation, indicative of hydroxylamine production[7]. While sequential inactivation of additional *H. pylori* genes, including *frxA* (a second NTR), contributes to even higher levels of MTZ^r (up to 250 µg/ml), resistance is always contingent upon mutation of *rdxA* first[8-10]. Direct enzymatic reduction of MTZ has not been demonstrated with native purified RdxA or with any other native NTR in vitro. Experimental evidence suggests that nitroreduction capacity of NTRs depends on the midpoint reduction potential of the FMN cofactor ($E_m \sim -190$ mV and low range of -380 mV)[11]. MTZ ($E_m -485$ mV) is clearly outside this range. Substrates exhibiting the highest rates of nitroreduction (e.g., nitrofurans) are in the -250 mV E_m range, whereas rates for substrates exhibiting very low midpoint potentials are orders of magnitude lower[11].

NTRs apparently possess intrinsic MTZ-reductase activity, since over-expression of enteric NTRs (NfsB and Cnr) also increases susceptibility to the drug[12]. Clinically, NTRs are considered attractive targets for nitroimidazole-based intervention therapies, that in addition to anaerobic bacteria also includes pathogens like *Mycobacterium tuberculosis*[13]. More recently, MTZ and related drugs (CB1954) are included with NTRs in gene-prodrug applications, either as research tools to direct selective tissue ablation or in novel treatments for certain cancers[14-16]. Thus, a mechanistic understanding of how NTRs activate MTZ is fundamental to implementation of these new applications and to the development of new redox-active prodrugs.

Historically, comparative studies of MTZs and MTZ^r strains of *H. pylori* revealed a correlation between MTZ resistance and decreased NAD(P)H oxidase activity in cell-free extracts[17,18]. Others proposed that NAD(P)H oxidase activity generated cellular pockets of low redox potential sufficient to catalyze nitroreduction[17]. Two results were taken as support for this hypothesis: (i) MTZ reductase activity, but not nitrofurazone reductase activity, was lost following cell lysis[19]; and (ii) MTZ^r *H. pylori* strains became more susceptible to MTZ under anaerobic or low oxygen conditions[20,21]. However, this hypothesis seemed to be undermined by the discovery of RdxA, assuming that RdxA directly reduced MTZ, and also because in general, NTRs exhibit very weak NAD(P)H oxidase activity [6,11].

Here we report that the *H. pylori* RdxA does possess potent NAD(P)H oxidase activity, and that this accounts for much of the NAD(P)H oxidase activity previously reported in cellular extracts. However, this oxidase activity does not contribute significantly to cellular hypoxia or to nonspecific MTZ reduction. Rather, we find that NAD(P)H-dependent MTZ-reductase activity of RdxA requires strictly anaerobic conditions. We conclude that molecular oxygen is a potent inhibitor of MTZ reductase activity of NTRs under normal atmospheric conditions.

RESULTS

Purification and properties of recombinant *H. pylori* RdxA

Initial efforts to obtain recombinant His₆-tagged RdxA by over-expression in *E. coli* resulted in the accumulation of protein in inclusion bodies[22]. While refolding efforts occasionally resulted in active fractions, these activities were spurious since refolded monomeric RdxA lacked flavin cofactors. We found that lowering the growth temperature and inducer concentration (IPTG) allowed more time for proper protein folding, resulting in better yields of native N-terminal His₆-tagged RdxA from *E. coli*. Purified His₆-RdxA was yellow and had a typical flavoprotein optical spectrum (see Figure 2A). The flavin cofactor of RdxA was confirmed as FMN by TLC following protein denaturation (data not shown). The molecular weight of the His₆-RdxA monomer was estimated at ~26 kDa by SDS-PAGE as expected from the sequence. However, as seen in Fig 2B, the estimated mass by gel filtration was 44 ± 1 kDa (slightly less than 52 kDa), indicating that RdxA is a typical member of the NTR class of homodimeric flavoproteins. For all kinetic analyses, RdxA is treated as a dimer with a molecular mass of 52,500 Da.

Substrate specificity

In general, NTRs catalyze $4e^-$ reductions of the nitro groups of nitroaromatic compounds to hydroxylamine adducts by a ping pong mechanism (reviewed in[23]). Usually, molecular oxygen does not intervene in NTR catalyzed reduction of substrates because it cannot access to the reaction pocket[24]. Therefore, initially no effort was made to exclude molecular oxygen from the enzyme assays and the initial (baseline) velocities due to endogenous NAD(P)H oxidase activity were subtracted from the total measured velocity recorded for each substrate (Table 1). Purified His₆-RdxA catalyzed NAD(P)H-dependent reductions of a range of nitro compounds, cytochrome *c*, ferricyanide, and BQ. The specific activities (k_{cat}) for nitrofuran substrates of RdxA ranged from 0.27 s^{-1} for furazolidone to 2.9 s^{-1} for nitrofurazone which are comparable with activities measured for His₆-NfsB of *E. coli* (5 - and 17 s^{-1} , respectively). However, RdxA lacked any MTZ reductase activity, regardless of buffer system used (Tris or phosphate) or range of pH or temperature. Initial efforts to decrease molecular oxygen concentrations by sparging cuvettes with N₂ or H₂ gas prior to assay did not appear to restore MTZ reductase activity (data not presented).

The NAD(P)H oxidase activity of RdxA was 70 times greater than the oxidase activity measured for NfsB ($k_{cat} = 0.04\text{ s}^{-1}$) (see Table 1). We considered the possibility that the high efficiency of oxygen consumption by RdxA might reflect both one and two electron transfer reactions generating superoxide and hydrogen peroxide, respectively. If RdxA was capable of single electron transfers to MTZ in the presence of molecular oxygen, futile cycling between the nitro anion intermediate and MTZ would result (typical of oxygen sensitive NTRs), thus explaining the absence of MTZ reduction under aerobic conditions[23]. However, as shown in Table 2, 94% of the oxygen consumed in the NAD(P)H-dependent oxidase reaction was reduced to hydrogen peroxide, a $2e^-$ transfer reaction typical for NTRs. In the horseradish peroxidase o-dianisidine assay, o-dianisidine was not a substrate for RdxA (data not presented). Only trace amounts of superoxide anions

were generated in the reaction, as additions of SOD did not decrease the rate of cytochrome *c* reduction as measured spectrophotometrically. Thus, RdxA is typical of other NTRs in catalyzing $2e^-$ transfers from NAD(P)H to molecular oxygen and producing hydrogen peroxide as product[11,25].

Cytoplasmic hypoxia hypothesis

While our previous studies indicated that RdxA catalyzed the reduction of MTZ to hydroxylamine in vivo[6-9], this activity was paradoxically absent in cell-free extracts or with purified protein. Based on the absence of any direct evidence of in vitro reduction of MTZ by either RdxA or NfsB, we reevaluated whether NAD(P)H-oxidase activity of RdxA might uniquely contribute to cytoplasmic hypoxia through removal of molecular oxygen from the bacterial cytoplasm, and thus lower cellular reduction potentials to levels that would cause spontaneous MTZ reduction. We reasoned that if cellular redox was indeed as low as -485 mV, then molecules of similar oxidation reduction potential should be equally reduced. Paraquat (PQ, also called methyl viologen, $E_m \sim -450$ mV) is often used as a low redox-potential electron acceptor in anaerobic enzyme assays[26]. When reduced, PQ produces an intense blue color that can be readily measured between 546 - 600 nm. Moreover, PQ is reduced by single electron transfers, and its re-oxidation by molecular oxygen generates superoxide anions, the basis for its use in studies of oxidative stress[27]. If the hypoxia hypothesis is correct, RdxA-expressing bacteria should become more susceptible to killing by PQ than bacteria not expressing RdxA. NfsB, which is also involved in MTZ activation[28], but lacks appreciable NAD(P)H oxidase activity[11], was used for comparison. Results from disk diffusion assays showed that expression of RdxA or NfsB in *E. coli* BL21(DE3) increased their susceptibility to MTZ, but not to PQ (Fig. 3A). Similarly in *H. pylori* G27, the *rdxA::cat* mutant was clearly much more resistant to MTZ killing than the wild type (RdxA⁺) strain; yet, both were equally susceptible to PQ (Fig. 3B). While it is possible that RdxA might have rendered the cytoplasm sufficiently anaerobic as to prevent PQ oxidation, direct spectrophotometric assays of PQ reduction in whole *E. coli* cells tended to rule out this possibility. Thus, the apparent selective reduction of MTZ by RdxA-expressing cells was taken as evidence for RdxA-MTZ substrate specificity rather than nonspecific reduction due to local hypoxia as suggested by others[18-20].

Reduction of MTZ requires strict anaerobic conditions

We next focused on the possibility that molecular oxygen might be inhibitory to MTZ-reductase activity, either by direct competition or by causing changes to the flavin or protein that might affect substrate specificity. We had noted in previous studies of pyruvate:ferredoxin oxidoreductase activity that, despite sparging cuvettes with H₂ gas to remove dissolved oxygen, remaining traces of oxygen delayed the onset of measurable enzymatic activity by several minutes when compared to assays prepared under strict anaerobic (glove box) conditions[29]. To test this possibility for RdxA, we extended the reaction time to allow for the endogenous NAD(P)H oxidase activity of RdxA to consume residual traces of oxygen. Once the endogenous oxidase activity reached a plateau (~ 10 min), MTZ reductase activity was observed (see Table 3). While initial velocities could be calculated under these conditions, it was not possible to obtain kinetic constants, as much of the NAD(P)H was consumed in oxygen removal and the effects of product build up (e.g., peroxide and NADP) on enzyme activity could not be determined. In the absence of an anaerobic glove box, we employed a glucose oxidase/catalase oxygen scavenging system to remove residual oxygen from the reaction mixtures[30,31]. The anaerobic generation system yielded similar rates of MTZ reduction for RdxA, but more importantly, enabled measure of the much lower MTZ reductase activities of NfsB (see Fig. 4). No MTZ-reductase activity was detected in the absence of NTRs or NADPH. Similarly, no activity was observed if either of the oxygen-scavenging enzymes was left out of the system (until the oxidase

activity of RdxA had rendered the system anaerobic). Based on specific activities, RdxA was ~60-times more active than NfsB in reducing MTZ (Table 3), which is consistent with *in vivo* results[7]. Neither enzyme reduced PQ under these strict anaerobic conditions (data not shown), which indicated that the glucose oxidase/catalase system did not appreciably lower the redox potential of the reaction mixture. In addition, nitrofurazone ($E_m \sim -250$ mV) was not chemically reduced by the anaerobic generation system and the specific activities for RdxA with nitrofurazone were equivalent under both anaerobic and aerobic conditions (data not presented). We conclude that the anaerobic generating system does not interfere with measures of MTZ-reductase activity. These results showed that the *in vitro* reduction of MTZ by nitroreductases requires strict anaerobic conditions, such as would be present in the cytoplasm of intact bacteria.

Kinetic analysis of RdxA

The kinetic constants determined with MTZ and other substrates are presented in Table 4. The reduction of MTZ by RdxA displayed Michaelis-Menten kinetics, though the kinetic constants determined for MTZ and other substrates assume an infinite concentration of NADPH. The mean apparent K_m for MTZ of 122 ± 58 μ M (determined over a range of fixed NADPH concentrations) was in line with values reported with nitrofurazone for NfsB (~160 μ M) [25] and confirmed in this study (data not presented). Importantly, MTZ reduction by RdxA appeared to conform to a ping pong mechanism, based on constant k_{cat}/K_m rates determined over a range of NADPH substrate concentrations ($1.92 \pm 0.24 \times 10^3$ $\text{M}^{-1}\text{s}^{-1}$). When NAD(P)H substrate concentrations were >300 μ M, the K_m and k_{cat} values for RdxA grew progressively higher ($K_m \geq 360$ μ M and $k_{cat} \geq 0.62$ s^{-1} , respectively) even though the k_{cat}/K_m rate remained constant. Similar results have been reported for NfsB with nitrofurazone and NADPH[25].

Kinetic analysis of NADPH oxidase activity revealed a K_m for O_2 for RdxA of 57 ± 14 μ M and a k_{cat} of 2.4 ± 0.2 s^{-1} . To determine if MTZ could inhibit the NADPH oxidase component of RdxA, initial velocities for NADPH oxidase activity over a range of oxygen concentrations was determined at a fixed concentration of MTZ at 200 μ M. While not depicted, the Lineweaver-Burk plot of the initial velocities versus substrate concentrations (with and without inhibitor) was within experimental error (superimposed), indicating that MTZ had no measurable inhibitory effect on the NADPH oxidase activity of RdxA (see Table 4).

RdxA exhibited much lower K_m values for nitrofurazone, cytochrome *c*, and BQ than for MTZ (K_m 1.7 ± 0.36 -, 5 ± 0.2 -, and 26 ± 4 μ M, respectively). Specific activities for these substrates were also 10-50 fold higher than for MTZ (see Table 4) and these rates were unaffected by oxygen. RdxA was particularly active in reducing CB1954, a prodrug used in conjunction with NfsB in cancer chemotherapy[16]. The bimolecular rate constant (k_{cat}/K_m) of RdxA in reducing CB1954 (3×10^5 $\text{M}^{-1}\text{s}^{-1}$) was about 10 times greater than for NfsB (2.6×10^4 $\text{M}^{-1}\text{s}^{-1}$). These results indicate that RdxA is similar to other NTRs in catalyzing reductions of a wide range of substrates, but may be unique in its ability to efficiently activate MTZ and CB1954.

DISCUSSION

We have investigated the molecular basis for MTZ activation by the RdxA nitroreductase of *H. pylori*. Previous studies established that loss of function mutations in *rdxA* were both necessary and sufficient to produce clinically significant resistance to this drug[6]. However, direct enzymatic reduction of MTZ by native purified RdxA, or any native NTR, had not been previously demonstrated. Here we report that NAD(P)H-dependent MTZ-reductase activity for RdxA and for the *E. coli* NfsB NTR requires strictly anaerobic conditions. Based

on competitor studies, MTZ is not detectably bound by RdxA, even when only trace amounts of molecular oxygen are present. MTZ reduction by RdxA was consistent with a ping pong catalytic mechanism (2 moles of NAD(P)H oxidized per mole of MTZ reduced) with a K_{cat} of 0.24 s^{-1} and a K_m of $122 \text{ }\mu\text{M}$. While the k_{cat} of RdxA for MTZ is relatively low compared with other substrates, the MTZ reductase activity of RdxA was ~60 fold higher than for NfsB, which is consistent with differences in susceptibility between *E. coli* expressing RdxA and NfsB (Fig. 3A) and [32].

As a typical NTR, RdxA exhibited a broad substrate preference for nitroaromatic and quinone compounds. The kinetic results presented in Table 4 were calculated at substrate concentrations in which RdxA activities fit the classical Michaelis-Menten equation. Accordingly, the k_{cat}/K_m rate constants for the substrates tested showed that nitrofurazone, 1,4-benzoquinone and CB1954 were the preferred substrates for RdxA. Interestingly, the k_{cat}/K_m for RdxA in reducing anti-cancer prodrug CB1954 ($3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) was about 10 times greater than determined for NfsB. It is noteworthy that the NfsB-CB1954 system, which employs a virus/gene direct-enzyme prodrug therapy (GDEPT) approach, has reached phase III clinical trials [33,34]. Several biochemical properties may make RdxA more suitable than NfsB for a GDEPT approach, such as its 10-fold higher activity with CB1954 and 60-fold greater ability to reductively activate MTZ, which might be useful in combination therapies due to the intrinsic toxicity of CB1954.

While the effect of oxygen on MTZ reduction by RdxA may be attributable to its 20-fold greater reduction rate (k_{cat}/K_m) over MTZ, the underlying mechanism for inhibition is not known. It is possible that oxygen indirectly influences redox state of the protein and/or of the FMN cofactor as alteration of the charge distribution over the FMN by reduction is suggested to affect substrate binding and activity [30]. A simple competition model in which oxygen and MTZ compete for binding to RdxA was eliminated by inhibitor studies showing that MTZ did not inhibit NADPH oxidase activity (see Table 4). In contrast, with substrates like the nitrofurans, nitroreduction was catalyzed by RdxA in the presence of oxygen, necessitating correcting for the contribution of oxygen to the rate. The apparent absence of binding of MTZ by RdxA in the presence of oxygen may suggest that the FMN cofactor must first be reduced in order to permit MTZ binding. In this regard, Race et al [25] showed that nitrofurazone bound in a catalytically unfavorable orientation to the oxidized flavin of NfsB and in the proper orientation to the reduced FMN. Since the effect of oxygen on MTZ reductase activity appears to be common to NTRs in general, it is likely that the redox status of the FMN cofactors is important for both binding of MTZ and for the two successive $2e^-$ transfers to produce the hydroxyl amine product.

We had speculated in an earlier study that both pI and number of cysteine residues (6 in RdxA and 1 in NfsB and Cnr) might contribute to the ~60-fold higher rate for MTZ reduction by RdxA over other NTRs [6]. While cysteine residue 87 (MVVCS in RdxA and 85 in VVFCA of NfsB and Cnr) is within a highly conserved region, stoichiometric redox titrations of the enteric NTR found no evidence for participation of other redox groups including cysteine in FMN reduction [43]. We cannot rule out the possibility that three additional residues clustered in a conserved region of the C-terminal half of RdxA might influence protein structure and indirectly affect FMN reactivity. It should be noted that none of the cysteine residues of RdxA are in a CXXC motif (thioredoxin fold) that in some flavoenzymes such as lipoyl dehydrogenase and glutathione reductase contributes to redox activity [24]. Whether cysteine residues in RdxA influence substrate specificity of the FMN domains will require further study.

We conclude that most NAD(P)H oxidase activity of MTZ^s strains and not in MTZ^r strains [17,18] is due to RdxA function. However, the question of whether RdxA oxidase activity

contributed, if any, to lowering cellular redox was tested by comparing PQ toxicity to that of MTZ in both *H. pylori* and *E. coli* (RdxA⁺ versus RdxA⁻) matched strains. Since no differences in PQ toxicity were found between RdxA⁺ and RdxA⁻ strains, compared with dramatic differences with MTZ (see Figure 2B), we concluded that RdxA did not contribute to cellular hypoxia as had been proposed by others[17,18,20]. On the contrary, these results provided strong support for substrate specificity of RdxA for MTZ in vivo.

Given these findings, what is the physiological role of RdxA in microaerophiles such as *H. pylori*? First, why are *rdxA* mutants rendered susceptible to MTZ at lower oxygen tensions or when briefly incubated under anaerobic conditions? One possibility is that the efficiencies of other enzymes for activating MTZ might also improve under hypoxic conditions. These might include FrxA, a second NTR of *H. pylori* that also activates MTZ in our anaerobic system (data not presented, but similar to NfsB), and FqrB, a flavin quinone oxidoreductase that reduces flavodoxin in the presence of NADPH and whose over-expression in *E. coli* also increases MTZ susceptibility[29,32]. The concept that many redox-active proteins can contribute to MTZ susceptibility is illustrated by studies by Albert et al [10], who used a hybridization-based comparative-genome sequencing strategy (NimbleGen) to map mutations to many additional genes of *H. pylori* that are associated with very high levels of resistance. Thus, the microaerophiles, like the anaerobes must contain redox-active enzymes and other cellular components that can activate MTZ under hypoxic conditions. Second, we considered what the role of RdxA might be under conditions of oxidative stress as might be encountered in highly inflamed tissue. Ordinarily, the respiratory chain of bacteria sufficiently reduces oxygen at the internal surface of the cytoplasmic membrane to limit the diffusion of oxygen into the anaerobic cytoplasm. In *E. coli*, oxidative stress (e.g., hydrogen peroxide) raises the redox potential above -180 mV, activating OxyR and SoxR that respond in turn by activating oxidative defense genes[35]. Since *H. pylori* lacks these regulatory proteins and has a rather inefficient respiratory system, perhaps RdxA and many other flavoproteins provide a compensatory function by removing molecular oxygen in the form of hydrogen peroxide. Ordinarily, accumulation of hydrogen peroxide in the cytosol would be considered deleterious, but in the case of *H. pylori*, its alkyl hydroperoxide reductase is particularly efficient in scavenging hydrogen peroxide[36]. We and others have noted that AhpC provides an essential function under higher oxygen conditions, by reducing cytosolic peroxides to water[36,37]. Thus, RdxA and AhpC might represent additional examples of functional adaptations in Epsilon proteobacteria that enable them to efficiently remove transient concentrations of molecular oxygen in the absence of global regulators of oxidative stress.

In summary, we have purified and characterized the RdxA nitroreductase of *H. pylori* and defined conditions under which this enzyme and other NTRs can be directly assessed for MTZ-reductase activity. The activity of NTRs is highly susceptible to molecular oxygen regardless of the level of oxidase activity. As NTRs are increasingly being used in gene therapy systems with MTZ and other prodrugs, it is likely that enzymes can be tailored to more efficiently activate these drugs under conditions that favor hypoxia and new redox active drugs can now be more efficiently screened in vitro. Future mechanistic and crystallographic studies of RdxA will likely contribute to protein engineering strategies to improve catalytic efficiency with MTZ and other redox-active drugs.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains used in this study include *E. coli* BL21CodonPlus(DE3)-RIL (Novagen, Inc.) and *H. pylori* strains G27 and SS1 (laboratory collection). The G27 *rdxA::cat* mutant was constructed as previously described[6]. *E. coli* strains were routinely grown in LB

medium [38] at 37°C. *H. pylori* strains were grown under humid microaerobic conditions at 37°C on Brucella-based medium supplemented with 7.5 % newborn calf serum (Gibco Laboratories) and antimicrobials as previously described[8].

Plasmid construction

DNA isolation and all recombinant DNA manipulations were carried out using standard methods[38]. Plasmid pET15b-rdxA5 in which the *rdxA* gene is expressed under the control of the T7 promoter, was constructed by cloning the PCR-amplified *rdxA* genes from *H. pylori* SS1 chromosomal DNA into vector pET15b (Novagen, Inc.). The following primers were used to create PCR fragments flanked by NdeI - BamHI sites, with restriction sites underlined: 5RdxA_NdeI (5'-GGGAATTCCATATGGAATTTTGGATCAAG) and 3RdxA_BamHI (5'-CGCGGATCCCTACAACCAAGTAATCGCATC). This construct allowed overexpression of the RdxA protein with N-terminal His₆ tag extensions to facilitate its purification. All DNA inserts were verified by automated DNA sequencing at the Biomolecular Research Facility at the University of Virginia School of Medicine.

Over-expression and purification of native RdxA and NfsB

The plasmids pET15b-rdxA5 and pET29b-nfsB (laboratory collection) were introduced into *E. coli* BL21CodonPlus(DE3)-RIL by transformation. Cells were then grown in LB broth supplemented with ampicillin (500 µg/ml) or kanamycin (20 µg/ml) at 37°C, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added in early log phase. The temperature was lowered to 20°C and the cells were allowed to grow overnight. The N-terminal His₆-tagged RdxA, and C-terminal His₆-tagged NfsB proteins were purified from cell extracts by Ni²⁺ affinity chromatography as described in the Novagen standard protocol. In brief, cells grown in 1 liter of LB were washed and suspended in 15 ml of binding buffer (50 mM NaPO₄, pH 8.0, 0.3 M NaCl, 10 mM imidazole, 12 mM β-mercaptoethanol, and 10% [vol/vol] glycerol) and disrupted by sonication for three 3-min periods, with 10-min intervals for cooling. Unbroken cells and particulate material were removed by centrifugation at 20,000 × g for 30 min at 4°C, and the cell extract was applied onto an HIS-Select Nickel Affinity Gel (Sigma) column with 1 ml of resin volume. The resin was washed with 8 volumes of wash buffer (binding buffer + 10 mM imidazole), and adsorbed His-tagged proteins were eluted with 5 ml of binding buffer + 100 mM imidazole. Eluted proteins were dialyzed against 2 liters of storage buffer (50 mM NaPO₄, pH 8.0, 0.3 M NaCl, 1 mM dithiothreitol (DTT), and 10% [vol/vol] glycerol), and stored at 4°C. Yields of RdxA proteins were in the range of 5 to 12 mg per liter of culture, NfsB - up to 30 mg per liter of culture, and a purity of 90 to 95% was estimated by Coomassie brilliant blue staining following sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Spectral analysis of RdxA and cofactor determination

The optical spectrum of RdxA nitroreductase was measured between 250 and 650 nm in a modified Cary-14 spectrophotometer (OLIS Instruments Co, Bogart, Ga). The flavin cofactors were identified by thin-layer chromatography (TLC) following protein denaturation at 70°C[39].

Gel filtration

Gel filtration was carried out in 0.8-×-45 cm column of Sephadex G-100 (Pharmacia). The Sephadex G-100 column was equilibrated in 0.1 M NaPO₄ (pH 8.0), 1 mM DTT buffer at a flow rate of 0.2 ml/min. The standard proteins used for the estimation of molecular mass included bovine albumin (66 kDa), carbonic anhydrase (31 kDa), and horse heart cytochrome C (12.4 kDa). The elution profiles were monitored by UV absorption at 280 nm (BioLogic DuoFlow, Bio-Rad Laboratories).

Disk assay for sensitivity to metronidazole and paraquat

LB or BA plates were uniformly streaked with 0.1 ml of cell suspensions adjusted to the OD₆₀₀ of 0.01 - 0.1. Sterile 7 mm filter paper disks saturated with 7 µl of MTZ (20 mg/ml) or PQ (20 mM) were placed onto the plates. The cells were incubated for 24 h (*E. coli*) or 72 h (*H. pylori*) before the zones of the inhibition were measured[40].

Steady-state kinetic experiments

Specific activity measurements were performed in 1-cm-path-length quartz cuvettes in buffer A [10 mM Tris-HCl pH 7.5 containing NAD(P)H (6 - 420 µM) and appropriate substrate (6 - 300 µM)]. The reactions were initiated by addition of an appropriate dilution of enzyme to a final reaction volume of 1 ml. The enzymatic activities were monitored spectrophotometrically in an OLIS Cary-14 spectrophotometer at 23°C, by following the decrease in absorbance at 340 nm resulting from the oxidation of NAD(P)H ($\epsilon=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Substrates assayed by following the oxidation of NAD(P)H included 1,4-benzoquinone, 2,4 dinitrotoluene, 3,5 dinitrobenzoate, methyl 4-nitrobenzoate, and oxygen. Corrections were used where reduced products absorbed in the 340 nm range. In addition, the reduction of various substrates was also monitored at appropriate wavelengths, which include the following: nitrofurazone, 400 nm ($\epsilon=12.6 \text{ mM}^{-1} \text{ cm}^{-1}$); nitrofurantoin, 405 nm ($\epsilon=12.1 \text{ mM}^{-1} \text{ cm}^{-1}$); furazolidone, 400 nm ($\epsilon=18.8 \text{ mM}^{-1} \text{ cm}^{-1}$); metronidazole, 320 nm ($\epsilon=9.0 \text{ mM}^{-1} \text{ cm}^{-1}$); horse heart cytochrome C, 550 nm ($\epsilon=18.9 \text{ mM}^{-1} \text{ cm}^{-1}$); CB1954, 420 nm ($\epsilon=1.2 \text{ mM}^{-1} \text{ cm}^{-1}$); and ferricyanide, 535 nm ($\epsilon=10.8 \text{ mM}^{-1} \text{ cm}^{-1}$). Kinetic constants (K_m and k_{cat}) were determined from plots of initial velocity versus substrate concentration by nonlinear regression analysis using the Prism 4 (GraphPad Software, Inc). Values for k_{cat} (s^{-1}) were estimated using the predicted dimeric molecular mass of 52,500 and 50,420 Da for N-terminally His₆-tagged RdxA and NfsB proteins, respectively. Initial-velocity kinetic assays were performed in triplicate. The reported error is a standard deviation.

NAD(P)H oxidase assays

NAD(P)H oxidase activity was measured with an oxygen electrode (Yellow Springs Instrument Company, Silver Springs, Ohio) inserted in a water-jacketed Gilson chamber as described previously[41]. Polarographic measurements were performed at 25°C using air saturated buffer A (~260 µM oxygen). To determine kinetic constants for oxygen, degassed anaerobic buffer was added to the 1.35 ml chamber in the presence of 300 µM NADPH and 28 µg of His₆-RdxA protein. Following equilibration (2 min), measured concentrations of air saturated buffer were injected into the chamber through the glass capillary bore stopper and initial velocities of oxygen consumption were recorded and the data fitted to the Michaelis Menten equation. Inhibitor effects of MTZ on NAD(P)H oxidase activity were determined at a fixed concentration of MTZ (250 µM). All experiments were performed in triplicate and reported as mean and standard deviation.

Determination of products of NADPH oxidase

NADPH oxidase activity was determined aerobically by following the oxidation of NADPH at 340 nm. The reaction mixture in buffer A contained 300 µM NADH and His₆-RdxA (7.2 µg/ml of protein) and the reaction was started by the addition of protein. To assess superoxide anion participation in the reaction, cytochrome c (30 µM) and 100 units of superoxide dismutase (SOD) were added to the reaction mixture. The difference in the rates of cytochrome c reduction in the presence and absence of SOD was used to compute the rate of superoxide anion generation as previously described[29]. The production of hydrogen peroxide from NADPH oxidase activity was monitored by the addition of 30 ng/ml horseradish peroxidase and 80 µg/ml o-dianisidine to the reaction previously described[42].

Metronidazole reduction assay

The reduction of MTZ was determined under anaerobic conditions by either allowing the NADPH-oxidase activity of RdxA to render the contents of the cuvette anaerobic or by employing a glucose oxidase/catalase system to generate anaerobic conditions prior to addition of RdxA[30,31]. In the former method, all buffers were degassed and sparged with hydrogen; however an additional 10 min was required to enable RdxA oxidase activity to remove remaining oxygen. In this assay, the blank cuvette contained buffer A. For the anaerobic generation method, the standard assay mixture contained 25 mM glucose, 6 units/ml glucose oxidase (Sigma), 6 units/ml catalase (Sigma), NAD(P)H (18 - 300 μ M), MTZ (0 - 150 μ M) in 10 mM Tris-HCl pH 7.5 buffer in 1-cm-path-length quartz screw-capped cuvettes, at 23°C. The blank cuvette did not contain MTZ. After 5 min of initial glucose oxidase/catalase reaction, the anaerobic reduction of MTZ was initiated by addition of an appropriate dilution of RdxA enzyme to both experimental and blank cuvettes. The enzymatic activities were monitored spectrophotometrically at 23°C, by following the decrease in absorbance at 320 nm resulting from reduction of MTZ ($\epsilon_{320}=9.0 \text{ mM}^{-1} \text{ cm}^{-1}$) and oxidation of NAD(P)H ($\epsilon_{320}=4.5 \text{ mM}^{-1} \text{ cm}^{-1}$). The contribution of NADPH oxidation to the reaction (~50% of the initial velocity measured) was corrected for by adjusting the extinction coefficient to reflect 2 moles of NAD(P)H oxidized per mole of MTZ reduced ($\epsilon_{\text{MTZ}} + 2\epsilon_{\text{NAD(P)H}} = 18.0 \text{ mM}^{-1} \text{ cm}^{-1}$).

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Abbreviations

MTZ	metronidazole
NTR	nitroreductase
BQ	1,4-Benzoquinone
PQ	paraquat
SOD	superoxide dismutase

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Figure 1.
Chemical structure of metronidazole.

Figure 2.

A. Optical spectrum of RdxA. Spectra were collected at a protein concentration of 1.5 mg/ml in 50 mM NaPO₄, pH 8.0, 0.3 M NaCl, 1 mM DTT, and 10% [vol/vol] glycerol. The RdxA spectrum has peaks at 460, 365, and 275 nm. B. Gel filtration showing molecular mass of RdxA was carried out in 0.8 × 45 cm column of Sephadex G-100 equilibrated in 0.1 M NaPO₄ (pH 8.0), 1 mM DTT buffer at a flow rate of 0.2 ml/min. The standard proteins used for the estimation of molecular mass included bovine albumin (66 kDa), carbonic anhydrase (31 kDa), and horse heart cytochrome *c* (12.4 kDa). The elution profiles were monitored by UV absorption at 280 nm.

Figure 3.

Disk diffusion assays for metronidazole and paraquat sensitivity. LB (A) or BA (B) plates were uniformly streaked with 0.1 ml of *E. coli* (A) or *H. pylori* (B). Bacterial suspensions were adjusted to an OD₆₀₀ of 0.01 - 0.1. Sterile 7 mm filter paper disks saturated with 7 μ l of MTZ (20 mg/ml) or PQ (20 mM) were placed onto the plates. The plates were incubated for 24 h (*E. coli*) or 72 h (*H. pylori*) before the zones of the inhibition were measured. Three replicates were performed for each experiment.

Figure 4.

Metronidazole reduction assay. MTZ reductase activity was determined under anaerobic conditions as described in the text. The enzymatic activities were monitored spectrophotometrically at 23°C, by following the decrease in absorbance at 320 nm resulting from reduction of MTZ ($\epsilon=9.0 \text{ mM}^{-1} \text{ cm}^{-1}$) and oxidation of NAD(P)H ($\epsilon=4.5 \text{ mM}^{-1} \text{ cm}^{-1}$). Results reported for MTZ reduction are corrected for the contribution of NAD(P)H oxidation using a combined molar extinction coefficient ($\epsilon_{\text{MTZ}} + 2\epsilon_{\text{NAD(P)H}} = 18.0 \text{ mM}^{-1} \text{ cm}^{-1}$). No activity was observed in the absence of enzyme or NAD(P)H. Three replicates were performed for each experiment.

Table 1Electron acceptor specificities of RdxA under aerobic conditions^a.

Electron acceptors	$\mu\text{M Substrate s}^{-1}\mu\text{M Protein}^{-1}$	
	NADH	NADPH
CB1954 (100 μM)	3.6	3.5
Nitrofurazone (24 μM)	2.7	2.9
Nitrofurantoin (19 μM)	1.3	1.6
2,4-Dinitrotoluene (60 μM)	1.9	2.5
3,5-Dinitrobenzamide (60 μM)	3.9	6.0
Methyl 4-nitrobenzoate (60 μM)	2.5	3.3
Furazolidone (8 μM)	0.27	0.29
Cytochrome c (60 μM)	1.2	2.5
Ferricyanide (300 μM)	2.4	2.5
1,4-Benzoquinone (300 μM)	3.4	1.8
Oxygen	2.8	2.5
Metronidazole (45 μM)	<0.001	<0.001

^a Conditions: 23°C; 10 mM Tris HCl pH 7.5; 60 μM NAD(P)H. The activities presented means of at least 3 determinations per substrate.

Table 2

Oxygen reduction by RdxA

Reaction	k_{cat} (s ⁻¹)
NADPH oxidation	2.8 ± 0.1
H ₂ O ₂ production	2.6 ± 0.01
Superoxide production	< 0.09

Table 3Enzymatic reduction of metronidazole in the absence of oxygen ($k_{\text{cat}}(\text{s}^{-1})$)

	NADH	NADPH
RdxA	0.054 ± 0.001	0.063 ± 0.001
NfsB	8.2×10^{-4}	1.2×10^{-4}

Table 4

Kinetic parameters of RdxA and NfsB

Variable substrate	Fixed substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
<u>RdxA</u>				
Metronidazole ^a	NADPH	122 ± 58	0.22 ± 0.06	2.1×10^3
Metronidazole ^b	NADPH (300 μM)	363 ± 109	0.62 ± 0.12	1.8×10^3
Oxygen (0 - 250 μM) ^c	NADPH (430 μM)	57 ± 14	2.4 ± 0.2	4.2×10^4
Oxygen (0 - 250 μM) ^d	NADPH (430 μM)	38 ± 8	2.6 ± 0.4	6.8×10^4
Nitrofurazone (0 - 24 μM)	NADPH (30 μM)	1.7 ± 0.36	2.4 ± 0.4	1.4×10^6
1,4-benzoquinone (0 - 90 μM)	NADPH (540 μM)	26 ± 4	19.4 ± 1.6	8×10^5
CB1954 (0 - 90 μM)	NADPH (90 μM)	35 ± 8	10.4 ± 0.1	3×10^5
NADPH (0 - 90 μM)	CB1954 (90 μM)	89 ± 34	14.0 ± 0.3	1.6×10^5
Cytochrome c (0 - 60 μM)	NADPH (60 μM)	5 ± 0.2	0.54 ± 0.02	1.1×10^5
NADPH (0 - 60 μM)	Cytochrome c (60 μM)	5.5 ± 0.7	0.54 ± 0.02	1×10^5
<u>NfsB</u>				
CB1954 (0 - 60 μM)	NADPH (60 μM)	79 ± 22	2.0 ± 0.2	2.6×10^4

^a Spectrophotometric assay using anaerobic generation system. Data presented represent the mean and standard deviation of 3 independent experiments with MTZ as variable substrate (0 – 120 μM) at each of three fixed NADPH concentrations (30, 60 and 90 μM).

^b Spectrophotometric assay using anaerobic generation system, with MTZ as variable substrate (0-150 μM).

^c Polarographic assay.

^d Polarographic competition assay (200 μM MTZ).