Structure of RdxA – an oxygen-insensitive nitroreductase essential for metronidazole activation in Helicobacter pylori

Marta Martínez-Julve‡, Adriana L. Rojas‡, Igor Olekhnovich, Vladimir Espinosa Angarica, Paul S. Hoffman and Javier Sancho

1 Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Spain
2 Biocomputation and Complex Systems Physics Institute (BIFI) – Joint Unit BIFI-IQFR (CSIC), Universidad de Zaragoza, Spain
3 Structural Biology Unit, CIC bioGUNE, Bizkaia Technology Park, Derio, Spain
4 Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia Health System, Charlottesville, VA, USA

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Correspondence
P. S. Hoffman or J. Sancho, Department of Medicine, Division of Infectious Diseases and International Health, University of Virginia Health System, Charlottesville, VA 22908, USA; Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, 50009 Zaragoza, Spain
Fax: +434 982 4047; +34 976762123
Tel: +434 924 2893; +34 976761286
E-mail: psh2n@virginia.edu; jsancho@unizar.es

*These authors contributed equally to this work

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The RdxA oxygen-insensitive nitroreductase of the human gastric pathogen Helicobacter pylori is responsible for the susceptibility of this organism to the redox active prodrug metronidazole [2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol]. Loss-of-function mutations in rdxA are primarily responsible for resistance to this therapeutic. RdxA exhibits potent NADPH oxidase activity under aerobic conditions and metronidazole reductase activity under strictly anaerobic conditions. In the present study, we report the crystal structure of RdxA, which is a homodimer exhibiting domain swapping and containing two molecules of FMN bound at the dimer interface. We have found a gap between the side chain of Tyr47 and the isoalloxazine ring of FMN that appears to be appropriate for substrate binding. The structure does not include residues 97–128, which correspond to a locally unstable part of the NTR from Escherichia coli, and might be involved in cofactor binding. Comparison of H. pylori RdxA with other oxidoreductases of known structure suggests that RdxA may belong to a new subgroup of oxidoreductases in which a cysteine side chain close to the FMN cofactor could be involved in the reductive activity. In this respect, the mutation of C159 to A or S (C159A/S) has resulted in a loss of metronidazole reductase activity but not NADPH oxidase activity. The RdxA structure enables the interpretation of the many loss-of-function mutations described previously, including those affecting C159, a residue whose interaction with FMN is required for the nitrreduction of metronidazole. The present studies provide unique insights into the redox behaviour of the flavin in this key enzyme for metronidazole activation, including a potential use in gene therapy.

Database
Structural data have been deposited in the Protein Data Bank under accession number 3QDL.

Abbreviations
ACN, acetonitrile; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; MR, molecular replacement; MTZ, metronidazole [2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol]; NTR, nitroreductase; PDB, Protein Data Bank; TFA, trifluoroacetic acid.
Introduction

Nitroreductases (NTR) catalyze the reduction of nitro groups of structurally diverse aromatic and heterocyclic compounds, including nitrotoluens, nitrofurans and nitroimidazoles [1,2]. These enzymes can be subdivided into two general classes based on one- or two-electron transfer mechanisms [3,4]. Enzymes of the oxygen-sensitive class, represented by cytochrome P450 reductase and xanthine dehydrogenase, catalyze one-electron transfers (nitro anion) that, in the presence of molecular oxygen, produce superoxide anions [3]. Enzymes of the oxygen-insensitive class, represented by the homodimeric NAD(P)H/FMN NTRs commonly found in bacteria, catalyze a two-step, 4e\(^{-}\) reduction of the nitro group, producing DNA damaging and mutagenic hydroxylamine adducts [3–6]. These NTRs, represented by NfsB of Escherichia coli and RdxA of Helicobacter pylori, also catalyze the reduction of molecular oxygen in a two-electron transfer that produces hydrogen peroxide [7].

Studies performed in vitro indicate that NTRs differ widely in substrate preference, with enteric NTRs reducing prodrugs such as nitrofurans but not substrates of a much lower redox potential such as metronidazole [MTZ; 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol] [1]. Thus, enteric bacteria are generally resistant to the action of MTZ unless the NTR genes are overexpressed. For example, in the Salmonella typhimurium strains used in Ames testing, the overexpression of NfsB results in a higher mutation frequency, which is attributed to the increased reduction of MTZ [8]. By contrast, the RdxA NTR of H. pylori efficiently reduces MTZ and is the major activating enzyme in this species [5,7]. Direct enzyme assays performed under strictly anaerobic conditions, which simulate the highly anaerobic bacterial cytoplasm, have established that the MTZ reductase activity of RdxA is 60-fold higher than the rate measured for the NfsB of E. coli, as well as for FrxA, a second NTR of H. pylori [7]. Expression of RdxA in other bacteria that are not otherwise susceptible to MTZ renders them susceptible [5,7]. In allelic exchange bacterial genetic studies, plasmid borne rdxA serves as a suicide gene that can be selected against with MTZ (i.e. loss of plasmid vector) [9,10]. NTRs have been employed in adenovirus prodrug gene therapy systems to activate prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) in the treatment of prostate cancer [11]. Previous studies show that RdxA is 10-fold more efficient than NfsB in the activation of CB1954 [7] and might be amenable to protein engineering for the development of a more efficient enzyme.

The differences in nitroreduction capacity of an NTR likely depend on the midpoint reduction potential of the FMN cofactor \(E_m\) of approximately 190 mV and a low range of \(-380 \text{ mV}\) [12]. MTZ \(E_m\) \(-486\) [13] is clearly outside this range, and thus RdxA appears to be unique among the NTRs with respect to its capacity to reduce this prodrug. However, in other flavoproteins, such as flavodoxins, hydrophobic or negatively-charged amino acids can contribute to even lower redox potentials [14]. Studies conducted by Olekhnovich et al. [7] determined that RdxA was also a potent NADPH oxidase that reduced molecular oxygen to hydrogen peroxide. The same studies also revealed that trace amounts of oxygen in the enzyme assay mixture were sufficient to inhibit the MTZ reductase activity of RdxA [7]. In the original study on RdxA [5], it was noted that the pI for RdxA was approximately 8 and also that the protein contained six cysteine residues, whereas the NTRs of enteric bacteria generally have one or two cysteine residues and more acidic pI values (pI 5.4–5.6). It is reasonable to consider the possibility that amino acid composition and, in particular, the cysteine residues influence the local redox range of the FMN cofactor with respect to enabling the more efficient reduction of MTZ. Removal of molecular oxygen might allow the reduction of cysteine thiols and also the accompanying conformational changes required to lower the redox potential of FMN. To address the mechanistic questions of aerobic and anaerobic substrate specificity, we have solved the crystal structure of RdxA and used site-directed mutagenesis of a uniquely positioned cysteine residue near the FMN to establish the requirement for the anaerobic reduction of MTZ.

Results and Discussion

Overall structure of RdxA from H. pylori

Two functional homodimers are present in the asymmetric unit of the tetragonal crystals. Each monomer consists of 210 amino acids folded in an \(\alpha + \beta\) motif (Fig. 1) containing eight \(\alpha\)-helices and five \(\beta\)-strands. The dimer exhibits domain swapping and is organized into two folding domains. The central core of each domain is formed by a five-stranded sheet (\(\beta5\beta\beta\beta\beta\)) composed of four antiparallel strands from one monomer (strands \(\beta1\beta2\beta4\beta3\)) plus strand \(\beta5\) from the other monomer. Surrounding this sheet, there are three small \(\alpha\)-helices on one side \((\alpha3, \alpha4\) and \(\alpha7\) and two larger \(\alpha\)-helices on the other \((\alpha2\) and \(\alpha6\). Helices \(\alpha2\) and \(\alpha6\) together with \(\alpha1\), packed perpendicularly onto the C-terminus of helix \(\alpha6\) form the dimer interface. Helix
Additionally packs onto helix α2 and α6 of the other monomer. The C-terminal, comprising residues 198–210 of one monomer containing the small α8 and β5, surround part of the other monomer where they contact α2 and β1. Besides the two protein chains, there are two FMN molecules inserted in the interface of the dimer with their re faces accessible to the solvent. Each FMN is surrounded by the β3 strand and the loop connecting helices α1 and α2 from one monomer, plus the loop connecting helix α2 and the β1 strand from the other monomer. In the structure, no electronic density could be observed for residues 97–128 (chain A) and 90–133 (chain B).

Using the DALI server [15], the structure of RdxA has been aligned and compared with other homologous structures of NTRs showing no more than 29% sequence identity (Fig. 2). The alignment shown in Fig. 2 does not contain residues 97–128 of RdxA, nor the presumably equivalent residues in the other proteins. The homologous reductases belong to two distinct groups: NTRs and flavin reductases. The closest structural homologue of RdxA is the NTR from Streptococcus mutans [Protein Data Bank (PDB code: 3GAG)]. The other enzymes exhibiting high structural homology are also NTRs. Nevertheless, the Staphylococcus epidermidis FMN:FAD reductase presents more similarity in structure than some prokaryotic NTRs, including that of E. coli.

**FMN cofactor binding site**

The RdxA dimer contains two molecules of FMN. For each FMN, all the residues interacting with the phosphate moiety are located in the same monomer, whereas the isoalloxazine and the ribityl moieties are bound at the dimer interface and form hydrogen bonds and hydrophobic interactions with residues of both monomers (Fig. 3).

At the si face, the Ile160 O and side-chain atoms are packed onto the isoalloxazine ring of FMN, with the O atom located at 3.2 Å from N5 and N10. The position equivalent to that of Ile160 is occupied in most of the homologous reductases by a Pro residue (Fig. 2), with the exception of the NTR from S. pneumoniae, as well as FPase I, which bears Ile and Thr residues, respectively. In all cases, these residues form stacking contacts with the isoalloxazine ring. Also noticeable at the si face is the presence of Cys159, whose S atom points toward the flavin and is located at 3.6–5.2 Å from N1 (depending on the chain). At the re face, the side chain of Tyr47 from the other monomer lays parallel on the isoalloxazine ring (closest distance of 6.0 Å) and appears appropriately located to allow the intercalation of substrates, as observed in other flavoenzymes [16,17]. The equivalent position in the aligned NTR (Fig. 2) is not conserved.

The O2N3O4 edge of the isoalloxazine is stabilized by side-chain hydrogen bonds between Lys20 and O2, and between Asn73 and N3 and O4. In other reductase sequences, the equivalent position is occupied by Asn or Gln. The FMN O4 atom additionally forms a hydrogen bond with Gly163, whereas N5 is hydrogen bound to Gly162. Gly163 is highly conserved in NTRs and flavin reductases (Fig. 2), where its small volume has been proposed to allow the entrance of substrate.
in the space over the re face of the flavin [18]. At the other edge of the isoalloxazine ring, Ile142 contacts one of the methyl groups.

The FMN ribityl forms hydrogen bonds with OG of Ser18 of the same monomer, with the main chain of Ser45 of the other monomer and with two water molecules. Finally, the phosphoryl group is tightly bound to the structure through contact with the side chains of several residues of the same monomer (Arg16, Ser18 Lys198 and Arg200), plus hydrogen bonds with the N atom of Ser18 and a water molecule.

The missing region of RdxA

The absence of electron density for residues 97–128 (chain A) and 90–133 (chain B) is very likely a result of proteolysis during purification. MS data corresponding to dissolved RdxA crystals show a main peak at 12174 kDa, with two additional components at 12370 and 12530 kDa. Possible H2 peaks appear at 6086 and 6182 kDa. The main peak thus corresponds to approximately half the mass of the RdxA protein (24067 kDa according to the sequence). This indicates that proteolysis of the protein sample has taken place. However, a sample of the same protein preparation that was incubated with NADP+ (and did not crystallize under the same conditions) was shown by SDS/PAGE to have a mass of 26313 ± 10 Da, which is close to the theoretical mass of the protein. The missing segments are occupied by helices F and G in the search model: the NTR of E. coli [19]. In this NTR, helix F is part of a solvent-exposed channel at the dimer interface where FMN lies and helix G is assumed to convey substrate specificity. Helices F and G exhibit high B values in the structure, and it has been proposed that the mobility of helix F might be important for optimal binding and catalysis [20]. We have recently developed a method to identify locally unstable regions of proteins using computed polarity and packing density profiles of protein buried interfaces. The method successfully identifies the unstable segments of proteins for which structural information of partly unfolded conformations has been reported [21]. The method is based on the analysis of PDB files and therefore cannot be used directly to characterize the missing region in RdxA because no coordinates are available for this region. However, it can provide useful insight into this missing region from an analysis of the corresponding region of the search model. The polarity profile of the search model is displayed in Fig. 4. The more unstable region of E. coli NTR, characterized by its high content of buried polar area, encompasses residues A89–M139, corresponding to residues P91.

Fig. 2. (A) Structure-based sequence alignment of homologous prokaryotic reductases. Secondary structure for RdxA was assigned by the DSSP server [39] and is shown above its sequence. The parentheses indicate the position of missing P97–S128 residues. (B) Alignment of the H97–S128 region in RdxA with the corresponding sequences in other NTRs. This alignment was performed using CLUSTALW [40].
and I136 of *H. pylori* RdxA, which crudely mimics the missing segment of the structure. This suggests that the missing region in the structure of RdxA will also be locally unstable and more exposed to proteolysis. The proposal that helix F of *E. coli* NTR may exhibit high functional mobility appears to be extended to the equivalent helix of RdxA and also to helix G. Our observation that NADP⁺ protects RdxA against proteolytic removal of the missing segment agrees with the role assigned to helix F of *E. coli* NTR with respect to binding this cofactor [20].

**Classification of MTZ resistance mutations in RdxA based on their potential impact on RdxA stability, dimerization, FMN binding or enzyme activity**

MTZ resistance strongly correlates with mutations in RdxA [5, 22]. The structure of RdxA allows the impact of those mutations to be assessed and classified at the protein level. Mendz and Megraud [22] provided a compilation of mutations in RdxA, including those resulting in a loss of function reported by many laboratories, which we have grouped into five classes. Class I mutations are those expected to reduce the affinity of the apoprotein for the FMN cofactor. As explained above, RdxA binds FMN by a combination of hydrogen bonds and electrostatic and hydrophobic interactions. Thermodynamic analysis of the energetics of FMN binding in a model flavoprotein [23] indicates that point mutations of FMN binding side chains can significantly decrease the strength of the binding. In RdxA, mutations such as those affecting residues R16, S18, K20, N73, I142, G162 and K200 involved in FMN binding (see above) are expected to decrease the affinity of the apoprotein for FMN, which may be detrimental for function.

Class II mutations are those expected to destabilize dimer formation. RdxA is a tight dimer and the monomer is expected to be conformationally unstable. Thus, the mutation of residues that appear at the dimer interface, such as L42, S43, R41, Q50, V55, M56, I142, G145, K202 and L209, are expected to destabilize the protein. Class III includes mutations of residues not expected to be crucial for FMN binding or for dimerization (as judged from the structure) but potentially associated with the redox function of the enzyme, given their location near the FMN redox centre and their chemical properties. Examples include C19, Y47 and C159. Class IV comprises mutations expected to cause protein destabilization unrelated to dimerization. Obvious examples are the mutations of the buried G149 or those of H17, a residue that is hydrogen bonded with the C terminus of helix α1 [24].
Finally, there are mutations associated with MTZ resistance, such as those at A143 or V192, for which we find no obvious explanation. These mutations comprise class V. A review of the original citations used by Mendz and Megraud [22] for their compilation revealed that the RdxA alleles from most MTZ resistant strains also contained multiple mutations, many of which probably do not contribute to a loss of enzyme activity. To our knowledge, there has been no systematic directed mutational analysis of RdxA.

**A brief comparison of *H. pylori* RdxA with *H. pylori* FrxA and with structurally homologous reductases**

The structure of FrxA, a second NTR present in *H. pylori*, is available in the PDB (2HOU). Although this enzyme alone (regardless of its level of expression) does not contribute to MTZ susceptibility in *H. pylori* strains mutant in rdxA, double mutants are two-fold more resistant to MTZ, indicating that FrxA is contributing to MTZ susceptibility [25,26]. The FrxA sequence is approximately 25% identical to that of RdxA and the two proteins are structurally related. Superposition of the FrxA and RdxA structures shows an rmsd of 1.15 Å for 125 Cα (Fig. 5). Interestingly, the missing region of RdxA (residues 97–128) is seen in FrxA to fold into two long α helices (112–124 and 93–103). Conversely, residues 57–76, which are missing in the FrxA structure, are seen in RdxA to be folded into two helices (80–67 and 73–79) with one of them contributing to FMN binding. Overall, the binding of the FMN cofactor is similar in the two NTRs, although more water molecules appear to be bound to the FMN in FrxA. However, within the very conserved segment approaching the re face of the isoalloxazine ring, Tyr47 of RdxA is replaced by an Ile residue in FrxA. The important Cys159 in RdxA (see below) is also present in FrxA (Cys161), similarly positioned close to the FMN N1 atom; thus, it possibly also plays a redox role, which should be mutationally tested. By contrast to cysteine-rich RdxA, where no pair of Cys residues appear to be appropriately located to form a disulphide bond (see below), the sulfur atoms of the only two Cys residues in FrxA (i.e. Cys161 and Cys193) are at 5.3 Å, and might form a disulphide bond at some stage of the catalytic cycle. Thus, the possible involvement of a disulphide bond in the catalytic cycle of FrxA is worthy of testing. If this bond is found to form, Cys159 in RdxA and Cys161 in FrxA, although structurally equivalent, would serve different roles in catalysis, which might contribute to the different specificity observed for the two *H. pylori* oxidoreductases. Further differences between the two enzymes that may contribute to the tailoring of their redox potentials include the presence of three aromatic residues close the FMN in RdxA (the putative substrate binding Tyr47, Tyr141 at 4.5 Å from FMN C7M, and also Phe72 at 3.8 Å from FMN N3) and the presence of Glu164 at 3.3 Å from FMN C7M in FrxA.

There are more than 30 structures of FMN containing NTRs deposited in the PDB (3R6W, 2WQF, 2WZV, 3N2S, 3BM1, 1YKI, 1ZCH, 1KQB, 1F5Y, 1YWQ, 2B67, 2I7H, 2R01, 3BEM, 3E10, 3E39, 3EK3, 3EO7, 3EO8, 3GAG, 3GE5, 3GE6, 3GFA, 3GR3, 3H4O, 3HJ9, 3HOI, 3K6H, 3KWK, 3M5K, 3OF4, 3PXV, 4DN2), many of which have been deposited by the Joint Center for Structural Genomics. The superfamily server [27] identifies RdxA as a member of the NADH oxidase/flavin reductase family, of which the server includes 13 members with a known 3D structure (1BKJ, 1F5V, 1KQB, 1NEC, 1NOX, 1V5Y, 1VFR, 1YWQ, 1ZCH, 2B67, 2FRE, 2I7H, 1DS7). The overall structural fold is relatively well preserved among all these structures, although the primary sequence is not. Based on various insertions and deletions around the FMN binding region, the NADH oxidase/flavin reductase family has been divided into three subclasses [28]. According to our crystallographic data, RdxA can now be included into the NOX subclass. Superposition of NOX and RdxA shows that all residues interacting with FMN are conserved except for Cys159, which, in NOX, corresponds to a valine residue (Fig. 6A). Interestingly, this valine is highly conserved among other NADH oxidase/flavin reductase structures (Fig. 6B). Thus, RdxA is the first protein of this family that shows a cysteine close to the FMN.

By using the DALI server, we have found more than 200 structures that exhibit an rmsd below 3.0 Å with RdxA. After aligning the first 200 structures, we found that only nine (2HAY, 3OF4, 2WZV, 3N2S, 2R01, 3BEM, 3EK3, 3EO8, 3GR3) present a Cys residue structurally homologous to Cys159 (Fig. 7C). Seven of these structures have been solved by the Joint Center for Structural Genomics with no associated publication. The only two publications related to these Cys residues containing NTRs, NfrA1 from *Bacillus subtilis* (PDB code: 3N2S) [29] and NfnB from *Mycobacterium smegmatis* (PDB code: 2WZV) [30], do not attribute a role to the cysteine at the FMN binding site, perhaps because it is not conserved in most FMN binding enzymes. Based on these findings, we propose that RdxA could be included as the representative member of a new subgroup of oxidoreductases that might use the cysteine close to the FMN cofactor to
perform its reductive activity. To test this hypothesis, it will be interesting to perform activity experiments with the cysteine mutants of the NTRs that contain this cysteine. We have begun this task by mutating Cys159, which is present in RdxA, followed by an analysis of the consequences with respect to the activity of the enzyme (see below).

**Structural and mutational insight into a possible role of cysteine residues in MTZ reduction**

RdxA contains six cysteine residues per subunit (Fig. 7), whereas other NTRs average approximately one cysteine (sequences are shown in Fig. 2). There is a possibility that the reported distinct redox behavior
of RdxA (efficient reduction of low potential MTZ plus high NADPH oxidase activity) is related to some of those cysteines. Figure 2 shows that none of the six cysteines is conserved among NTRs from other bacteria. Two of them, Cys87 and Cys159, appear in three of the other NTRs, although never simultaneously. Because the sequence comparison of structurally homologous NTRs offers no information on the potential significance of the RdxA cysteines, we have aligned the sequences of four RdxAs belonging to H. pylori and related species (Fig. 8) aiming to determine which sequences are conserved. In addition, we included the FrxA NTR, which not only is more closely related to NfsB, but also has been implicated in MTZ susceptibility and resistance [25]. Of the six cysteine residues in H. pylori RdxA, Cys19, 140, 159 and 184 are conserved in the other Helicobacter species, and two of them, Cys184 and 159, appear close to the isoalloxazine ring in the H. pylori RdxA structure (Fig. 7). However, only Cys159 points to the ring, with its S atom being close to the N1 FMN atom (at 3.6 Å in chain A). The proximity of RdxA Cys159 to FMN raises the possibility that this residue plays a role in MTZ reduction. In this respect, we propose that, because the N1 atom of FMN can become protonated in its 2e\\^\(-\) reduced hydroquinone form, Cys159 might donate a proton to N1 to facilitate the formation of the neutral hydroquinone. If this were the case, the resulting thiolate ion could destabilize the hydroquinone, thus decreasing the FMN redox potential, which is needed for RdxA to reduce MTZ. A similar key role in providing a proton to the N5 atom of FMN has been proposed for the single cysteine residue of the light receptor phototropin [31], although, in that case, the thiolate reacts to form a covalent adduct with FMN.

To test our proposal, we changed Cys159 to either alanine or serine. The mutations were confirmed by DNA sequencing and the purified proteins were analyzed for enzymatic activity. As shown in Table 1, RdxA C159S and RdxA C159A both exhibited increased NADPH oxidase activity (40% and 70%, respectively) compared to wild-type RdxA. This higher NADPH oxidase activity might reflect a greater access of molecular oxygen to the active site in the mutants. By contrast, there were no differences in NTR activity with either nitrofurazone or the prodrug CB1954 as substrates under aerobic conditions. However, under the strictly anaerobic conditions required for MTZ reductase activity, the RdxAC159S and RdxAC159A mutants showed 62% and 72% decreased MTZ reductase activity, respectively (Table 1, compared to wild-type enzyme activity. It is clear that Ala residues cannot donate protons and also that Ser resides are very unlikely to do so except under extreme pH conditions or very special protein environments. The diminished MTZ reductase activity displayed by the Ser and Ala mutants, compared to Cys containing wild-type enzyme is in agreement with our proposed mechanism. It should be noted that the six cysteines present in each RdxA monomer are clustered on a plane approximately parallel to the FMN si face (Fig. 7). No disulfide bridge is formed in the structure. The two closest cysteine residues are Cys19 and Cys159, with their

![Fig. 7. Relative positions of the FMN and six cysteine residues in the RdxA monomer. The complete chain D is shown in a tube representation and the cysteine residues (S atoms in yellow) and FMN cofactor are shown using a stick model. The short distance (red dotted line and number) between the SG atom of Cys159 and the N1 atom of the isoalloxazine ring is also shown.](image)

**Table 1.** Effect of mutation of residue Cys159 on the RdxA NADPH oxidase and NTR activities. Conditions: 23 °C; 10 mM Tris/HCl, pH 7.5; 150 μM NADPH for aerobic assays and 30 μM for the anaerobic assay with MTZ. The activities presented are the mean of three determinations per substrate. For substrates other than oxygen, the reported activities are corrected for NADPH oxidase activity. Activity is given as: μM substrate·s\(^{-1}\)·μM protein\(^{-1}\). WT, wild-type.

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<th>Electron acceptor</th>
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<td>MTZ</td>
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sulfur atoms at 7.1–7.5 Å. This large distance, together with the results of our mutational analysis on Cys159, means that a disulfide bond between those two residues is unlikely to participate in RdxA catalysis. Finally, in light of the different MTZ activity of RdxA and NfsB NTR, future studies might explore the addition to NfsB NTR of a cysteine equivalent to Cys159, and NfsB NTR, in order to evaluate the effect of thiol on redox reactions either by allowing the NADPH-oxidase activity of RdxA to render the contents of the cuvette anaerobic or by using a glucose oxidase/catalase system, as described previously [7]. Specific activities are presented as μM substrate−s−1 μM protein−1.

Site-directed mutagenesis

The RdxA C159A and C159S mutants were generated by PCR using the primers: RdxA C159A, 5′-GGGAATTCCATATGGAATTTTTGGATCA-3′; and 3RdxA_BamHI, 5′-GGGAATTCCATATGGAATTTTTGGATCA-3′. The mutated genes were inserted into the pET29B HI sites of the pET29B HI vector. Mutated RdxA and FrxA genes were cloned into the pET29B HI sites of the pET29B HI vector, in order to evaluate the effect of thiol on redox potential and acquired MTZ reductase activity.

Experimental procedures

Protein purification and enzyme assays

Purification of the native and mutant RdxA proteins and enzyme assays were performed as described previously [7]. 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 (150 μM) and appropriate substrate (50–150 μM)] as described previously [7]. The reduction of MTZ was determined under anaerobic conditions either by allowing the NADPH-oxidase activity of RdxA to render the contents of the cuvette anaerobic or by employing a glucose oxidase/catalase system, as described previously [7]. Specific activities are presented as μM substrate−s−1 μM protein−1.

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and 1 μL of protein dissolved in 20 mM Hepes (pH 7.5) at 9 mg·mL⁻¹. After 3 days at 20 °C, square-shaped crystals appeared, belonging to the P4₁ tetragonal space group with unit cell parameters of: a = 49.4 Å, b = 49.4 Å, c = 304.5 Å. The crystals had a twin fraction of 0.458; therefore, they apparently belonged to the higher symmetry space group P4₂₁₂. Matthews’s coefficient [32] calculations for the P4₂₁₂ space group suggested the presence of a dimer of dimers (Fig. 1A) in the asymmetric unit with \( V_m = 1.94 \text{ Å}^3 \text{Da}^{-1} \) and a solvent content of approximately 37%. The crystals were cryoprotected by soaking in the mother liquor supplied with 20% glycerol and then flash-cooled under a nitrogen stream at 100 K. Two data sets at 37%. The crystals were cryoprotected by soaking in the mother liquor supplied with 20% glycerol and then flash-cooled under a nitrogen stream at 100 K. Two data sets at 2.0 Å and 3.4 Å were collected at beam lines ID23-2 and BM-14, respectively, of the synchrotron cooled under a nitrogen stream at 100 K. Two data sets at 2.0 Å and 3.4 Å were collected at beam lines ID23-2 and BM-14, respectively, of the synchrotron radiation facility ESRF in Grenoble (France). Images were collected with 0.1° of rotation given the length of the c-axis of the unit cell. Data reduction was conducted using MOSFLM [33] and SCALA [34] (Table 2).

**Phase calculation and model building**

The low-resolution dataset processed in the P422 space group and the RdxA sequence of *H. pylori* were submitted to the BALBES [35] server for molecular replacement (MR) in the eight possible space groups. Using the RdxA sequence, BALBES found 21 structures with > 15% identity but only the top six were used to generate 15 models for the MR search. The best solution was found in P4₁₂₁₂ using a *E. coli* NTR domain (PDB code: 1ICR) as a template. The selection of this domain, in which two helices were deleted, was found to be critical because crystal formation is driven by a proteolyzed form of RdxA, as demonstrated only after solving the structure. The solution found with BALBES was used as a search-model for MR with PHASER software (http://www.phaser.com/) using the high-resolution data set processed in P4₁₂₁₂. Then, after several cycles of refinement with REFMAC [36], combined with automatic building using Buccaneer [37], the RdxA model resulted in a \( R_{\text{free}} \) of 0.33/0.38. Next, the model was manually completed with COOT [38] and refined in REFMAC [36] with an \( R_{\text{free}} \) of 0.26/0.33. The model was used later to perform MR with PHASER using the high-resolution dataset processed in P4₁. Finally, after applying the twin operator during refinement in REFMAC [36], the \( R_{\text{merge}} \) and \( R_{\text{free}} \) reached acceptable values of 0.18 and 0.22, respectively (Table 2). The final model has been deposited in the Protein Data Bank (PDB code: 3QDL).

**MS analysis**

Drops containing the crystals were dissolved [4 μL of crystal sample plus 2 μL of sinapinic acid, 70 : 30 acetonitrile (ACN): trifluoroacetic acid (TFA) 0.1%, matrix elution] over a ground Steel Massive 384 target plate (Bruker, Billerica, MA, USA). The sample was then desalted using the ZipTip C4 (Millipore, Billerica, MA, USA) desalting method. As a control, an RdxA sample containing NADP⁺ was acidified by adding 1% TFA and the protein was concentrated and desalted by passing it through ZipTip C18 columns in accordance with the manufacturer’s instructions. Protein was eluted with 50% ACN/0.1% TFA/H₂O followed by 70% ACN/0.1% TFA/H₂O. MALDI-TOF MS was performed using a 4800 Plus MALDI-TOF TOF (Applied Biosystems, Foster City, CA, USA) in the linear mode with an accelerating voltage of 20 kV, a mass range of 6000–80000 Da, 1000 shots per spectrum and a laser intensity of 4800. Spectra were calibrated externally using a standard protein mixture (Protein-Mass Protein MALDI-MS Calibration Kit MSCAL3; Sigma, St Louis, MO, USA).

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