Structural Basis of 5-Nitroimidazole Antibiotic Resistance

THE CRYSTAL STRUCTURE OF NimA FROM DEINOCOCCUS RADIODURANS*

Hanna-Kirsti S. Leiros‡, Sigrid Kozielski-Stuhrmann‡, Ulrike Kapp, Laurent Terradot,
Gordon A. Leonard, and Seán M. McSweeney§

From the Macromolecular Crystallography Group, European Synchrotron Radiation Facility, BP 220, 6, Rue Jules Horowitz, F-38043 Grenoble Cedex 09, France

5-Nitroimidazole-based antibiotics are compounds extensively used for treating infections in humans and animals caused by several important pathogens. They are administered as prodrugs, and their activation depends upon an anaerobic 1-electron reduction of the nitro group by a reduction pathway in the cells. Bacterial resistance toward these drugs is thought to be caused by decreased drug uptake and/or an altered reduction efficiency. One class of resistant strains, identified in Bacteroides, has been shown to carry Nim genes (NimA, -B, -C, -D, and -E), which encode for reductases that convert the nitro group on the antibiotic into a non-bactericidal amine. In this paper, we have described the crystal structure of NimA from Deinococcus radiodurans (drNimA) at 1.6 Å resolution. We have shown that drNimA is a homodimer in which each monomer adopts a β-barrel fold. We have identified the catalytically important His-71 along with the cofactor pyruvate and antibiotic binding sites, all of which are found at the monomer-monomer interface. We have reported three additional crystal structures of drNimA, one in which the antibiotic metronidazole is bound to the protein, one with pyruvate covalently bound to His-71, and one with lactate covalently bound to His-71. Based on these structures, a reaction mechanism has been proposed in which the 2-electron reduction of the antibiotic prevents accumulation of the toxic nitro radical. This mechanism suggests that Nim proteins form a new class of reductases, conferring resistance against 5-nitroimidazole-based antibiotics.

Antibiotic resistance is an increasing problem throughout the developed world, and knowledge about different resistance mechanisms is important for efficient treatment of bacterial infections. One important class of antibiotics, the 5-nitroimidazole (5-Ni) drug derivatives, includes metronidazole (MTR),

dimetridazole (DMZ), and tinidazole (TNZ). MTR is extensively used in the treatment of anaerobic infections caused by Trichomonas vaginalis, Entamoeba histolytica, Enterococcus species, Giardia lamblia, Clostridium species, and Bacteroides (1–4) and is also a critical ingredient of modern multidrug therapies for Helicobacter pylori eradication regimes used to control ulcers (5). The mode of action for the 5-Ni antibiotics, as illustrated in Fig. 1, has been shown to be similar in different pathogens (6–8). The inactive prodrug enters cells by simple diffusion and is then reduced in a 1-electron reduction into the toxic compound, the short-lived radical anion R–N/O2. This reaction is mediated by ferredoxin, which receives an electron from the pyruvate-ferredoxin oxidoreductase (PFOR) complex via conversion of pyruvate to acetyl coenzyme A (9). The resulting nitro radical anion probably causes DNA strand breaks, DNA helix destabilization, unwinding of DNA, and finally cell death (1, 2, 10, 11), and damage to other vital cell systems is also possible (6).

The success of such drugs depends on the reductive activation of the nitro group on the 5-Ni drug, which is controlled by the redox system of the target cell. As a consequence, species with altered, absent, or elevated redox potential pathways are resistant to 5-Ni drugs (see Ref. 12 and references therein). For H. pylori, the most convincing data regarding MTR resistance relate to inactivation of the RdxA gene, which encodes an oxygen-insensitive NADPH nitroreductase (13). Still, resistance has been found in H. pylori strains with an intact RdxA gene (14). In T. vaginalis, a reduced amount of available ferredoxin as an electron acceptor/donor is thought to be responsible for drug resistance (15), but strains with knock-out ferredoxin genes are not resistant under aerobic or anaerobic conditions (16). Therefore, it is likely that multiple pathways lead to both activation and resistance of the MTR and other 5-Ni drugs.

The 5-Ni resistance of some of Bacteroides fragilis strains was shown to be mediated by specific genes, named Nim, located either on the chromosome (NimB) or on small mobilizable plasmids, e.g. pIP417 (NimA), pIP419 (NimC), and pIP421 (NimD) (17–20). A fifth Nim gene, NimE, was discovered that confers resistance to high MTR concentrations in strains from Bacteroides thetaiotaomicron, B. fragilis, and Bacteroides ovatus (21). The enzymatic activity of the Nim gene products was deduced by comparing the metabolism of a 5-Ni-susceptible strain with the same strain harboring a plasmid containing the NimA sequence from B. fragilis (22). In the sensitive strain, the classic reduction of DMZ to its nitro radical anion was observed in agreement with the general scheme (see Fig. 1). However, in the resist-

*MES, 4-morpholinethanesulfonic acid; PNPO, pyridoxine 5’-phosphate oxidase; Pyr, pyruvate, Lac, lactate; r.m.s.d., root mean square deviation.
against these antibiotics. The generation of non-toxic derivatives and confers resistance to the reduction of 5-Ni compounds by Nim enzymes that leads to their function as reductases. We propose a mechanism for related enzymes, our observations suggest that Nim proteins do indeed function as reductases. Following the increasing availability of new bacterial genome sequences, it emerges that Nim homologues are present in other genera of bacteria, including Deinococcus radiodurans, B. fragilis, Helicobacter hepaticus, Clostridium sp., Salmonella typhi, Streptomyces avermitilis, as well as in Archaea Methanosarcina sp. Although the underlying physiological function of the Nim homologues in these organisms is unknown, it seems likely that the Nim family as a whole. Taken together with previous studies, it is clear that Nim proteins are 5-Ni reductases, which can act as an electron donor through the PFOR complex with MTR (Fig. 1), increased the drug uptake (22). Hence, it was proposed that Nim proteins are 5-Ni reductases, which possibly use ferredoxin as the electron donor (22).

DNA into the Gateway destination vector pDEST17 (Invitrogen) by the company ProteinXpert SA, Grenoble, France. The correct sequence with an amino-terminal hexahistidine tag (sequence MSYHHHHHH-HHHLESTTLYKRAVGH1022) has been confirmed by DNA sequencing...

E. coli BL21(DE3)pLysS cells (Novagen) transformed with pDEST17-drNimA were grown at 37 °C in rich broth 2×YT medium (16 g/liter bacto-tryptone, 10 g/liter bacto-yeast extract, 10 g/liter NaCl) with 100 mg/liter ampicillin and 34 mg/liter chloramphenicol, and at an O.D. of 0.5, the cells were induced with 1 mM isopropyl- β-D-thiogalacto-pyranoside for 4 h at 37 °C. The harvested cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.2, 500 mM NaCl, 1 mM (imidazole) supplemented with DNaseI, Lysozyme, and Complete EDTA-free Protease Inhibitor Mixture (Roche) and lysed by sonication, and the soluble lysate was applied to nickel-nitrotriacetate acid resin (Qiagen). The protein was eluted with a linear gradient of imidazole at a concentration of from 10 to 500 mM. Fractions containing drNimA were de-salted (HiTrap desalting column, Pharmacia Corporation) and loaded onto a MonoQ column (Amersham Bioscience) and further eluted with a NaCl gradient (0–1 M) in which drNimA eluted at around 0.1 M NaCl. Fractions with drNimA were pooled and further purified by analytical gel filtration (Superdex 200, Pharmacia Corporation) in a buffer consisting of 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA. The peak fractions were concentrated to 20 mg/ml. Electrospray mass spectrometry of the purified drNimA confirmed the molecular mass of ~24 kDa (drNimA 21.9 kDa + His-tag 2.5 kDa).

Oligomeric State and Cross-linking—An additional gel filtration run (in 25 mM Tris-HCl, pH 8.0, 100 mM NaCl) was also carried out with drNimA and the standard molecular mass markers, albumin (67 kDa) and chymotrypsin (25 kDa), to correlate the elution volume of drNimA to the molecular mass of the protein in solution. One run with only the molecular mass marker, ovalbumin (43 kDa), was also performed. All four proteins were at concentrations of ~1 mg/ml.

Another analytical gel filtration run (Superdex 200 (Pharmacia Corporation) in 50 mM Hepes, pH 7.5, and 1 mM EDTA) with the pure drNimA protein was performed to change the buffer from Tris to Hepes, and this elution curve is given in Fig. 4a. The main peak from this run was used for the cross-linking experiments. A series of 0.25, 0.50, 1.0, and 5.0% mass cross-linker, ethylene glycol-bis (succinimido) A-chloro-succinimide ester (Sigma) in Me2SO was added to the protein and left for 20 min on the bench, and then Tris was added to a final concentration of 60 mM. The series was analyzed on a 12% SDS-PAGE gel (see Fig. 4b).

Crystallography—Crystallization, Soaking, Data Collection, and Structure Solution—Initial screening for suitable crystallization conditions was carried out by the hanging drop method using standard commercial screening solutions. The final crystallization conditions at 4 °C had 4-μl hanging drops consisting of a 1:1 mixture of protein (6 mg/ml) and reservoir solution with 0.65–0.9 M sodium acetate and 0.1 M sodium cacodylate, citrate, or MES buffered at pH 6.0–5.5. The crystals grew as rosettes with plate-like “fingers,” which were cracked, and the resulting plates with an approximate size of 100 × 30 × 5 μm³ were used for data collection. For structure solution purposes, a mercury derivative (drNimA-Hg) was prepared by soaking the crystal in a solution with 1 mM ethyl mercury thiosalyslate, 0.2 M sodium acetate, 0.1 M sodium cacodylate, pH 6.0, and 30% polyethylene glycol 4000 at 4 °C for 20 min.

The complex structures described in this paper were prepared by soaking the native crystals in solution containing 0.2 M sodium acetate, 0.1 M sodium cacodylate, pH 6.0, and 30% polyethylene glycol 4000 and 1.5, or 10 mM MTR or TNZ (both from Sigma) at 4 °C. As will be seen, soaking for 2 h in 10 mM MTR produced a structure with drNimA in complex with MTR (drNimA-MTR), whereas soaking for 20 h in 1 mM TNZ yielded a complex with covalently bound pyruvate (drNimA-Pyr), and soaking for 23 h in 1 mM MTR produced a complex with drNimA and covalently bound lactate (drNimA-Lac).

All diffraction data were collected at the European Synchrotron Radiation Facility, Grenoble, France, using crystals cooled to 100 K. Cryo-protection for the native crystals was effected by leaving the crystals for ~20 s in a solution with 0.2 M sodium acetate, 0.1 M sodium cacodylate, pH 6.0, and 30% polyethylene glycol 4000. Crystals of the mercury derivative and of the various complexes could all be frozen straight from their soaking solutions. The crystals of drNimA (including the substrate soaks) showed great sensitivity to ambient temperatures; therefore all manipulations described above and flash freezing in liquid nitrogen were performed in a cold room maintained at 4 °C.

All crystals belong to the space group C2, and the unit cell dimensions for the native crystal were a = 99.86 Å, b = 38.95 Å, c = 59.81 Å, and β = 114.25° (see Table 1 for further details). All data were integrated using MOSFLM, scaled with SCALA, and structure factors...
**FIG. 2.** Sequence alignment of the Nim amino acid sequences with the secondary structure elements of the drNimA assigned. Abbreviation, species for the sequences, and the TrEMBL entry in parentheses are as follows: drNimA, NimA from *D. radiodurans* (Q9RW27); bfNimA, NimA from *Bacteroides vulgatus* (Q45801); bfNimB, NimB from *B. fragilis* (Q45146); bfNimE, NimE from *B. fragilis* (Q9L4E6); mmNimA, NimA from *M. mazei* (Q8PT76); stNimA, NimA from *S. typhi* (Q8Z8F0); ctNimA, NimA from *C. tetani* (Q896U9); saHyp, hypothetical protein from *S. avermitilis* (Q827C5), and hhHyp, conserved hypothetical protein from *H. hepaticus* (Q7VG50). The figure was produced by ESPript 2.2 (prodes.toulouse.inra.fr/ESPript/ESPript/).

**TABLE I**

Statistics from the data collections

| Parameter | drNimA-Hg (EMTS) | drNimA (Native) | drNimA-MTR | drNimA-Pyr | drNimA-Lac |
|-----------|------------------|----------------|------------|------------|------------|
| X-ray statistics |                  |                |            |            |            |
| Beamline   | ID14-EH2         | ID14-EH1       | ID14-EH4   | ID14-EH2   | ID14-EH4   |
| Space group | C2               | C2             | C2         | C2         | C2         |
| PDB entry  |                  |                |            |            |            |
| Unit cell  | a = 100.09       | a = 99.86      | a = 99.59  | a = 99.94  | a = 99.80  |
| Resolution (Å) (highest bin) | 20–1.90 (2.21–2.10) | 20–1.80 (1.69–1.60) | 20–1.90 (2.00–1.90) | 30–1.80 (1.90–1.80) | 30–1.88 (1.98–1.88) |
| Multiplicity | 8.1 (8.2)        | 3.7 (3.7)      | 4.3 (4.4)  | 2.7 (2.7)  | 2.5 (2.5)  |
| Completeness (%) | 99.9 (100.0) | 99.9 (100.0) | 99.9 (99.9) | 99.0 (99.5) | 99.8 (100.0) |
| Intensity (I(h)) | 5.3 (1.5)       | 7.6 (1.5)     | 3.4 (1.7)  | 3.9 (0.8)  | 8.9 (1.5)  |
| Mean (I/σ(I)) | 15.4 (4.3)      | 11.2 (2.8)    | 9.3 (2.9)  | 9.5 (1.7)  | 9.9 (2.2)  |
| R_sym (%)   | 12.8 (46.2)     | 7.4 (44.8)    | 14.2 (37.9) | 8.90 (69.0) | 8.1 (46.6) |
| R_anom (%)  |                  |                |            |            |            |
| FOM_SIRAS  | 0.45 (to 2.1 Å) |                |            |            |            |
| FOM_sph   | 0.46 (to 1.6 Å) | 0.69 (to 2.3 Å) |            |            |            |

*R_sym = (ΣΣ |I(h) − (I(h))| / ΣΣ |I(h)|), where |I(h)| is the rth measurement of reflection h and (I(h)) is the weighted mean of all measurements of h.*

*R_anom = Σ(|I(h)| − (|I(h)|)) / Σ(|I(h)|), where (|I(h)|) is the mean intensity of the reflection.*

FOM_SIRAS = figure of merit after SIRAS phasing.

FOM_sph = figure of merit after solvent flattening.
Crystal Structure of NimA from D. radiodurans

obtained using TRUNCATE software (24).

The structure of drNimA was elucidated using the single isomor-
phous difference with anomalous scattering technique, with initial ex-
perimental phases based on the single ethyl mercaptan thiophosphate
derivative obtained to 2.1 Å using the software program SOLVE (25).
Phase improvement and extension to 1.6 Å resolution were carried out
with RESOLVE (26), and ARP/wARP software (27) was then used to
produce an initial model for drNimA (192 of the final 224 residues).
There is one monomer in the crystallographic asymmetric unit result-
ing in a solvent content of 43% and a Matthews coefficient of 2.2 Å3/Da.

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In the final native structure, the pyruvate is located between the
β2 and β3 strands, and O-1 in the carboxyl group is 2.38 Å
away from His-71 Ne-2. The three oxygen atoms of the pyruvate
are hydrogen bound to the four water molecules W-1, W-2,
W-3, and W-5. The amino acids Val-139 and Leu-107', along with
Phe-140 and Phe-98', also contribute to the binding site
(Figs. 3f and 6a).

Comparison of Sequence and Structural Homologues—To
search for sequence and structural homologues, the drNimA
sequence and coordinates were used with the BLAST (30) and
DALI servers (31). BLAST identified a number of sequence
homologues in the data base, which included sequences from the
Clostridium species, Bacteroides species, H. hepaticus and
S. typhi, and Archaea (Methanosarcina mazei). The sequence
identity of drNimA toward the sequences included in Fig. 2 is
14–23%, and the homology is 46–55%. Two sequence motifs
were found in the Conserved Domain Database (32): flavin
mononucleotide (FMN) binding and pyridoxamine 5’-phos-
phate oxidase.

The DALI server confirmed these results by identifying other
proteins with similar β-barrel fold as drNimA, which are all
FMN-binding proteins. They include the human pyridoxine
5’-phosphate oxidase (PNPO) (Protein Data Bank (PDB) code
1nrg, Z-score 4.0, root mean square deviation 2.15 Å over 97 Ca
atoms), the FMN-binding protein from Desulfovibrio vulgaris
(PDB code 1axj, Z-score 8.2, r.m.s.d. 2.3 Å over 83 Ca atoms),
and ferric reductase from Archaeoglobus fulgidus (PDB code
1i0r, Z-score 5.3, r.m.s.d. 2.5 Å for 83 Ca atoms). Comparison
of drNimA with a monomer of these structures shows that
drNimA contains some unique structural elements, namely the
orientation of helix a2 and a3 and the extension of a4, a5, and
a6 (compare Fig. 3, a and b, which are in the same orientation).
In drNimA, the helices a2 and a3 are involved in the pyruvate
binding site, whereas the strand extensions stabilize the ho-
momider (Fig. 3a). Superposition of these enzymes reveals that
the location of the active sites are all on the same side of the
barrel, and this region in drNimA contains His-71, which ap-
pears to be important for enzymatic activity of the Nim
enzymes.

Interestingly, the PNPO structure displays a similar dimer
organization (33) to that observed in drNimA. Although the
sequence identity between the two enzymes is low (15% iden-
tity and 44% homology), the β-barrel folds and location of the
barrels in the dimers are very similar, as shown in Fig. 3, c and
d, of the drNimA and PNPO dimers. Further, the MTR
binding site (as described under "Complex Structures") overlaps
with the isoalloxazine rings of the FMN (compare Fig. 3, a–d).

Thus it appears that drNimA shares a structural scaffold
with a broad family of β-barrel-containing enzymes in which
the β-barrel fold seems important for the electron transfer
abilities of these enzymes. Because drNimA has this fold and
His-71 overlaps with the active sites of the other enzymes, this
supports the hypothesis that drNimA is a reductase in which
His-71 is important. Remarkably, the two active sites of both
drNimA and PNPO are composed of residues from both mon-
omers and are located at the same place at the dimer interfaces.

Complex Structures—To gain further insight into the antibi-
optic binding site of the Nim enzymes, drNimA crystals were
soaked to obtain substrate-bound states of the structure, and
here three structures are presented. Refinement statistics for
all structures discussed are summarized in Table II.

In the second structure (drNimA-MTR; 2-h soak with MTR),
a MTR molecule could clearly be distinguished sitting in a cleft
at the monomer–monomer interface, with a water accessible
surface of 42 Å2. The antibiotic is sandwiched between Pro-56
and Tyr-111' (Fig. 3f), two residues that are highly conserved

RESULTS

Structure, Fold, and Dimerization of drNimA—The crystal
structure of drNimA comprises one monomer in the asymmet-
tric unit, and the final model of the native structure (drNimA)
comprises 10 of the 21 residues in the amino-terminal His-tag,
including the six histidines, 194 of 195 residues of the protein
itself, one acetate ion, one pyruvate molecule, and 334 water
molecules. The approximate dimensions of the monomer (Fig.
3a) are 65 × 45 × 55 Å3. The structure consists of a central
six-stranded anti-parallel β-barrel with strand order β1, β2,
β3, β6, β5, β4. The β-barrel is non-symmetric with β-strands
β4, β5, and β6 elongating the barrel toward both the amino
and carboxyl termini of the protein. These three extended strands
are perpendicular to the helix α1 (from Asp-24 to Arg-33) that
locks the bottom of the β-barrel as shown in Fig. 3a. Opposite
the expanded strands, two helices (α2, α3) flank the β-barrel.

Analytical gel filtration and cross-linking experiments clearly
indicate that drNimA is a homodimer (Fig. 4). This homodimer is,
in the crystals, formed by the crystallographic 2-fold axis. When
the dimer is being formed, it buries a surface area of 2314 Å2
that is 18% of the area in a monomer. In the dimer, the long β-strands
(β4, β5, β6) of one monomer grip onto the β-barrel of the other
monomer forming a β-propeller with ten strands (Fig. 3, c and e)
with approximate dimensions of 75 × 47 × 43 Å3. The two
drNimA monomers are held together by 10 hydrogen bonds,
including a salt bridge between Arg-38 and the carboxyl group
of Glu-91, some aromatic interactions, and several water-mediated
interactions at the dimer interface.

Native Structure—During the refinement of the drNimA
structure, a flat, X-shaped portion of difference electron density
was observed in a solvent-exposed pocket on the monomer-
monomer interface (Fig. 5a). This density is at hydrogen-bind-
ing distance to the absolutely conserved residue His-71 (Fig. 2).
To form an interpretation of this electron density, several pos-
sible molecules were tested for suitability. Only molecules sat-
sifying the constraints of size and shape placed by the differ-
ence electron density, and also conforming to the chemical
environment available, were considered. Ultimately, the most
satisfactory explanation of the residual difference density was
obtained by the incorporation of a pyruvate moiety into the
model. The assignment of a pyruvate molecule here seems to be
reasonable, because all three oxygen atoms are involved in
hydrogen binding networks (Fig. 5b), and the hydrophilic
methyl group (of the pyruvate) is facing Phe-140 from one
molecule and Phe-98' from the second monomer (Fig. 5c), where the
‘—’ implies residues from the second monomer in the ho-
momider). The refined pyruvate moiety fits within the observed
electron density (Fig. 6a), it has reasonable bond lengths and
bonds angles, and no difference density was observed when the
structure refinement was completed.
Fig. 3. a, ribbon diagram of the drNimA monomer with the active site His-71, Pro-56, Tyr-111, the pyruvate, and the antibiotic metronidazole, all depicted as ball-and-stick atoms. The structure is color-coded from blue to red when going from the amino terminus to the carboxyl terminus. Secondary structure elements are labeled corresponding to the sequence alignment (Fig. 1). The acetate ion inside the barrel is also included in...
in the sequence alignment (Fig. 2). The aromatic ring of Tyr-111 and the imidazole ring of MTR are almost parallel, separated by 3.2 Å (between the plane of the two rings; see Fig. 3), indicating that \( \pi \)-orbital interactions between the two rings systems are important. Although Pro-56 is strictly conserved, Tyr-111 is substituted in some sequences by a phenylalanine, suggesting that an aromatic ring at position 111 is required to interact with the imidazole ring of the drug.

The structural comparison of drNimA and drNimA-MTR shows several differences between the two structures. Both the pyruvate and the MTR are now found at the interface of the \( \beta \)-barrels, sandwiched between the two protein monomers, and interact with residues from both monomers. The O-1 atom in the carboxyl group of the pyruvate is moved slightly closer toward His-71 Ne-2 (compare Fig. 6, a and b). The binding of MTR results in other minor structural changes in and around the active sites: a water molecule (W-75) is displaced, Pro-56 and Tyr-111 are moved further away from each other, and residues Asn-20 to Ser-23, Ser-113, and Ser-171 to Asn-175 are displaced. The RMS displacements between drNimA and drNimA-MTR (main chain residues 2–195) are 0.269 Å.

In the third structure (drNimA-Pyr; 20-h soak with TNZ), no antibiotic could be found bound to the protein; however, we could clearly distinguish the pyruvate molecule covalently attached to His-71 Ne-2. In the finally refined structure, a well defined His-71-Pyr residue was therefore included, with a bond in the figure. b, shown is the PNPO monomer labeled with secondary structure elements according to Musayev et al. (33), and the flavin (FMN) and pyridoxal-5’-phosphate (PLP) are also depicted. The orientation and color coding are the same as for the drNimA in panel a). Shown also are the drNimA (c) and the PNPO dimers (d). The active residues are shown in both panels, which are in the same orientations and have same sizes. The location of the two \( \beta \)-barrels and the two active sites are indicated with arrows and boxes. e, the drNimA dimer folded as a \( \beta \)-propeller as shown by the magenta and green monomers. Residues His-71, Pro-56, Tyr-111, the pyruvate, and the antibiotic metronidazole are all depicted as ball-and-stick atoms for the two active sites. f, a close view down one of the antibiotic binding sites in the drNimA dimer. Residues involved are color-coded in magenta and green according to the monomer of origin. The pyruvate and the antibiotic metronidazole are shown in white and some hydrogen bonds are given. All figures were made with MOLSCRIPT (39) and Raster-3D (40).
distance from O-1 (pyruvate) to N/H9280 of 1.47 Å (Fig. 6c). No other structural changes could be found between drNimA-Pyr, drNimA-MTR, and drNimA (RMS displacements are 0.22 Å²).

In the fourth structure (drNimA-Lac; 23-h soak with MTR), the modeling of a His-71-Pyr residue left a significant peak of difference electron density (Fo/Fc). After consideration of the chemical environment and shape of the density, (most of) this electron density could be described when a His-71-Lac residue was used in the refinement (Fig. 6d). However, the final refinement showed some positive Fo/Fc density overlapping with the His-71-Pyr residue in drNimA-Pyr (Fig. 6d). It was therefore concluded that the full reduction of pyruvate into lactate was not achieved. Finally, we chose to include only a His-71-Lac residue (with full occupancy) in the drNimA-Lac structure, because that was the major conformation of this residue.

**DISCUSSION**

The 5-Ni-based drug, MTR, is still one of the most effective drugs against infections caused by anaerobic bacteria, particularly when treating those caused by *e.g.* *T. vaginalis* and *Bacteroides* (1, 2, 34). 5-Ni drugs are inactive prodrugs that enter the cell by simple diffusion and are thereafter reduced in a 1-electron reduction by ferredoxin into its toxic radical anion (R–NO₂⁻RN) (6) as shown in Fig. 1. Resistance toward these compounds has been encountered in several bacteria, based on an altered or deficient reduction mechanism by which the prodrug could not be transformed into the toxic radical (13, 15, 16). In *B. fragilis*-resistant strains, the resistance mechanism appears to be related to a specific family of genes named Nim. Nim family proteins are thought to be 5-nitroimidazole reductases that transform the nitro group of the prodrug into its non-toxic amine derivative (22).

The structure of drNimA from *D. radiodurans* presented here is the first available structure of a Nim family protein. The dimeric structure has the same basic β-barrel scaffold used by other enzymes involved in electron transfer (33, 35–37) but displays a simpler active site in which the antibiotic and the pyruvate interact with residues from both monomers.

**Putative Mechanism and Action of Pyruvate**—Consistent with previous evidence (22), the four structures presented allow us to propose how drNimA might function as a reductase and reveal the essential role played by pyruvate in the reaction mechanism. In the native state of the protein, a pyruvate ion was found within hydrogen-binding distance to the conserved His-71. Upon binding of MTR, the pyruvate moiety initially became more tightly bound to His-71, and finally a modified His-71-Pyr residue forms the oxidation product of His and pyruvate with the release of two electrons and one H⁺ (Fig. 7, Step ❶). The electrons can be shuttled via a water molecule (*e.g.* W-1) to the antibiotic. Thus, the first step of the antibiotic reduction becomes R–NO₂⁻ → RN=O (Fig. 7, Step ❷), similar to other bacterial nitro reductases (38). Importantly, this 2-electron reduction avoids formation of the toxic nitro radical anion (Fig. 1) and leads to resistance against the antibiotic.

In this proposed mechanism, the drNimA-MTR structure is an intermediate structure, which appears in between the native state and the drNimA-Pyr structure (Fig. 7). The reduction of pyruvate into lactate, from the drNimA-Pyr structure to the drNimA-Lac structure, may provide an idea as to how the enzyme is recycled back to its native state. To reduce pyruvate into lactate, 2H⁺ and 2e⁻ are needed. In *vivo*, putative electron sources are available for the Nim enzymes (*e.g.* NAD(P)(H)), but in the drNimA-Lac crystal structures, water is one possible electron donor (H₂O → ½O₂ + 2H⁺ + 2e⁻) that could explain how pyruvate was reduced to lactate. To recycle the pyruvate/lactate cofactor, the lactate to the His-71 bond must be broken, but our crystallographic study does not give any details on this process.

The refined structures of drNimA show that His-71 and pyruvate can be oxidized into a His-71-Pyr residue (and release...
FIG. 6. Electron density maps. A, the final $\sigma A$-weighted $2F_o - F_i$ electron density map (1.2$\sigma$) of the native drNimA structure with only the pyruvate in the active site. B, the active site of the complex with pyruvate and the MTR antibiotic (drNimA-MTR). The $2F_o - F_i$ map (1.0$\sigma$) is contoured together with some inter-atomic distances. The orientation is slightly different from panel A, but the sizes are identical. C, shown are the soaks, which resulted in a covalently bound pyruvate (drNimA-Pyr) with the corresponding $2F_o - F_i$ map (1.2$\sigma$). D, shown is the covalently bound lactate molecule (drNimA-Lac) with its $2F_o - F_i$ map (1.1$\sigma$). In panel D, the His-71-Pyr residue of the drNimA-Pyr structure is included as solid black bonds. The orientation of panels B–D are identical, but the sizes are bigger in panels C and D to focus on the covalent links. All figures have $F_o - F_i$ maps at $+4\sigma$ (green) and $-4\sigma$ (red) and were made using BobScript (42).
Proposed antibiotic resistance mechanism.

During this study, other Nim homologues were found in several species. It is theoretically possible that the proposed mechanism awaits further biochemical and structure studies, the structure of drNimA reported here should provide a first step toward the design of new or modified 5-Ni drugs.

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