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Commentary

Flavin oxidation state impacts on nitrofuran antibiotic binding orientation in nitroreductases

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Nitroreductases catalyse the NAD(P)H-dependent nitro reduction in nitrofuran antibiotics, which activates them into cytotoxic molecules leading to cell death. The design of new effective nitrofuran antibiotics relies on knowledge of the kinetic mechanism and nitrofuran binding mode of microbial nitroreductases NfsA and NfsB. This has been hampered by multiple co-crystallisation studies revealing ligand binding in non-electron transfer competent states. In a recent study by Day et al. (2021) the authors investigated the likely reaction mechanism and mode of nitrofurantoin binding to NfsA using potentiometry, global kinetics analysis, crystallography and molecular dynamics simulations. Their findings suggest nitrofurantoin reduction proceeds via a direct hydride transfer from reduced FMN, while the crystallographic binding orientation is an inhibitory complex. Molecular dynamics simulations suggest ligand binding orientations is dependent on the oxidation state of the FMN. This study highlights the importance of utilising computational studies alongside traditional crystallographic approaches, when multiple stable ligand binding orientations can occur.

Nitroaromatics are a diverse array of primarily synthetic compounds [1,2] that are released into the environment through industrial processes. These compounds include nitrofurans, nitrobenzenes and nitropyrenes, and are commonly used as pesticides, explosives, pharmaceuticals, food additives and antimicrobial agents [3]. *In vivo* enzymatic degradation of these anthropogenic pollutants releases highly reactive NO_x species, which attack a variety of intracellular macromolecules [4]. Therefore, nitroaromatics are reputedly indirect toxins and carcinogens, and are considered to be very polluting [5,6].

Microbial degradation of nitroaromatics are primarily performed by two families of FMN-containing nitroreductases (NRs), which catalyse the NAD(P)H-dependent reduction of a diverse range of nitroaromatic and nitro heterocyclic compounds [7]. Type I, or oxygen-insensitive NRs, catalyse nitro group reduction in compounds such as nitrofurans, nitrobenzenes, nitroimidazoles and nitrotoluenes. This is performed via the successive transfer of two electrons from NAD(P)H without free radical formation [2,8]. These enzymes are further subdivided into two main sub-groups NfsA and NfsB, based on their resemblance to *Escherichia coli* NRs [2,9]. NfsA preferentially uses NADPH as the coenzyme, while NfsB can use either NADH or NADPH with similar affinities [10,11]. Type II NRs are oxygen sensitive, and reduce the nitro group of substrates aerobically by one-electron transfer to form a negatively charged nitro group. This subsequently reacts with an oxygen molecule, yielding a superoxide radical and reforming the original nitro substrate [12]. The accumulation of superoxide radicals from these futile redox cycles leads to oxidative stress, although Type II NRs can also catalyse Type I-like two-electron transfers under strictly anaerobic conditions [12].

A key medicinal role played by bacterial NfsA and NfsB homologues is the activation of antibiotics and antimicrobial agents. Nitrofurantoin is a commonly prescribed antibiotic for urinary tract infections, while nitrofurazone is used as a topical treatment for skin infections in burns patients [9,13]. Activation

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of these compounds by NRs is via the formation of nitroso compounds, which are later converted into the respective hydroxylamine derivatives (Figure 1a) that attack a wide variety of biological macromolecules leading to microbial cell death [14,15]. The production of cytotoxic hydroxylamines *in vivo* is also used in cancer gene therapy (prodrug CB1954) [16], selective cell ablation [17–19] and explosives bioremediation [20,21].

While resistance to nitrofuran antibiotics remains low, their poor solubility limits their medicinal applications. Detailed structural analysis of the binding of nitrofurantoin and/or nitrofurazone to microbial nitroreductases NfsA/NfsB would greatly assist in facilitating the design of new soluble nitrofuran derivatives for biomedical purposes. Until recently, crystal structures of *E. coli* NfsA [23] and seven other homologues were available [24,25]. However, only one contained an active site ligand; the inhibitor NAD^+. The crystal structure

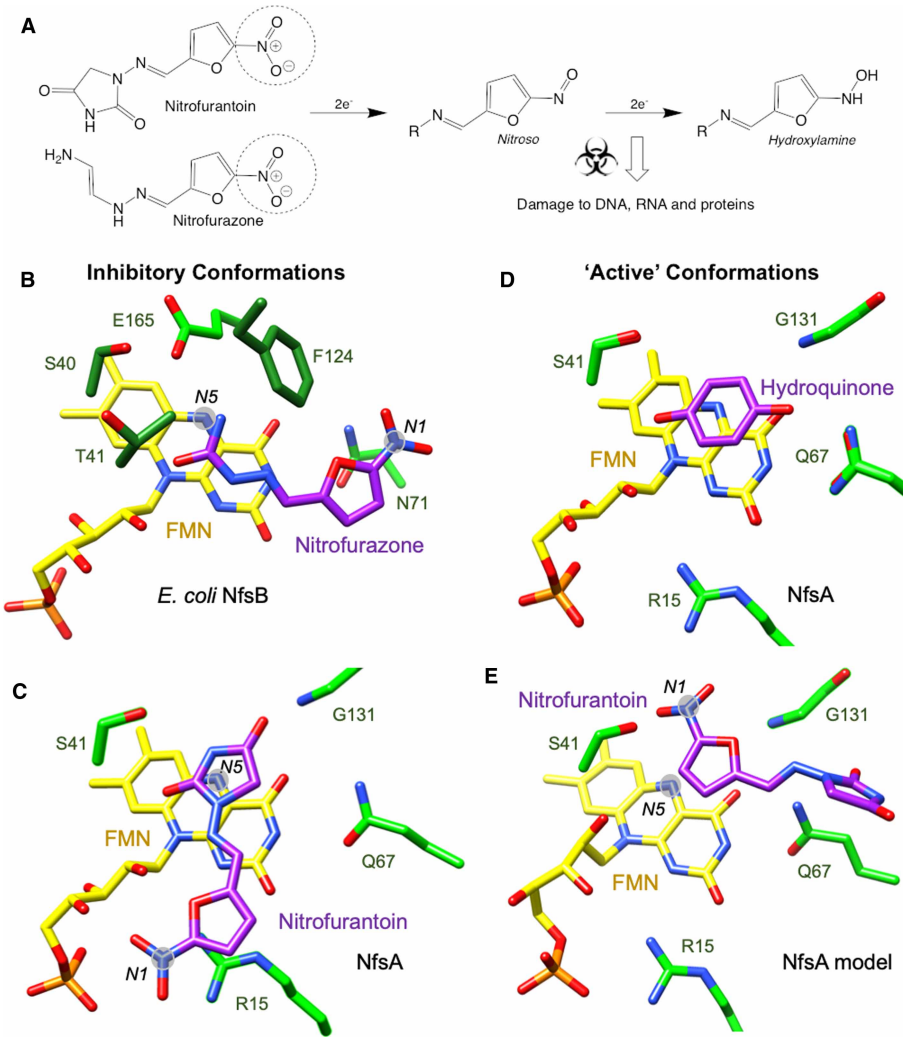

Figure 1. Activity and binding of antibiotics nitrofurantoin and nitrofurazone with nitroreductases NfsA and NfsB.
(**A**) Nitroreductase-catalysed reduction of the nitro-group to the nitroso and hydroxylamine moieties in nitrofurantoin [9]. Crystal structure of the active site of (**B**) nitrofurazone-bound *E. coli* NfsB [4] in an inhibitory binding mode and (**C**) nitrofurantoin-bound NfsA [9]. (**D**) Crystal structure of hydroquinone-bound NfsA [9] and (**E**) model of the active site of nitrofurantoin-bound NfsA in active binding modes. For parts **B**–**E**, the images were generated in Chimera [22], with residues depicted in sticks with green carbons, FMN with yellow carbons and the antibiotics with purple carbons. The FMN N5 and ligand nitro group N1 atoms are indicated by transparent grey circles with labels in italics. The Protein Data Bank accession codes used to generate images **B**–**D** were 1YKI, 7NB9 and 7NMP, respectively. The coordinates for a potential active conformation of reduced NfsA-nitrofurantoin (**E**) was one of the models deposited in modelarchive (modelarchive.org.doi) as project ma-9z55z, which was kindly supplied by Dr Eva I. Hyde and reproduced with permission [4,9,22].

of nitrofurazone-bound *E. coli* NfsB is known, but the ligand is bound in an inhibitory conformation (Figure 1b), with the nitro moiety too distant from the FMN N5 atom to facilitate hydride transfer [4]. Other NfsB homologue co-crystal structures contained either cofactor derivatives or other ligands [26–28]. Therefore, no known structures exist that show the mode of binding of nitrofuran antibiotics to either NfsA or NfsB in an electron transfer competent state.

Progress in understanding the binding mode of nitrofurantoin to nitroreductases was seen in recent crystallographic, modelling and kinetics studies by Day et al. [9]. The crystal structure of nitrofurantoin-bound NfsA was solved to a high resolution. Like previous studies, however, the antibiotic was bound in a flipped or inactive conformation with the furan ring near the FMN N5 atom instead of the nitro group (Figure 1c). A second, in this case productive, structure was generated between NfsA and a product hydroquinone (Figure 1d), but this ligand is unrelated to nitrofuran antibiotics.

This prevalence of flipped binding orientations of substrates within co-crystal structures of NRs is also seen in the unrelated FMN-containing Old Yellow Enzyme (OYE) family [29,30]. Examples include co-crystal structures of pentaerythritol tetranitrate reductase (PETNR) with steroids [29] or hydroxy substituted 1-aryl-2-nitropropenes [30], and xenobiotic reductase A (XenA) bound with coumarin derivatives [31]. Interestingly, this family also catalyses nitro reduction in α- or β-alkyl-β-arylnitroalkenes as a side reaction to the classical activated C=C reduction with the substrate bound in an alternate conformation [30,32]. In this case, α,β-substituted arylnitroalkene nitro reduction usually proceeds via dehydration to the equivalent nitroso intermediate, followed by alkene reduction and tautomerisation to form the oxime product [30]. Subsequent steps yield an unstable imine species, which spontaneously hydrolyses to form a carbonyl compound and ammonia, in what has been described as a biological equivalent of the Nef reaction [32]. This highlights two unrelated families of enzymes that show multiple binding conformations of nitro-containing substrates, with both containing FMN and catalysing nitro reduction, albeit with different final products.

Alternate hypotheses have arisen to explain the prevalence of non-productive binding conformations seen within co-crystal structures of FMN-containing oxidoreductases. One less favoured model for NRs is that an initial electron transfer from FMN occurs instead of hydride transfer [33,34]. This would be followed by proton transfer from a bound water molecule, with the subsequent transfer of a second electron and proton almost simultaneously. For such a mechanism to occur, only the electron orbitals of FMN and the substrate need to overlap, suggesting the crystal structure with the ligand in the flipped orientation could potentially represent an active conformation. This model was supported by early MD simulations of *E. coli* NfsB with prodrug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) [33] and *Enterobacter cloacae* NR with nitrobenzene [35]. Later studies disputed this by showing small kinetic H/D isotope effects with nitrobenzoate reduction. More recently, the redox potential of NfsA was determined, which found the two single electron transfer steps have redox potentials very close together (E1 = −272 ± 7 V and E2 = −268 ± 10 V) [9]. This suggests a simultaneous two-electron reduction is more likely over two single electron transfer steps, although redox potentials can change with substrate presence.

The prevailing model for nitrofurantoin reduction by NfsA is a direct hydride transfer from the N5 of reduced FMN to a nitro group oxygen atom [9]. This is consistent with earlier studies on the NfsA-catalysed reduction in quinones and nitroaromatic compounds, which demonstrated a general 'ping-pong' reaction scheme, with nitroaromatic substrate degradation showing a linear dependence on a single-electron reduction potential [36]. Studies further showed that data on the reduction in quinones by NfsA also supports a single-step hydride transfer mechanism [36]. Based on these observations, the 'flipped' binding modes seen in NR co-crystal structures would be indicative of stable non-reactive complexes that predominate when FMN is in the oxidised state [9]. Following NAD(P)H-dependent FMN reduction, the active conformation(s) may become more prevalent due to electronic redistribution around the FMN. However, visualisation of this by the generation and cryo-trapping of two-electron reduced anaerobic NR crystals soaked with a substrate analogue or product is likely to be technically challenging, although this approach has been demonstrated for other enzyme systems [37].

Precedent for alterations in the active site architecture between oxidised and reduced FMN-containing enzymes is seen with earlier crystallisation studies with PETNR [29]. The active site of two-electron reduced PETNR showed the absence of an anion (e.g. acetate) normally seen in oxidised enzyme structures. This is consistent with an increased electron density in the pyrimidine moiety of FMN. In addition, there was a more pronounced butterfly bending of the isoalloxazine ring around the N5-N10 axis [29]. Taken together, both structural and electronic changes in the active site environment upon FMN reduction may be sufficient to induce significant changes in how ligands bind to the enzyme. This was demonstrated by X-ray crystallographic and Raman spectroscopy studies of the Michaelis complex of both oxidised and reduced XenA in the presence

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of coumarin ligands [31]. In this case, crystal structures showed distinct differences in the orientation of 8-hydroxycoumarin between the oxidised and reduced co-crystal structures, with both displaying a flattened structure. However, only the reduced complex displayed the rich vibrational band pattern between the reduced flavin and the substrate, which likely accelerates the reaction [31].

The use of computational approaches, such as molecular dynamics (MD) simulations [38,39], is becoming common place as an alternative to investigating potential enzyme-ligand interactions in the absence of crystallographic data with ligands bound in a catalytically competent orientation. This approach was taken recently to investigate a range of potential binding conformations of nitrofurantoin with oxidised and reduced forms of NfsA. Initial MD simulations with the 'flipped' nitrofurantoin-bound oxidised dimeric NfsA structure showed the ligand orientation was maintained for over 200 nm in both monomers, suggesting the crystallographic snapshot is a stable conformation [9]. Replication of the NfsA simulations with a reduced FMN showed that neither monomer showed stable nitrofurantoin retention in the flipped binding mode. This suggests that nitrofurantoin preferentially binds in different conformations within the oxidised and reduced states of NfsA [9]. These data are consistent with analogous MD simulations of *E. cloacae* NR with p-nitrobenzoate [27], which also showed a difference in orientation of the ligand between the oxidised and reduced states of the enzyme [34].

A computational model of nitrofurantoin-bound reduced NfsA was generated that was consistent with a direct hydride transfer mechanism (Figure 1e), with one of the nitro oxygen atoms of the substrate ~3.6 Å from the N5 of $FMNH^-$ [9]. Molecular dynamics simulations showed the formation of multiple hydrogen bonds between the protein and both the nitro group and the furan ring of the substrate. A good binding enthalpy of the complex was calculated ($\max \sim -22$ kcal mol^{-1}), however the exact distance between the N5 atom of $FMNH^-$ and the nearest ligand nitro oxygen atom fluctuated during the simulation [9]. The latter suggests either further improvements in the modelled orientation of the bound ligand are required, or a range of possible electron transfer competent states may be possible. However, given the poor retention of the crystallographically determined ligand position during simulations with the reduced enzyme, this model could represent a realistic approximation of the binding mode of nitrofurantoin to NfsA during the normal course of the reaction.

In conclusion, recognition of the role of the electronic state of cofactors in substrate binding adds an additional dimension to our understanding of the dynamics of ligand binding in NRs and other oxidoreductases. Crystal structures give static 'snapshots' of enzyme-ligand states with some dynamic information. Contributions by NMR solution structures and computational models can assist greatly when multiple conformational states are apparent or suspected. Simulating ligand binding in both the oxidised and reduced forms of NRs could inform on the relative likelihood of whether new analogues will act as substrates or inhibitors within the reduced enzyme. For NR-dependent biomedical drug development, this collaborative approach is a valuable addition to the toolbox for predicting potential active conformations and ranking the likely effectiveness of newly designed target nitrofuran derivatives.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

FMN, flavin mononucleotide; $FMNH^-$, two-electron reduced flavin mononucleotide; MD, molecular dynamics; NAD(P)H, nicotinamide adenine dinucleotide (phosphate) reduced form; NfsA and NfsB, major oxygen-insensitive nitroreductases from *E. coli*; NO_x, nitrogen oxides; NRs, nitroreductases; PETNR, pentaerythritol tetranitrate reductase; XenA, xenobiotic reductase A.

References

1 Bryant, C. and Luca, M. (1991) *Chemistry and Biochemistry of Flavoenzymes. II*, CRC Press, Boca Raton, FL
2 Bryant, D.W., McCalla, D.R., Leeksma, M. and Laneuville, P. (1981) Type I nitroreductases of *Escherichia coli*. *Can. J. Microbiol.* **27**, 81–86 https://doi.org/10.1139/m81-013
3 Boddu, R.S., Perumal, O. and K, D. (2020) Microbial nitroreductases: a versatile tool for biomedical and environmental applications. *Biotechnol. Appl. Biochem.* https://doi.org/10.1002/bab.2073

4 Race, P.R., Lovering, A.L., Green, R.M., Ossor, A., White, S.A., Searle, P.F. et al. (2005) Structural and mechanistic studies of *Escherichia coli* nitroreductase with the antibiotic nitrofurazone: reversed binding orientations in different redox states of the enzyme. *J. Biol. Chem.* **280**, 13256–13264 https://doi.org/10.1074/jbc.M409652200
5 Spain, J.C. (1995) Biodegradation of nitroaromatic compounds. *Annu. Rev. Microbiol.* **49**, 523–555 https://doi.org/10.1146/annurev.mi.49.100195.002515
6 Traversi, D., Degan, R., De Marco, R., Gilli, G., Pignata, C., Villani, S. et al. (2009) Mutagenic properties of PM2.5 urban pollution in the northern Italy: the nitro-compounds contribution. *Environ. Int.* **35**, 905–910 https://doi.org/10.1016/j.envint.2009.03.010
7 Roldán, M.D., Pérez-Reinado, E., Castillo, F. and Moreno-Vivián, C. (2008) Reduction of polynitroaromatic compounds: the bacterial nitroreductases. *FEMS Microbiol. Rev.* **32**, 474–500 https://doi.org/10.1111/j.1574-6976.2008.00107.x
8 Olekhnovich, I.N., Goodwin, A. and Hoffman, P.S. (2009) Characterization of the NAD(P)H oxidase and metronidazole reductase activities of the RdxA nitroreductase of *Helicobacter pylori*. *FEBS J.* **276**, 3354–3364 https://doi.org/10.1111/j.1742-4658.2009.07060.x
9 Day, M.A., Jarrom, D., Christofferson, A.J., Graziano, A.E., Anderson, J.L.R., Searle, P.F. et al. (2021) The structures of *E. coli* NfsA bound to the antibiotic nitrofurantoin; to 1,4-benzoquinone and to FMN. *Biochem. J.* **478**, 2601–2617 https://doi.org/10.1042/BCJ20210160
10 Anlezark, G.M., Melton, R.G., Sherwood, R.F., Coles, B., Friedlos, F. and Knox, R.J. (1992) The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)—I purification and properties of a nitroreductase enzyme from *Escherichia coli*—a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). *Biochem. Pharmacol.* **44**, 2289–2295 https://doi.org/10.1016/0006-2952(92)90671-5
11 Zenno, S., Koike, H., Tanokura, M. and Saigo, K. (1996) Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *Escherichia coli*, similar in biochemical properties to FRase I, the major flavin reductase in *Vibrio fischeri*. *J. Biochem.* **120**, 736–744 https://doi.org/10.1093/oxfordjournals.jbchem.a021473
12 Peterson, F.J., Mason, R.P., Hovsepian, J. and Holtzman, J.L. (1979) Oxygen-sensitive and -insensitive nitroreduction by *Escherichia coli* and rat hepatic microsomes. *J. Biol. Chem.* **254**, 4009–4014 https://doi.org/10.1016/S0021-9258(18)50687-6
13 Guay, D.R. (2001) An update on the role of nitrofurans in the management of urinary tract infections. *Drugs* **61**, 353–364 https://doi.org/10.2165/00003495-200161030-00004
14 McCalla, D.R., Olive, P., Tu, Y. and Fan, M.L. (1975) Nitrofurazone-reducing enzymes in *E. coli* and their role in drug activation *in vivo*. *Can. J. Microbiol.* **21**, 1484–1491 https://doi.org/10.1139/m75-220
15 McOsker, C.C. and Fitzpatrick, P.M. (1994) Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. *J. Antimicrob. Chemother.* **33**, 23–30 https://doi.org/10.1093/jac/33.suppl_A.23
16 Williams, E.M., Little, R.F., Mowday, A.M., Rich, M.H., Chan-Hyams, J.V.E., Copp, J.N. et al. (2015) Nitroreductase gene-directed enzyme prodrug therapy: insights and advances toward clinical utility. *Biochem. J.* **471**, 131–153 https://doi.org/10.1042/BJ20150650
17 Clark, A.J., Iwobi, M., Cui, W., Crompton, M., Harold, G., Hobbs, S. et al. (1997) Selective cell ablation in transgenic mice expressing *E. coli* nitroreductase. *Gene Ther.* **4**, 101–110 https://doi.org/10.1038/sj.gt.3300367
18 Curado, S., Anderson, R.M., Jungblut, B., Mumm, J., Schroeter, E. and Stainier, D.Y.R. (2007) Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev. Dyn.* **236**, 1025–1035 https://doi.org/10.1002/dvdy.21100
19 Kaya, F., Mannioui, A., Chesneau, A., Sekizar, S., Maillard, E., Ballagny, C. et al. (2012) Live imaging of targeted cell ablation in *Xenopus*: a new model to study demyelination and repair. *J. Neurosci.* **32**, 12885–12895 https://doi.org/10.1523/JNEUROSCI.2252-12.2012
20 Hannink, N., Rosser, S.J., French, C.E., Basran, A., Murray, J.A.H., Nicklin, S. et al. (2001) Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase. *Nat. Biotechnol.* **19**, 1168–1172 https://doi.org/10.1038/nbt1201-1168
21 Panz, K. and Miksch, K. (2012) Phytoremediation of explosives (TNT, RDX, HMX) by wild-type and transgenic plants. *J. Environ. Manage.* **113**, 85–92 https://doi.org/10.1016/j.jenvman.2012.08.016
22 Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. et al. (2004) UCSF chimera–a visualization system for exploratory research and analysis. *J. Comput. Chem.* **13**, 1605–1612 https://doi.org/10.1002/jcc.20084
23 Kobori, T., Sasaki, H., Lee, W.C., Zenno, S., Saigo, K., Murphy, M.E.P. et al. (2001) Structure and site-directed mutagenesis of a flavoprotein from *Escherichia coli* that reduces nitrocompounds: alteration of pyridine nucleotide binding by a single amino acid substitution. *J. Biol. Chem.* **276**, 2816–2823 https://doi.org/10.1074/jbc.M002617200
24 Tanner, J.J., Barbour, L.J., Barnes, C.L., Tu, S.C. and Krause, K.L. (1999) Unusual folded conformation of nicotinamide adenine dinucleotide bound to flavin reductase P. *Protein Sci.* **8**, 1725–1732 https://doi.org/10.1110/ps.8.9.1725
25 Tanner, J.J., Lei, B., Tu, S.-C. and Krause, K.L. (1996) Flavin reductase P: structure of a dimeric enzyme that reduces flavin. *Biochemistry* **35**, 13531–13539 https://doi.org/10.1021/bi961400v
26 Haynes, C.A., Koder, R.L., Miller, A.-F. and Rodgers, D.W. (2002) Structures of nitroreductase in three states: effects of inhibitor binding and reduction. *J. Biol. Chem.* **277**, 11513–11520 https://doi.org/10.1074/jbc.M111334200
27 Pitsawong, W., Haynes, C.A., Koder, R.L., Rodgers, D.W. and Miller, A.-F. (2017) Mechanism-informed refinement reveals altered substrate-binding mode for catalytically competent nitroreductase. *Structure* **25**, 978–987.e974 https://doi.org/10.1016/j.str.2017.05.002
28 Lovering, A.L., Hyde, E.I., Searle, P.F. and Scott, A.W. (2001) The structure of *Escherichia coli* nitroreductase complexed with nicotinic acid: three crystal forms at 1.7 A 1 A and 2.4 A resolution. *J. Mol. Biol.* **309**, 203–213 https://doi.org/10.1006/jmbi.2001.4653
29 Barna, T.M., Khan, H., Bruce, N.C., Barsukov, I., Scrutton, N.S. and Moody, P.C.E. (2001) Crystal structure of pentaerythritol tetranitrate reductase: "flipped" binding geometries for steroid substrates in different redox states of the enzyme. *J. Mol. Biol.* **310**, 433–447 https://doi.org/10.1006/jmbi.2001.4779
30 Toogood, H.S., Fryszkowska, A., Hulley, M., Sakuma, M., Mansell, D., Stephens, G.M. et al. (2011) A site-saturated mutagenesis study of pentaerythritol tetranitrate reductase reveals that residues 181 and 184 influence ligand binding, stereochemistry and reactivity. *ChemBioChem* **12**, 738–749 https://doi.org/10.1002/cbic.201000662
31 Werther, T., Wahlefeld, S., Salewski, J., Kuhlmann, U., Zebger, I., Hildebrandt, P. et al. (2017) Redox-dependent substrate-cofactor interactions in the Michaelis-complex of a flavin-dependent oxidoreductase. *Nat. Commun.* **8**, 16084 https://doi.org/10.1038/ncomms16084
32 Durchschein, K., Ferreira-da Silva, B., Wallner, S., Macheroux, P., Kroutil, W., Glueck, S.M. et al. (2010) The flavoprotein-catalyzed reduction of aliphatic nitro-compounds represents a biocatalytic equivalent to the Nef-reaction. *Green Chem.* **12**, 616–619 https://doi.org/10.1039/b922691e

Biochemical Journal (2021) **478** 3423–3428
https://doi.org/10.1042/BCJ20210489

33 Christofferson, A. and Wilkie, J. (2009) Mechanism of CB1954 reduction by *Escherichia coli* nitroreductase. *Biochem. Soc. Trans.* **37**, 413–418 https://doi.org/10.1042/BST0370413
34 Christofferson, A.J. (2020) Asymmetric ligand binding in homodimeric *Enterobacter cloacae* nitroreductase yields the Michaelis complex for nitroaromatic substrates. *J. Mol. Model.* **26**, 28 https://doi.org/10.1007/s00894-020-4288-9
35 Isayev, O., Crespo-Hernández, C.E., Gorb, L., Hill, F.C. and Leszczynski, J. (2012) *In silico* structure–function analysis of *E. cloacae* nitroreductase. *Proteins* **80**, 2728–2741 https://doi.org/10.1002/prot.24157
36 Valiauga, B., Williams, E.M., Ackerley, D.F. and Čėnas, N. (2017) Reduction of quinones and nitroaromatic compounds by *Escherichia coli* nitroreductase A (NfsA): characterization of kinetics and substrate specificity. *Arch. Biochem. Biophys.* **614**, 14–22 https://doi.org/10.1016/j.abb.2016.12.005
37 Masgrau, L., Roujeinikova, A., Johannissen, L.O., Hothi, P., Basran, J., Ranaghan, K.E. et al. (2006) Atomic description of an enzyme reaction dominated by proton tunneling. *Science* **312**, 237–241 https://doi.org/10.1126/science.1126002
38 Hess, B., Kutzner, C., Spoel, D… and Lindahl, E. (2008) GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theory Comput.* **4**, 435–447 https://doi.org/10.1021/ct700301q
39 Silva, A.W.S.. and Vranken, W.F. (2012) ACPYPE: AnteChamber PYthon Parser interfacE. *BMC Res. Notes* **5**, 367 https://doi.org/10.1186/1756-0500-5-367