

Changing the electron donor improves azoreductase dye degrading activity at neutral pH

Qi, Jingxian; Paul, Caroline E.; Hollmann, Frank; Tischler, Dirk

DOI

[10.1016/j.enzmictec.2017.02.003](https://doi.org/10.1016/j.enzmictec.2017.02.003)

Publication date

2017

Document Version

Accepted author manuscript

Published in

Enzyme and Microbial Technology: biotechnology research and reviews

Citation (APA)

Qi, J., Paul, C. E., Hollmann, F., & Tischler, D. (2017). Changing the electron donor improves azoreductase dye degrading activity at neutral pH. *Enzyme and Microbial Technology: biotechnology research and reviews*, 100, 17-19. <https://doi.org/10.1016/j.enzmictec.2017.02.003>

Important note

To cite this publication, please use the final published version (if applicable). Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights. We will remove access to the work immediately and investigate your claim.

1 Changing the electron donor improves azoreductase dye degrading activity at neutral pH

2 Jingxian Qi^{a,‡}; Caroline E. Paul^b; Frank Hollmann^b; Dirk Tischler^{a,‡};

3
4 ^a Institute of Biosciences, TU Bergakademie Freiberg, Leipziger Str. 29, Freiberg 09599,
5 Germany

6 ^b Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ
7 Delft, The Netherlands

8
9 [‡] Corresponding authors: JQ and DT, Institute of Biosciences, TU Bergakademie Freiberg,
10 Leipziger Str. 29, Freiberg 09599, Germany. Phone: +49(0)3731/394151; Fax:
11 +49(0)3731/393012. E-mail address: jingxianqi@gmail.com or dirk-tischler@email.de

13 **Abstract:**

14 The oxygen-insensitive azoreductase AzoRo originating from *Rhodococcus opacus* 1CP was
15 found to be most active at low pH (ca. 4) and high temperature (ca. 50 °C). AzoRo is not an
16 efficient biocatalyst when used at low pH due to stability problems. To overcome this issue, we
17 discovered that AzoRo accepts an alternative electron donor, 1-benzyl-1,4-dihydronicotinamide
18 (BNAH), which allows fast turnover at neutral pH. In order to screen this nicotinamide coenzyme
19 mimic as a source of electrons, AzoRo-catalysed reactions were run under neutral conditions,
20 under which typically slow rates are observed with NADH. For the reduction of 1 azo bond by
21 azoreductases 2 moles nicotinamide coenzyme are needed. AzoRo displayed Methyl Red (MR)
22 reduction activities with NADH and NADPH of 5.49 ± 0.14 U mg⁻¹ and 4.96 ± 0.25 U mg⁻¹,
23 respectively, whereas with BNAH it displayed 17.01 ± 0.74 U mg⁻¹ (following BNAH oxidation)
24 and 7.16 ± 0.06 U mg⁻¹ (following MR reduction). Binding of BNAH to AzoRo was determined
25 with a K_m of 18.75 ± 2.45 μM (BNAH oxidation) and 12.45 ± 0.47 μM (MR reduction). In order to
26 show applicability of this system an upscaled reaction was performed using 78.6 μg of purified
27 AzoRo to convert 2.96 μmol of MR (total reaction volume: 40 ml) within a 1 hour reaction.

28
29 **Key words:** nicotinamide cofactor mimics, *Rhodococcus*, Methyl Red degradation, 1-benzyl-1,4-
30 dihydronicotinamide, azoreductase, azo dyes

31
32 **Running title:** BNAH-driven azoreductase

34 **Highlights:**

35 - AzoRo accepts nicotinamide-like cofactors.

36 - BNAH allows azo dye degradation at neutral pH.

37 - Upscaling of MR-reduction is possible.

38

39 **Background**

40 Azo dyes contain one or more azo bonds ($R_1-N=N-R_2$) and are frequently employed in various
41 industries such as food, chemical, textile among others [1-2]. These azo dyes have been
42 released in the environment due to human activities, and microorganisms have evolved or
43 adapted metabolic routes to deal with them. Another group of specific enzymes directly involved
44 in azo dye removal via azo bond reduction has been reported [3-4]. Recently, we described a
45 novel member of this enzyme family as an oxygen-insensitive NADH-dependent FMN utilizing
46 azoreductase (AzoRo) [5]. The AzoRo enzyme belongs to a distinct subtype of azoreductases
47 related to AzoR which originates from *E. coli* [6]. These azoreductases prefer Methyl Red (MR,
48 2-(*N,N*-dimethyl-4-aminophenyl)), as a substrate and use the nicotinamide coenzyme NADH as
49 an electron donor [5-6].

50 One challenge with using the recently discovered AzoRo is the low pH required (pH 4) for rapid
51 and efficient dye degradation (MR degradation activity 141.25 U mg^{-1}) [5]. However, under those
52 conditions the enzyme was found to be rather unstable, and the best use of a related enzyme
53 was only possible through immobilization [7]. The required NADH cofactor is also unstable at
54 acidic pH [8-9]. Thus a highly efficient regeneration system is necessary. Alternatively, a less
55 expensive reductant, a synthetic nicotinamide cofactor analogue, can be used [10-12]. These
56 coenzyme biomimetics have become more relevant over the last decades to investigate
57 oxidoreductases. The most employed mimic is the 1-benzyl-1,4-dihydronicotinamide (BNAH),
58 which can be easily synthesized and used in stoichiometric amounts, or with a regeneration
59 system [13-14].

60 Here we aimed to investigate the possibility to first employ BNAH as an electron donor with
61 AzoRo for the reduction of azo dyes (Scheme 1), secondly to check the efficiency of AzoRo at a
62 more neutral pH to overcome enzyme stability issues encountered at acidic pH. Finally, the
63 reaction process was upscaled to show potential applications.

64

65 *Scheme 1. Azoreductase-catalysed degradation of azo dye methyl red with 1-benzyl-1,4-*
66 *dihydronicotinamide (BNAH) as an electron donor.*

67 **Methods**

68 The enzymatic activity of AzoRo was measured by a UV-vis spectrophotometer at the maximum
69 absorption wavelength of substrates at ambient temperature (22 °C). Standard reaction
70 conditions involved starting with 1.97 µg of purified AzoRo in 1 ml final volume containing 20 mM

71 Tris-HCl buffer at pH 7.2, 150 μM BNAH, 50 μM FMN and 30 μM MR in a quartz cuvette of 1 cm
72 path length at 430 nm ($\epsilon_{\text{MR}} = 23.36 \text{ (mM cm)}^{-1}$) [5]. Triplicate measurements were performed for
73 each assay. Two control assays were conducted: one was performed without enzyme and
74 another was conducted without FMN and enzyme. In addition to BNAH, NADH and NADPH
75 were used as electron donors for comparison.

76 The maximum absorbance wavelength of BNAH is 358 nm in 20 mM Tris-HCl at pH 7.2. Initial
77 velocities of the enzymatic reaction performed by varying concentrations of BNAH were
78 measured at 358 nm for BNAH consumption ($\epsilon_{\text{BNAH}} = 6.18 \text{ (mM cm)}^{-1}$) and at 430 nm for MR
79 consumption. The BNAH concentrations varied from 10 to 125 μM in BNAH consumption assays
80 and from 10 to 200 μM in MR consumption assays. Apparent kinetic parameters (K_m and V_{max})
81 were obtained from nonlinear Michaelis-Menten assumption. One unit (U) of AzoRo activity was
82 defined as the amount of protein required to degrade 1 μmol substrate per minute whereas the
83 reductant or MR could be considered as a substrate.

84 In addition to MR as a substrate, BNAH was further used with AzoRo to reduce other azo dyes
85 such as Methyl Orange (MO) and Brilliant Black (BB) which could be degraded by AzoRo
86 utilizing NADH [5]. A 10 min standard assay was conducted utilizing appropriate substrates (30
87 μM MO, 30 μM BB) at 465 nm ($\epsilon_{\text{MO}} = 23.25 \text{ (mM cm)}^{-1}$) and 570 nm ($\epsilon_{\text{BB}} = 56.5 \text{ (mM cm)}^{-1}$),
88 respectively.

89 AzoRo-catalysed MR reduction was upscaled to 40 ml to degrade 84 μM MR, starting with 150
90 μM BNAH and 50 μM FMN. Samples were taken every 15 min over 1 hour. An additional 150
91 μM of BNAH was added after 30 min.

92 **Results and Discussion**

93 The enzyme AzoRo was successfully produced as described before and stored at $-20 \text{ }^\circ\text{C}$ in
94 storage buffer [5]. Previous investigations revealed the enzyme is most active (141.25 U mg^{-1} ;
95 determined at 525 nm following MR reduction) at pH 4 and accepts NADH as an electron donor
96 while using FMN as a shuttle in order to reduce the azo group of MR. Furthermore, MR was
97 determined as the favored substrate and thus biochemical as well as phylogenetic data allowed
98 to classify AzoRo of the strain 1CP with AzoR-like azoreductases, which have the azoreductase
99 from *E. coli* as prototype [5-6].

100 All attempts to determine accurate kinetic parameter (v_{max} and K_m) at neutral pH (7.2) failed with
101 AzoRo. There was no clear tendency measurable that would allow fitting according to
102 Michaelis-Menten (not shown). Data fitting was only possible at a pH of 6 by following the NADH
103 oxidation to provide a v_{max} of 51.38 U mg^{-1} and K_m of 9.71 μM [5]. However, it was possible to
104 determine a maximum MR reducing activity under neutral pH conditions (20 mM Tris-HCl buffer,
105 pH 7.2, 50 μM FMN and 150 μM electron donor; following MR reduction) for NADH (5.49 ± 0.14

106 U mg⁻¹), NADPH (4.96 ± 0.25 U mg⁻¹) and BNAH (6.76 ± 0.13 U mg⁻¹). Those activities show that
107 among the natural nicotinamide cofactors, NADH is slightly favored over NADPH (90% activity of
108 NADH). Considering the errors this difference is rather small and might indicate that at neutral
109 pH AzoRo does not differentiate between these two electron donors. This result fits to the
110 previously made classification of AzoRo [5], and the capability to employ both NADH and
111 NADPH as an electron donor is typically for AzoR-like azoreductases [6]. The maximum activity
112 achieved with the above concentrations is in congruence to the previously reported data (2.50 U
113 mg⁻¹ in phosphate buffer, pH 7.0) [5]. The artificial electron donor BNAH was better suited to
114 reduce MR (123% activity of NADH), and this is the first report that azoreductases can actually
115 use synthetic nicotinamide analogues as cofactors.

116 BNAH, like NADH and NADPH, is unstable at acidic pH but stable at neutral pH, therefore we
117 tried to determine AzoRo dependency on this artificial cofactor at pH 7.2. Because of the higher
118 activity with BNAH and the better stability of AzoRo at neutral pH, it was now possible to
119 generate data sets suitable for an analysis according to Michaelis-Menten (Fig. 1). And it need to
120 be mentioned that BNAH does not reduce MR in the absence of AzoRo under herein employed
121 assay conditions at pH 7.2.

122
123 *Fig. 1: Kinetic analysis of AzoRo utilizing BNAH at various concentrations as the electron donor.*
124 *A) assay at 358 nm; B) assay at 430 nm. Prior each assay the standard assay as described*
125 *previously [5] had been determined as a reference point. Then the assay for the herein*
126 *determined kinetics have been prepared accordingly with 1.97 µg AzoRo in 1 ml cuvette*
127 *containing Tris-HCl buffer (20 mM, pH 7.2), 50 µM FMN and 30 µM MR. Reagents were*
128 *incubated to have proper temperature (22 °C) and the assay was started by adding enzyme.*
129 *Standard deviations of triplicates were included (< 8.1%). According to the reaction scheme 1*
130 *AzoRo employs 2 moles of BNAH in order to reduce 1 mole of MR.*

131
132 The results obtained clearly demonstrate that BNAH allows characterization of the enzyme
133 AzoRo at a neutral pH at which it is more stable. The binding of BNAH to AzoRo and the specific
134 activities were investigated while following BNAH oxidation as well as MR reduction (Fig. 1;
135 Scheme 1). Therefore, enzyme and FMN concentrations were set constant and the BNAH
136 concentration was varied. In the case of BNAH oxidation, a v_{max} of 17.01 ± 0.74 U mg⁻¹ and a K_m
137 of 18.75 ± 2.45 µM were obtained; the k_{cat} was 7.19 s⁻¹ and k_{cat}/K_m was 0.38 µM⁻¹·s⁻¹. The
138 experiment was repeated, this time following the reduction of MR giving a v_{max} of 7.16 ± 0.06 U
139 mg⁻¹ and a K_m of 12.45 ± 0.47 µM; the k_{cat} was 3.02 s⁻¹ and k_{cat}/K_m was 0.24 µM⁻¹·s⁻¹. Compared
140 to NADH as the electron donor, the activity of AzoRo for MR reduction at optimal pH 4 is faster

141 by a factor of about 20, but cannot be measure at pH 7.2. At this stage it is worth mentioning that
142 at pH 4 high activity was observed with BNAH reducing MR without the enzyme. No difference in
143 activity for MR reduction was observed with or without AzoRo.

144 Theoretically the enzyme AzoRo needs 2 mol of BNAH to efficiently reduce 1 mol of MR, or it
145 may simply need more BNAH due to an uncoupling reaction [15]. From the results obtained it
146 becomes clear that AzoRo consumes about 2.4 mol BNAH per mol MR, which implies AzoRo
147 has some uncoupling reaction under the conditions applied. The BNAH oxidation activity of
148 AzoRo in the absence of MR is up to $1.66 \pm 0.3 \text{ U mg}^{-1}$ (9.8 %), which clearly demonstrates the
149 uncoupling. This means AzoRo unproductively oxidizes BNAH as previously observed for NADH
150 [5]. In the case of NADH, with a different buffer and pH, unproductive NADH oxidation amounted
151 to $1.81 \pm 0.11 \text{ U mg}^{-1}$ (3.5 %). This indicates NADH might be a better substrate for the reductive
152 half reaction. The unproductive oxidation of NADH or BNAH results in reduced FMN which may
153 undergo certain reaction yielding most likely hydrogen peroxide as product [15]. Taking the
154 uncoupling and the errors determined into account the ratio BNAH oxidation to MR reduction is
155 almost 2 according to the supposed reaction (Scheme 1).

156 AzoRo, in combination with BNAH as a cost-effective cofactor mimic [16], were thereafter
157 employed to degrade MO and BB as well. The activity of MO degradation was 0.96 U mg^{-1} , 14.2 %
158 of MR degradation activity. However, it turned out that BNAH itself could react with BB, causing
159 obvious degradation at 570 nm. Thus clearly BNAH was identified as a powerful reductant which
160 itself can degrade azo dyes (as seen above with MR at pH 4). Hence, at this stage further
161 substrates for AzoRo with BNAH were not screened. The AzoRo-catalysed reduction of MR was
162 upscaled, monitoring MR reduction at 430 nm. A final amount of 2.96 μmol of MR was degraded
163 by 78.6 μg of AzoRo within one hour; the total removal efficiency was 88%.

164 In conclusion, AzoRo accepts the artificial cofactor BNAH as an electron donor, which enables
165 the degradation of azo dyes at moderate pH and temperature. A substrate test and an upscaling
166 were successfully achieved (MR conversion of $20 \text{ mg l}^{-1} \text{ h}^{-1}$ by 2 mg l^{-1} AzoRo). It now gives the
167 possibility studying the combination of AzoRo and nicotinamide cofactor mimics. The ability of
168 BNAH to directly reduce azo dyes will be further investigated.

169 **Acknowledgement**

170 JQ was supported by a pre-doctoral scholarship from China Scholarship Council
171 (201206400019) and DT was funded by the European Social Fund (ESF) and the Saxonian
172 Government (GETGEOWEB: 100101363). CEP acknowledges her NWO Veni (number
173 722.015.011) for financial support.

174 **References**

- 175 [1] F. Rafii, J.D. Hall, C.E. Cerniglia, Mutagenicity of azo dyes used in foods, drugs and
176 cosmetics before and after reduction by *Clostridium* species from the human intestinal
177 tract, *Food Chem. Toxicol.* 35 (1997) 897-901.
- 178 [2] S. Pricelius, C. Held, S. Sollner, S. Deller, M. Murkovic, R. Ullrich, M. Hofrichter, A.
179 Cavaco-Paulo, P. Macheroux, G.M. Guebitz, Enzymatic reduction and oxidation of fibre-
180 bound azo-dyes, *Enzyme Microb. Technol.* 40 (2007) 1732-1738.
- 181 [3] A. Stolz, Basic and applied aspects in the microbial degradation of azo dyes, *Appl.*
182 *Microbiol. Biotechnol.* 56 (2001) 69-80.
- 183 [4] R.L. Singh, P.K. Singh, R.P. Singh, Enzymatic decolorization and degradation of azo
184 dyes – A review, *Int. Biodeter. Biodegr.* 104 (2015) 21-31.
- 185 [5] J. Qi, M. Schlömann, D. Tischler, Biochemical characterization of an azoreductase from
186 *Rhodococcus opacus* 1CP possessing methyl red degradation ability, *J. Mol. Catal. B:*
187 *Enzym.* 130 (2016) 9-17.
- 188 [6] M. Nakanishi, C. Yatome, N. Ishida, Y. Kitade, Putative ACP phosphodiesterase gene
189 (*acpD*) encodes an azoreductase, *J. Biol. Chem.* 276 (2001) 46394-46399.
- 190 [7] W. Lang, S. Sirisansaneeyakul, L. Ngiswara, S. Mendes, L.O. Martins, M. Okuyama, A.
191 Kimura, Characterization of a new oxygen-insensitive azoreductase from *Brevibacillus*
192 *laterosporus* TISTR1911: Toward dye decolorization using a packed-bed metal affinity
193 reactor, *Bioresour. Technol.* 150 (2013) 298-306.
- 194 [8] O.H. Lowry, J.V. Passonneau, M.K. Rock, The stability of pyridine nucleotides, *J. Biol.*
195 *Chem.* 236 (1961) 2756-2759.
- 196 [9] J.T. Wu, L.H. Wu, J.A. Knight, Stability of NADPH - Effect of various factors on the
197 kinetics of degradation, *Clinical Chemistry* 32 (1986) 314-319.
- 198 [10] P. Karrer, F.J. Stare, N-Alkyl-O-dihydro-nicotinic acid-amide, *Helv. Chim. Acta* 20 (1937)
199 418-423.
- 200 [11] C.E. Paul, I.W.C.E. Arends, F. Hollmann, Is simpler better? Synthetic nicotinamide
201 cofactor analogues for redox chemistry, *ACS Catal.* 4 (2014) 788-797.
- 202 [12] C.E. Paul, F. Hollmann, A survey of synthetic nicotinamide cofactors in enzymatic
203 processes, *Appl. Microbiol. Biotechnol.* 100 (2016) 4773-4778.
- 204 [13] Y. Okamoto, V. Köhler, C.E. Paul, F. Hollmann, T.R. Ward, Efficient in situ regeneration
205 of NADH mimics by an artificial metalloenzyme, *ACS Catal.* 6 (2016) 3553-3557.
- 206 [14] C.E. Paul, S. Gargiulo, D.J. Opperman, I. Lavandera, V. Gotor-Fernández, V. Gotor, A.
207 Taglieber, I.W.C.E. Arends, F. Hollmann, Mimicking nature: synthetic nicotinamide
208 cofactors for C=C bioreduction using enoate reductases, *Org. Lett.* 15 (2013) 180-183.

- 209 [15] V. Massey, Activation of molecular oxygen by flavins and flavoproteins, *J. Biol. Chem.*
210 269 (1994) 22459-22462.
- 211 [16] C.E. Paul, D. Tischler, A. Riedel, T. Heine, N. Itoh, F. Hollmann, Nonenzymatic
212 regeneration of styrene monooxygenase for catalysis, *ACS Catal.* 5 (2015) 2961-2965.





