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

The oxygen-insensitive azoreductase AzoRo originating from *Rhodococcus opacus* 1CP was 14 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 discovered that AzoRo accepts an alternative electron donor, 1-benzyl-1,4-dihydronicotinamide 17 18 (BNAH), which allows fast turnover at neutral pH. In order to screen this nicotinamide coenzyme mimic as a source of electrons, AzoRo-catalysed reactions were run under neutral conditions, 19 under which typically slow rates are observed with NADH. For the reduction of 1 azo bond by 20 21 azoreductases 2 moles nicotinamide coenzyme are needed. AzoRo displayed Methyl Red (MR) reduction activities with NADH and NADPH of 5.49 \pm 0.14 U mg⁻¹ and 4.96 \pm 0.25 U mg⁻¹, 22 respectively, whereas with BNAH it displayed 17.01 \pm 0.74 U mg⁻¹ (following BNAH oxidation) 23 and 7.16 \pm 0.06 U mg⁻¹ (following MR reduction). Binding of BNAH to AzoRo was determined 24 25 with a K_m of 18.75 ± 2.45 μ M (BNAH oxidation) and 12.45 ± 0.47 μ M (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.

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

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32 Running title: BNAH-driven azoreductase

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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 in azo dye removal via azo bond reduction has been reported [3-4]. Recently, we described a 44 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 related to AzoR which originates from E. coli [6]. These azoreductases prefer Methyl Red (MR, 47 2-(N,N-dimethyl-4-aminophenyl)), as a substrate and use the nicotinamide coenzyme NADH as 48 an electron donor [5-6]. 49

One challenge with using the recently discovered AzoRo is the low pH required (pH 4) for rapid 50 51 and efficient dye degradation (MR degradation activity 141.25 U mg⁻¹) [5]. However, under those conditions the enzyme was found to be rather unstable, and the best use of a related enzyme 52 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), which can be easily synthesized and used in stoichiometric amounts, or with a regeneration 58 59 system [13-14].

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

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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

The enzymatic activity of AzoRo was measured by a UV–vis spectrophotometer at the maximum absorption wavelength of substrates at ambient temperature (22 °C). Standard reaction conditions involved starting with 1.97 µg of purified AzoRo in 1 ml final volume containing 20 mM Tris-HCl buffer at pH 7.2, 150 μ M BNAH, 50 μ M FMN and 30 μ M MR in a quartz cuvette of 1 cm path length at 430 nm ($\epsilon_{MR} = 23.36 \text{ (mM cm)}^{-1}$) [5]. Triplicate measurements were performed for each assay. Two control assays were conducted: one was performed without enzyme and another was conducted without FMN and enzyme. In addition to BNAH, NADH and NADPH 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 ($\varepsilon_{BNAH} = 6.18$ (mM cm)⁻¹) and at 430 nm for MR consumption. The BNAH concentrations varied from 10 to 125 µM in BNAH consumption assays 79 and from 10 to 200 μ M in MR consumption assays. Apparent kinetic parameters (K_m and V_{max}) 80 were obtained from nonlinear Michaelis-Menten assumption. One unit (U) of AzoRo activity was 81 defined as the amount of protein required to degrade 1 µmol substrate per minute whereas the 82 reductant or MR could be considered as a substrate. 83

In addition to MR as a substrate, BNAH was further used with AzoRo to reduce other azo dyes such as Methyl Orange (MO) and Brilliant Black (BB) which could be degraded by AzoRo utilizing NADH [5]. A 10 min standard assay was conducted utilizing appropriate substrates (30

465 nm ($ε_{MO}$ = 23.25 (mM cm)⁻¹) and 570 nm ($ε_{BB}$ = 56.5 (mM cm)⁻¹), respectively.

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

92 Results and Discussion

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

All attempts to determine accurate kinetic parameter (v_{max} and K_m) at neutral pH (7.2) failed with AzoRo. There was no clear tendency measureable that would allow fitting according to Michaelis-Menten (not shown). Data fitting was only possible at a pH of 6 by following the NADH 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 determine a maximum MR reducing activity under neutral pH conditions (20 mM Tris-HCl buffer, pH 7.2, 50 µM FMN and 150 µM electron donor; following MR reduction) for NADH (5.49 ± 0.14

U mg⁻¹), NADPH (4.96 \pm 0.25 U mg⁻¹) and BNAH (6.76 \pm 0.13 U mg⁻¹). Those activities show that 106 107 among the natural nicotinamide cofactors, NADH is slightly favored over NADPH (90% activity of NADH). Considering the errors this difference is rather small and might indicate that at neutral 108 pH AzoRo does not differentiate between these two electron donors. This result fits to the 109 110 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 mg⁻¹ in phosphate buffer, pH 7.0) [5]. The artificial electron donor BNAH was better suited to 113 reduce MR (123% activity of NADH), and this is the first report that azoreductases can actually 114 115 use synthetic nicotinamide analogues as cofactors.

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

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

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

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

Theoretically the enzyme AzoRo needs 2 mol of BNAH to efficiently reduce 1 mol of MR, or it 144 145 may simply need more BNAH due to an uncoupling reaction [15]. From the results obtained it becomes clear that AzoRo consumes about 2.4 mol BNAH per mol MR, which implies AzoRo 146 147 has some uncoupling reaction under the conditions applied. The BNAH oxidation activity of AzoRo in the absence of MR is up to $1.66 \pm 0.3 \text{ U mg}^{-1}$ (9.8 %), which clearly demonstrates the 148 149 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 to $1.81 \pm 0.11 \text{ U mg}^{-1}$ (3.5 %). This indicates NADH might be a better substrate for the reductive 151 half reaction. The unproductive oxidation of NADH or BNAH results in reduced FMN which may 152 153 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 employed to degrade MO and BB as well. The activity of MO degradation was 0.96 U mg⁻¹, 14.2 % 157 of MR degradation activity. However, it turned out that BNAH itself could react with BB, causing 158 obvious degradation at 570 nm. Thus clearly BNAH was identified as a powerful reductant which 159 itself can degrade azo dyes (as seen above with MR at pH 4). Hence, at this stage further 160 161 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 by 78.6 µg of AzoRo within one hour; the total removal efficiency was 88%. 163
- In conclusion, AzoRo accepts the artificial cofactor BNAH as an electron donor, which enables the degradation of azo dyes at moderate pH and temperature. A substrate test and an upscaling were successfully achieved (MR conversion of 20 mg l⁻¹ h⁻¹ by 2 mg l⁻¹ AzoRo). It now gives the possibility studying the combination of AzoRo and nicotinamide cofactor mimics. The ability of BNAH to directly reduce azo dyes will be further investigated.

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Concentration BNAH (µM)

