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

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## 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  $5.49 \pm 0.14 \text{ U mg}^{-1}$  and  $4.96 \pm 0.25 \text{ U mg}^{-1}$ ,  
23 respectively, whereas with BNAH it displayed  $17.01 \pm 0.74 \text{ U mg}^{-1}$  (following BNAH oxidation)  
24 and  $7.16 \pm 0.06 \text{ U mg}^{-1}$  (following MR reduction). Binding of BNAH to AzoRo was determined  
25 with a  $K_m$  of  $18.75 \pm 2.45 \text{ } \mu\text{M}$  (BNAH oxidation) and  $12.45 \pm 0.47 \text{ } \mu\text{M}$  (MR reduction). In order to  
26 show applicability of this system an upscaled reaction was performed using 78.6  $\mu\text{g}$  of purified  
27 AzoRo to convert 2.96  $\mu\text{mol}$  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.

- BNAH allows azo dye degradation at neutral pH.

- Upscaling of MR-reduction is possible.

## Background

Azo dyes contain one or more azo bonds ( $R_1-N=N-R_2$ ) and are frequently employed in various industries such as food, chemical, textile among others [1-2]. These azo dyes have been released in the environment due to human activities, and microorganisms have evolved or 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 novel member of this enzyme family as an oxygen-insensitive NADH-dependent FMN utilizing 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, 2-(*N,N*-dimethyl-4-aminophenyl)), as a substrate and use the nicotinamide coenzyme NADH as an electron donor [5-6].

One challenge with using the recently discovered AzoRo is the low pH required (pH 4) for rapid and efficient dye degradation (MR degradation activity  $141.25 \text{ U mg}^{-1}$ ) [5]. However, under those conditions the enzyme was found to be rather unstable, and the best use of a related enzyme was only possible through immobilization [7]. The required NADH cofactor is also unstable at acidic pH [8-9]. Thus a highly efficient regeneration system is necessary. Alternatively, a less expensive reductant, a synthetic nicotinamide cofactor analogue, can be used [10-12]. These coenzyme biomimetics have become more relevant over the last decades to investigate 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 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.

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

## 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  $\mu$ M BNAH, 50  $\mu$ M FMN and 30  $\mu$ 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.

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

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  $\mu$ M MO, 30  $\mu$ M BB) at 465 nm ( $\epsilon_{MO} = 23.25 \text{ (mM cm)}^{-1}$ ) and 570 nm ( $\epsilon_{BB} = 56.5 \text{ (mM cm)}^{-1}$ ), respectively.

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

## Results and Discussion

The enzyme AzoRo was successfully produced as described before and stored at -20  $^{\circ}$ C in storage buffer [5]. Previous investigations revealed the enzyme is most active (141.25 U  $\text{mg}^{-1}$ ; 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  $\text{mg}^{-1}$  and  $K_m$  of 9.71  $\mu$ M [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  $\mu$ M FMN and 150  $\mu$ M electron donor; following MR reduction) for NADH ( $5.49 \pm 0.14$

U mg<sup>-1</sup>), NADPH (4.96 ± 0.25 U mg<sup>-1</sup>) and BNAH (6.76 ± 0.13 U mg<sup>-1</sup>). Those activities show that 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 pH AzoRo does not differentiate between these two electron donors. This result fits to the previously made classification of AzoRo [5], and the capability to employ both NADH and NADPH as an electron donor is typically for AzoR-like azoreductases [6]. The maximum activity achieved with the above concentrations is in congruence to the previously reported data (2.50 U mg<sup>-1</sup> in phosphate buffer, pH 7.0) [5]. The artificial electron donor BNAH was better suited to reduce MR (123% activity of NADH), and this is the first report that azoreductases can actually 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.

*Fig. 1: Kinetic analysis of AzoRo utilizing BNAH at various concentrations as the electron donor. A) assay at 358 nm; B) assay at 430 nm. Prior each assay the standard assay as described previously [5] had been determined as a reference point. Then the assay for the herein determined kinetics have been prepared accordingly with 1.97 µg AzoRo in 1 ml cuvette containing Tris-HCl 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. Standard deviations of triplicates were included (< 8.1%). According to the reaction scheme 1 AzoRo employs 2 moles of BNAH in order to reduce 1 mole of MR.*

The results obtained clearly demonstrate that BNAH allows characterization of the enzyme AzoRo at a neutral pH at which it is more stable. The binding of BNAH to AzoRo and the specific 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 concentration was varied. In the case of BNAH oxidation, a  $v_{max}$  of 17.01 ± 0.74 U mg<sup>-1</sup> and a  $K_m$  of 18.75 ± 2.45 µM were obtained; the  $k_{cat}$  was 7.19 s<sup>-1</sup> and  $k_{cat}/K_m$  was 0.38 µM<sup>-1</sup>·s<sup>-1</sup>. The experiment was repeated, this time following the reduction of MR giving a  $v_{max}$  of 7.16 ± 0.06 U mg<sup>-1</sup> and a  $K_m$  of 12.45 ± 0.47 µM; the  $k_{cat}$  was 3.02 s<sup>-1</sup> and  $k_{cat}/K_m$  was 0.24 µM<sup>-1</sup>·s<sup>-1</sup>. Compared to NADH as the electron donor, the activity of AzoRo for MR reduction at optimal pH 4 is faster

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 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 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 uncoupling. This means AzoRo unproductively oxidizes BNAH as previously observed for NADH [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 half reaction. The unproductive oxidation of NADH or BNAH results in reduced FMN which may undergo certain reaction yielding most likely hydrogen peroxide as product [15]. Taking the uncoupling and the errors determined into account the ratio BNAH oxidation to MR reduction is almost 2 according to the supposed reaction (Scheme 1).

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  $0.96 \text{ U mg}^{-1}$ , 14.2 % of MR degradation activity. However, it turned out that BNAH itself could react with BB, causing obvious degradation at 570 nm. Thus clearly BNAH was identified as a powerful reductant which itself can degrade azo dyes (as seen above with MR at pH 4). Hence, at this stage further substrates for AzoRo with BNAH were not screened. The AzoRo-catalysed reduction of MR was upscaled, monitoring MR reduction at 430 nm. A final amount of 2.96  $\mu\text{mol}$  of MR was degraded by 78.6  $\mu\text{g}$  of AzoRo within one hour; the total removal efficiency was 88%.

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  $20 \text{ mg l}^{-1} \text{ h}^{-1}$  by  $2 \text{ mg l}^{-1}$  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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