APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2011, p. 4223–4225 Vol. 77, No. 12
0099-2240/11/$12.00 doi:10.1128/AEM.02289-10

BTI1, an Azoreductase with pH-Dependent Substrate Specificity▽†

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Received 24 September 2010/Accepted 8 April 2011

The group II azoreductase BTI1 utilizes NADPH to directly cleave azo bonds in water-soluble azo dyes, including quenchers of fluorescence. Unexpectedly, optimal reduction was dye specific, ranging from a pH of <5.5 for Janus green B, to pH 6.0 for methyl red, methyl orange, and BHQ-10, to pH >8.3 for flame orange.

Azo dyes are vivid colorants that consist of aromatic rings connected by one or more azo bonds. Thanks to the lack of native fluorescence, the DAB(C/S)YL and Black Hole Quencher (BHQ) azo dyes can be used as true dark quenchers of fluorescence in genetic assays, such as real-time quantitative PCR (5). Three groups of azoreductases (EC 1.7.1.6) cleave azo dyes into their colorless aromatic amines. Group II flavodoxin-related enzymes have been described from *Bacillus* sp. strain OY1-2, *Bacillus subtilis,* and others. They exist in a dimer-tetramer equilibrium, utilize flavin mononucleotide (FMN) as a noncovalently bound cofactor, and spend NADPH for direct reduction (1, 2, 4, 10, 11, 13). To date, azoreductase substrate specificities have been assessed mostly around physiological pH, which possibly underestimates optimal reactivity. Data on the pH-dependent reduction by the group II BTI1 azoreductase (Biosearch Technologies Inc.) of water-soluble azo dye quenchers are here presented.

Enzyme. The cloning of BTI1, based on the *Bacillus* OY1-2 azoreductase (11), as well as expression and verification of the functionalized BTI10 variant used throughout this study as a tetrameric FMN-containing azoreductase is described in the supplemental material.

Azoreductase activity measurements. Potential substrates (Fig. 1) in fresh double-distilled water (ddH_2O) at 1.0 or 0.10 mM were kept at +4°C until used at 20 to 40 μM for substrate susceptibility testing and at 1.0 to 20 μM for kinetics measurements. Stock solutions of β-NADH and β-NADPH (5 mM) in ddH_2O were prepared fresh weekly, stored at −20°C (12), and used at 200 μM. Reduction was carried out in 200-μl reaction mixtures in 96-well plates, with 0.5 to 2 μg of enzyme for substrate susceptibility testing and 10.0 ng to 1.0 μg of enzyme for kinetics measurements. Reaction rates were calculated by fitting the initial part of the absorbance decay by linear regression to find the slope. Extinction coefficients and absorbance maxima (λ_{max}) are in Table 1. All experiments were repeated at least three times, with similar results. The data were processed and plotted and kinetic parameters calculated by using Origin 6.0 (OriginLab, Corp., Northampton, MA).

Reaction conditions. The initial reduction by BTI10 of the test dye methyl orange (MeO) in 20 mM sodium phosphate (pH 7.0) buffer, 250 μM β-NADPH at room temperature, and a 20 μM concentration of the substrate (11) was slow (0.80 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein) and failed to reach completion. Addition of more NADPH, but not NADH, extended the reaction but did not affect the rate, verifying hydride donor depletion (4, 7). Upon cessation of reduction, the absorbance increased, suggesting azo bond reformation from a short-lived hydrazo intermediate (13). The effect of pH on the reaction was explored in buffers at a 20 mM final concentration (see the supplemental material). BTI10 optimally reduced MeO at pH 6.0 (Fig. 2).

Substrate specificity is influenced by pH. Known and potential azo fluorescence quenchers (Fig. 1) expected to be water soluble were tested. The disappearance of absorbance at the dyes' λ_{max} was monitored at pHs 5.5 to 8.3 over time. Several dyes could not be tested due to insufficient solubility or sensitivity to NADPH (see the supplemental material). Ammonium-azo-1 (Am-Azo-1), citrus yellow, orange G, Ponceau BS, and ruby red were inert to reduction with BTI10 (pHs 5.5 to 8.3). A lack of reduction of the last two is surprising because they have been reduced by other azoreductases (2, 3, 8, 9). At pH 6, BTI10 efficiently reduced MeO, *o*-methyl red (*o*-MeR), and BHQ-10 but flame orange (FO) and Janus green B (JGB) only poorly. The reduction rate was found to be both dye and pH specific (Fig. 2); BHQ-10 and *o*-MeR reduction peaked at pHs 6.0 to 6.5, similarly to that of MeO, whereas the FO reduction rate increased up to at least pH 8.3, and reduction of JGB in a 20-min endpoint assay could be observed only at pHs 5.5 and 6.0.

Kinetic analysis. Michaelis-Menten and Lineweaver-Burke kinetic analyses were applied to determine the V_{max} and the K_m values for MeO, *o*-MeR, and BHQ-10 at pH 6.0 and for FO at pH 8.3 (Table 1). Kinetic parameters for the reduction of JGB could not be obtained due to slow reduction. The V_{max} of reduction (μM concentration of dye reduced $s^{-1} \cdot mg^{-1}$ enzyme) ranged from 131 for *o*-MeR to 508 for MeO. The *o*-MeR reduction rate is comparable to that published for

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† Supplemental material for this article may be found at http://aem.asm.org/.
▽ Published ahead of print on 29 April 2011.

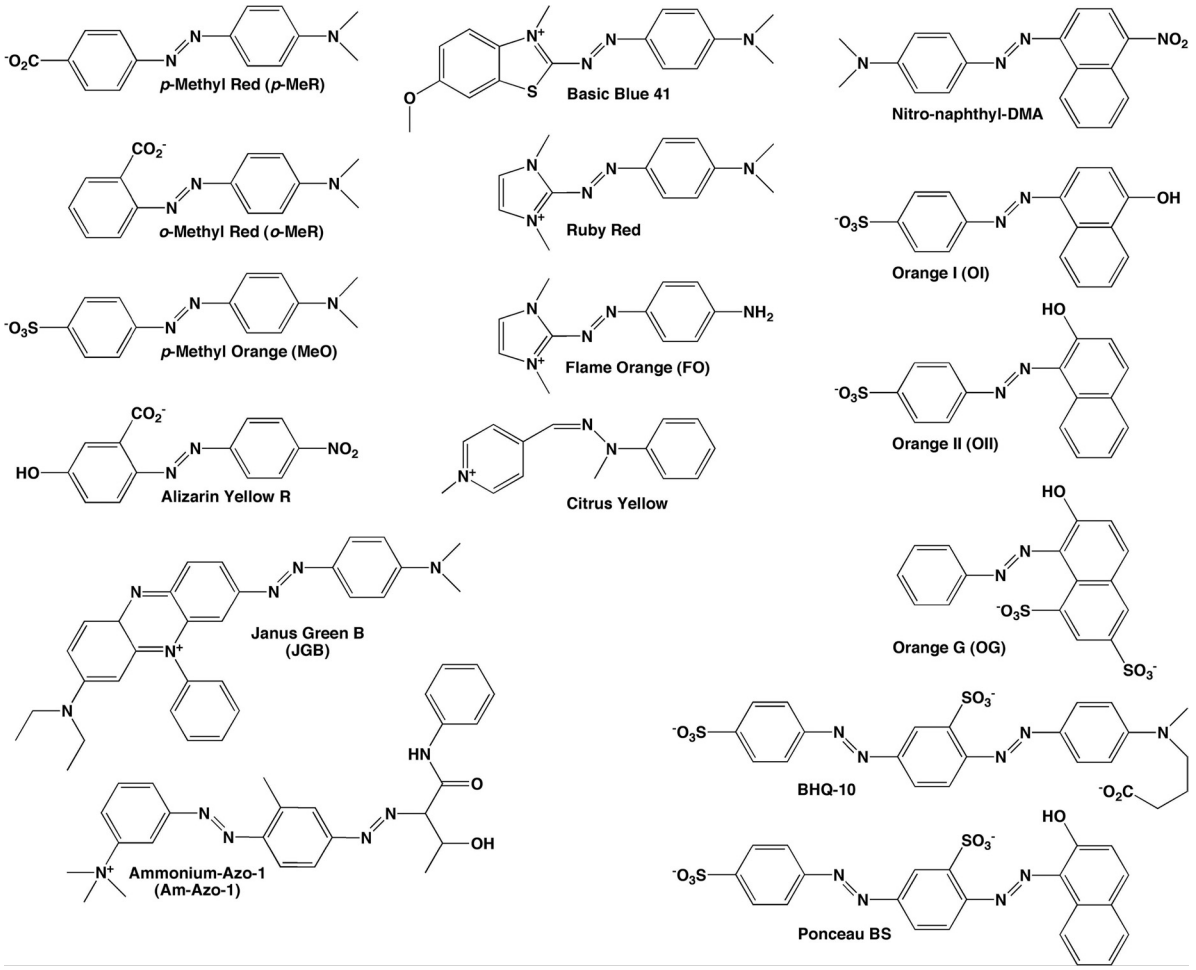

FIG. 1. Structures of dyes tested for reduction. The full names and details on the synthesis of BHQ-10 are given in the supplemental material.

Rhodobacter sphaeroides azoreductase at pH 7.0 (408 μM $s^{-1} \cdot mg^{-1}$ enzyme) (6) but considerably faster than those reported at pH 7.5 for *B. subtilis* YhdA, *Geobacillus stearothermophilus,* and *Bacillus* OY1-2 azoreductases (12.0, 13.1, and 10.4, respectively) (10). The measured K_m values (μM) showed a wide (53-fold) range, from 9.7 for *o*-MeR to 511 for FO. The reported K_m for *o*-MeR at pH 7.0 is 270 μM for *R. sphaeroides* azoreductase (6) and at pH 7.5 is 91.7 μM for *B. subtilis* YhdA, 134 μM for *G. stearothermophilus* azoreductase, and 395 μM for *Bacillus* OY1-2 azoreductase (10, 11). Efficient *o*-MeR reduction by BTI10 may thus be due to tight substrate binding.

TABLE 1. Absorption and kinetic data of dyes reduced by BTI10

Substrate	λ_{max} (nm)	ε ($M^{-1} \cdot cm^{-1}$)	V_{max} (μM · $s^{-1} \cdot mg^{-1}$)[b]	K_m (μM)	V_{max}/K_m
BHQ-10	515	35,600	290	59	4.9
Janus green B	594[a]	38,000	ND	ND	ND
Flame orange	483	28,359	150	510	0.29
Methyl orange	466	24,846	510	300	1.7
o-Methyl red	435	23,400	130	9.7	13.5

[a] Loss of absorbance was measured at 660 nm.
[b] ND, not determined.

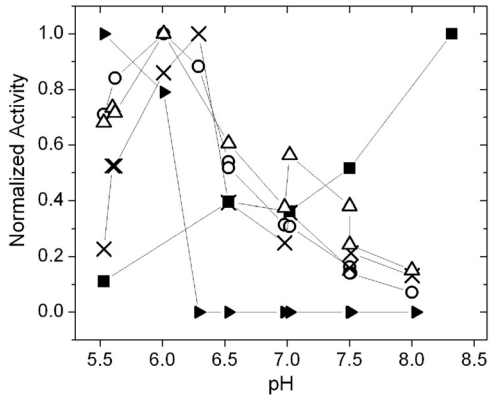

FIG. 2. Reduction by BTI10 as a function of pH of JGB (filled triangles), *o*-MeR (open triangles), MeO (open circles), BHQ-10 (×), and FO (filled squares). The data were normalized for each dye to its maximal activity (μmol dye reduced $min^{-1} \cdot mg^{-1}$ protein) at optimal pH (for BHQ-10, 1.35 at pH 6.29; for MeO, 0.95 at pH 6.01; for *o*-MeR, 0.95 at pH 6.01; and for FO, 0.168 at pH 8.32). The maximal activity for JGB was empirically defined as a 25.1% loss of absorbance at 660 nm and at pH 5.5 after 20 min.

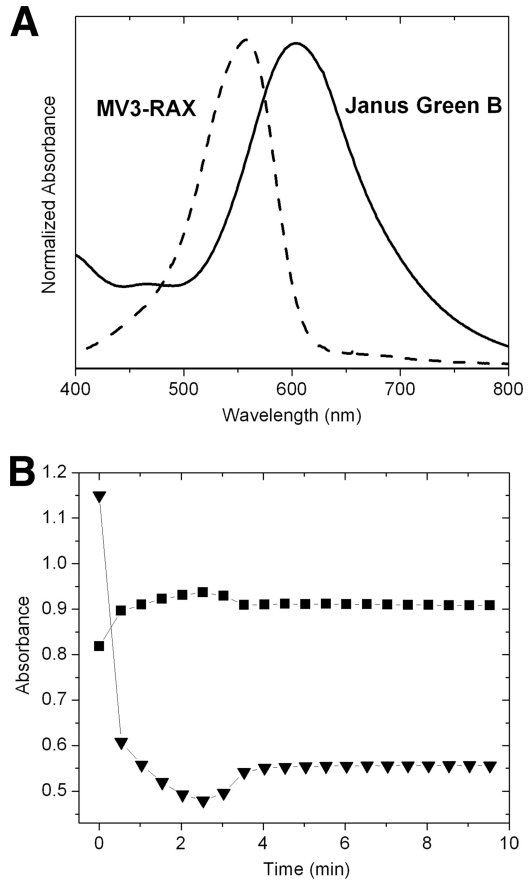

FIG. 3. Reduction at a suboptimal pH. (A) Absorbance spectra of JGB and MV-3RAX recorded at pH 6.0. (B) The effect of NADPH depletion on the reduction of JGB (30 μM) by BTI10 (73 ng · liter^{-1}) in MES (morpholineethanesulfonic acid; pH 6.0) with 200 μM NADPH. Change in absorbance at the JGB λ_{max} (594 nm) (triangles) and at the MV-3RAX λ_{max} (557 nm) (squares) was monitored over 10 min. Shown are the results from a representative experiment (out of three).

Reaction at suboptimal pH. Reduction of the JGB (λ_{max}, 594 nm) azo bond releases 4-(dimethyl-amino)-aniline and methylene violet 3RAX (MV-3RAX) (λ_{max}, 557 nm) (Fig. 3A), enabling simultaneous observation of substrate reduction and product formation at wavelengths distinct from those of the cofactors. Upon addition of azoreductase at the suboptimal pH of 6.0 and an amount of NADPH insufficient for complete reduction, the A_{594} rapidly decreased for less than a minute and then slowly decreased for up to 2.5 min, after which it increased slightly and then leveled off at 4 min (Fig. 3B). Reciprocal changes were seen at 557 nm. While no distinct absorption spectrum for the semireduced product intermediate was observed, its spectrum is expected to be very similar to that of MV-3RAX, also lacking azo bond conjugation. The coupled absorbance changes for JGB and MV-3RAX upon NADPH depletion is consistent with the reformation of JGB from a semireduced hydrazo intermediate.

The results obtained in the present study with BTI10 revealed a profound pH-dependent substrate specificity. Although the active substrates differ both in structure and charge, their absorption spectra were refractory to a change in pH from 5.5 to 8.3, consistent with their known and calculated pK_as. Conversely, a lack of color change is consistent with maintained charge and tautomer equilibria and can thus not explain the pH-dependent substrate specificity, leaving pH-dependent dye-enzyme interactions as the most likely explanation.

Alex Herrault is thanked for assistance with mass spectrometry, and Jerry Ruth is thanked for support.

The project described was supported by award number R43/R44GM076843 from the National Institute of General Medical Sciences.

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