The BTI1 Azoreductase Colorimetric and Fluorometric Reporter System

Mary K. Johansson, Albert C. Wong, Eliana S. Armstrong, Erik J Peterson, Richard E. Grant, Mark V. Reddington, Ronald M. Cook, Hans E. Johansson

BIOSEARCH TECHNOLOGIES
Advancing Nucleic Acid Technology℠

81 Digital Drive
Novato, CA 94949
www.biosearchtech.com

Abstract

Program # 502.17, Abstract # 6822

Reliable duplex and multiplex assays remain a challenge for current fluorescence reporter enzyme technology. To this end we have developed a system that takes advantage of pro-fluorescent probes and a novel azoreductase.

The BTI1 azoreductase gene was cloned from a soil sample, optimized and used to bacterially express the protein. Biochemical characterization of the purified enzyme in solution revealed a tetramer carrying a non-covalently bound flavin mononucleotide cofactor. BTI1 readily reduced an array of carboxylated and sulfonated azo dyes including methyl orange (DABSYL) and the Black Hole Quencher (BHQ™) dyes. Enzyme kinetic parameters of the four-hydride transfer reaction were consistent with the bi-bi ping-pong mechanism of related oxidoreductases. In our optimized colorimetric assay, the detection limit of the BTI1 azoreductase was >10 fold lower than that of β-galactosidase.

Reduction of the azo-quenchers in pro-fluorescent substrates containing BHQs and a range of fluorophores readily released fluorescence in an NADPH-dependent manner with robust signal to noise ratios.

In short, by matching BTI azoreductase with pro-fluorescent substrates we have created a simple, yet adaptable, reporter system that generates diverse fluorescence signals.

Background and Goals

Reporter enzyme assays are employed to assess the effect of cellular stimuli with high sensitivity. The preferred signal output is highly amplified, long lasting and free from background noise. Enzymes that release fluorescent signals are well suited for reporter amplification. A persistent limitation lies in the need for a compatible internal control enzyme assay. To complement existing fluorogenic assays with limited wavelength readouts, we set out to construct a reporter system that yields fluorescent signals over the visible spectrum.

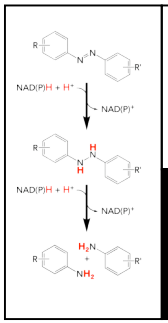
There are >10,000 man-made aromatic color fast azodyes used for textile, food and hair coloring. Azo dyes have broad absorption spectra with no fluorescence and are ideally suited as quencher dyes. With this in mind, Biosearch Technologies has developed a series of Black Hole Quenchers (BHQs) with a polyaromatic azo-backbone that have found widespread use in multiplex quantitative cDNA and genomic DNA PCR assays.

Azo dyes present an environmental burden in locals heavy with textile industries. To address this issue much research has gone into bacterial and fungal decolorization. Out of this research bacterial NAD(P)H-azoreductases have been isolated and characterized.

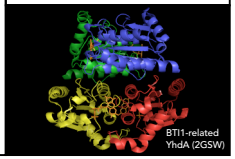
BHQs with different absorption ranges can readily be linked to fluorophores throughout the spectrum. The azoreductase reporter enzyme acting on such dark pro-fluorescent substrates can therefore be combined with other fluorogenic reporter assays.

Here is described the procurement and biochemical characterization of the BTI1 azoreductase and our efforts of assembling a novel reporter enzyme system.

Enzymatic azoreduction



- Reaction proceeds in a bi-bi, ping-pong fashion where two substrates are oxidized and reduced in two steps at one or more active sites.
- Hydride donor: NAD(P)H + H⁺
- Flavin (FAD or FMN) in active site serves as hydride intermediary
- Reduction of dye results in azo-bond cleavage, accompanied by loss of color and quenching ability

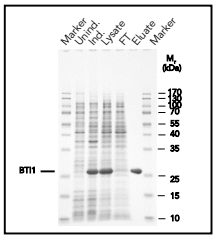


Materials and Methods

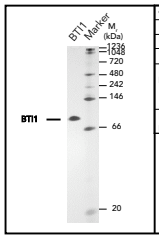
Cloning and expression The BTI1 azoreductase clone SO1-2 was isolated from a local soil sample by use of degenerate PCR. The azoreductase (C70V) open reading frame (GenBank ID: EU664999) was codon-optimized for expression in *E. coli* and mammalian cells and equipped with peptide tags for immunorecognition (myc) and affinity purification (His_6). Tagged BTI1 (C70V) was expressed and purified making use of the C-term His_6 tag from *E. coli* BL21(DE3) pLysS essentially as recommended (Novagen). Routinely, 50 ml cultures yielded 2-5 mg of >97 % pure protein.
Gel-electrophoresis Samples for SDS-PAGE were denatured in Laemmli loading buffer and separated on a 14% Tris-Gly gel (Invitrogen). Samples in Tris-Cl glycerol were separated on a 14 % native polyacrylamide gel (Invitrogen). Proteins in the gels were stained with GelCode (Pierce). The size markers were: PAGE-Ruler (Fermentas), Native-Mark (Invitrogen).
HPLC and Mass Spectrometry The absorption spectra and the elution profiles in anion exchange HPLC of native and denatured BTI1(C70V) as well as of standard flavin co-factors were recorded on an HP diode array spectrometer and a Waters HPLC. BTI1(C70V) was subjected to ESI-MS (Waters) to determine the mass of the denatured polypeptide and that of the yellow non-covalently bound cofactor.
Pro-fluorescent substrate synthesis and purification Pro-fluorescent probes were synthesized on a Biosearch 8700 DNA synthesizer using standard phosphoramidite reagents. Samples were purified by AX-HPLX, and salts removed on a reverse phase cartridge. Purity was assessed by RP- and AX-HPLC. The absence of absorption from free amines (quencher degradation products) confirmed dye integrity.
Quantitative azoreduction All buffers were at 200 mM and NADPH at 200 µM. In both absorbance and fluorescence assays, 200 µl samples were placed in 96-well plates. Typically 0.5-2.0 µg of enzyme was used per reaction. Apparent K_M and V_{max} values were determined by varying the concentration of azo dye substrates (0.5-50 µM) and making Lineweaver-Burk plots. Reaction rates were calculated by fitting the initial part of the absorbance decay by linear regression to find the slope. Absorption spectra were recorded in a HP 8453 diode array spectrophotometer, and absorbance measurements were recorded in a Tecan Safire plate reader.

Results

Expression and purification



Biophysical characterization



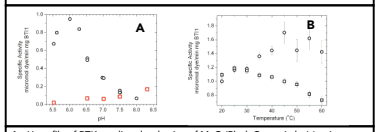
Component	Abs signature (nm)	AX-HPLC Elution time (min)	M_{calc} (g•mol⁻¹)	M_{obs} (g•mol⁻¹)
Riboflavin	268, 369, 441	0.9	376.36	377.08
FMN	268, 369, 445	3.2	456.34	**457.06**
FAD	266, 369, 446	4.2	758.55	786.00
BTI1(C70V) bound	274, 369, 446 (native) 268, 369, 447 (denatured)	8.6 3.2	N/A	**457.10**
BTI1(C70V) polypeptide	N/A	N/A	22,209.5	22,210.8

Figure: The M_r in native gel-electrophoresis (90±10kDa) is consistent with a homo-tetrameric BTI1(C70V) (M_{calc} 90.7 kDa).

Table: Left panel: Flavin absorption signatures and HPLC elution times consistent with non-covalently bound FMN cofactor.
Right panel: M_{obs} of flavins, BTI1 co-factor and polypeptide by ESI-MS, all consistent with M_{calc} polypeptide and FMN cofactor.

Enzymology

- Test substrates:
  – Methyl Orange (MeO; Orange III); Water soluble pH indicator; pK_a 3.7; A_{max} (pH > 3.7) 466 nm (ε: 35900 M^{-1}•cm^{-1})
  – Flame Orange (Basic Orange 31, BO31); Water soluble hair dye (Ciba); pK_a > 8.5; A_{max} (pH < 8.3) 483 nm (ε: 28360 M^{-1}•cm^{-1})

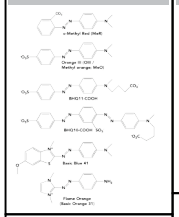


A pH-profile of BTI1-mediated reduction of MeO (Black Open circles) (optimum 6.0), and FO (Red Open circles) (optimum > 8.3)
B (Open circles) Effective temperature range for consistent azoreduction (at pH 6.0): 25-40 °C. (Open squares) Effective thermostability range <20-45 °C

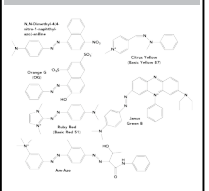
- pH optimum substrate- and enzyme- dependent
- NADPH preferred hydride donor (not shown)
- Mono- or di-valent cations not required (not shown)
- Tolerates moderate concentrations of cell extraction detergents and organic solvents (not shown)

Substrate specificity

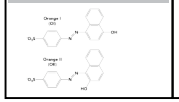
Efficient substrates



Non-substrates

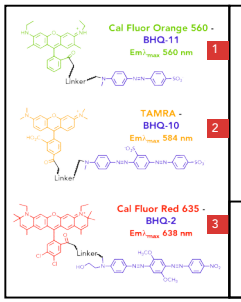


Intermediate substrates



- BTI1 reduces a diverse set of azodyes with a range of pKas, but seems to discriminate against nitro- and non-substituted phenyl compounds.
- Note BB41 is reduced by NADPH alone.

Fluorescence release



Normalized Fluorescence Intensity

	1	2	3
Buffer	1.4	0.42	1.1
Buffer, NADPH	1	1	1
Buffer, NADPH, BTI1	40	72	2.5
Dithionite	28	180	21

- Reduction of quenched fluorophore probes (4 µM) by BTI1 (10 ng / µl) or dithionite (10 mM) pH 6.0, 30 min.
- Reactions excited (>365 nm) and fluorescence imaged (Wratten yellow filter No. 8 and a Canon IS 600 camera).

- Efficient ground state quenching -> Wide range of Quencher-Fluorophore pairs
- Soluble quencher more efficient BTI1-substrate
- High signal to noise ratios

Summary

- The BTI1 azoreductase is a homo-tetrameric flavo-enzyme that uses a non-covalently attached FMN
- Black Hole Quenchers and other azodyes are efficiently reduced by BTI1 over wide pH and temperature ranges
- BTI1 releases fluorescent signals with high signal to noise ratios
- The enzyme's broad temperature optimum and and ability to reduce substrates with different pKas and absorption maxima will enable duplex and multiplex assays with reporter enzymes like β-galactosidase and alkaline phosphatase

References

1. RM Cook, M Lyttle & D Dick. (2006) Dark Quenchers for donor-acceptor energy transfer. US Patents 7019129 and 7109312.
2. Y Suzuki, T Yoda, A Ruhul & W Sugiura. (2001) Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus* sp. OY1-2 isolated from soil. *J Biol Chem* **276**(12), 9059-65.
3. RM Cook, ES Armstrong & HE Johansson. (2006) Inducible fluorescence assay. WO 2006/010038 A2, US 2006/0194218 A1, pending.
4. MK Johansson, RM Cook. (2003) Intramolecular dimers: A new design strategy for fluorescence-quenched probes. *Chemistry (Eur J)* **9**(15), 3466-71.
5. MK Johansson. (2007) Choosing reporter-quencher pairs for efficient quenching through formation of intramolecular dimers. *Meth Mol Biol* **335**, Chpt. 2, pp. 17-29.
6. C Brownlee. (2007) Choosing industry: Biotech makes its mark. *ACS Chem Biol* **2**(6), 372–6.
7. H Chen. (2006) Recent advances in azo dye degrading enzyme research. *Curr Protein Pept Sci* **7**(2), 101-11.
8. A Pandey, P Singh, L. Iyengar. (2007) Bacterial decolorization and degradation of azo dyes. *Int Biotedeer Biodegr* **59**(2), 73-84.
9. MV Reddington & M Lyttle. (2008) Xanthine dyes US Patent 7344701.

Notes

- Current addresses: EJP: Department of Psychology, Colorado State University, Fort Collins, CO; ESA: Achaogen, Inc., South San Francisco, CA; REG: University of Wisconsin Biotechnology Center, Madison, WI
- We gratefully acknowledge the support from NIH (SBIR R43/R44 GM076843)
- We thank Margaret Roy for initial work on the project and Dr. Jerry Ruth for thoughtful suggestions.