A short-chain dehydrogenase/reductase from *Vibrio vulnificus* with both blue fluorescence and oxidoreductase activity†

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Received (in Cambridge, MA, USA) 16th November 2006, Accepted 23rd January 2007
First published as an Advance Article on the web 12th February 2007
DOI: 10.1039/b616763b

A dual function blue fluorescent protein from *Vibrio vulnificus* is also an NADPH-dependent oxidoreductase, rendering it a useful tool for biophysical studies.

Fluorescent proteins, such as the green fluorescent protein from the jellyfish species *Aqueora victoria*, have revolutionized protein biophysics by providing an efficient means to track the folding status of proteins.[1] In 2001, Su *et al.* reported the identification of a blue fluorescent protein (BFP) in a clinical isolate of *Vibrio vulnificus* that belonged to the short-chain dehydrogenase/reductase (SDR) family, rather than featuring the typical β-can structure of GFP and its homologs. The fluorescence of BFP is generated by binding NADPH and magnifying the innate level of fluorescence of this compound, such that *E. coli* cells expressing the protein are blue to the eye when excited at 283 or 352 nm.[2] The likelihood that the natural function of BFP is in biofluorescence is very slim, given its unique fold and mechanism of fluorescence and the fact that it is present only in certain clinical isolates. More likely, the enzyme functions in the oxidation or reduction of substrates encountered by the organism, using NADP(H) as a cofactor. It is also possible that impairment of turnover of NADPH by accumulated mutations has led to the visible level of fluorescence.

A protein with both fluorescence and catalytic activity would be a useful addition to existing tools for the biophysical study of proteins as it would allow separate measurement of binding events (*via* fluorescence) and substrate turnover (*via* activity). Therefore, we tested several standard substrates for members of the SDR family to try to identify the natural activity of BFP.

The gene sequence of BFP (AAG41118) was codon optimized for expression in *E. coli*, synthesized from oligonucleotides designed with DNAWorks,[3] and cloned into pET28a in frame with the C-terminal histidine tag. The plasmid was transformed into *E. coli* BL21 cells and overexpressed with the addition of 0.1 mM IPTG at room temperature for 4 hours. The protein was purified by immobilized metal affinity chromatography (Ni-NTA) and analyzed by SDS-PAGE. Fluorescence was confirmed at both excitation wavelengths upon addition of varying amounts of NADPH using a SpectraMax Gemini (BMG, Offenburg, Germany) multiwell fluorimeter.

Previous work used directed evolution to create mutants with increased fluorescence. The most fluorescent mutant in that work had seven point mutations E40K, V76A, L77I, V83M, S124C, G176S and E179K, leading to approximately 4-fold increased fluorescence. A single mutant G176S was also identified that had 2-fold increased fluorescence.[4] Increased fluorescence could result either from decreased K_M value for NADPH or from the impairment of catalytic turnover. To test which of these operates in the case of BFP, the seven-fold mutant (BFP-7) and a double mutant G176S/E197K (BFP-2) were also created *via* point mutagenesis, expressed, purified, and tested along with the wild-type BFP.

The specific activity of BFP on a panel of typical substrates for ketoreductases (Table 1) was measured by monitoring appearance or disappearance of NADPH at 340 nm on a DU800 spectrophotometer at 25 °C (Beckman). Saturated solutions were prepared by adding 100 μL of the organic compound per 20 mL of buffer solution (50 mM phosphate buffer, pH 8.0). The solution was then stirred vigorously for 15 minutes at room temperature. After settling, the buffer phase was carefully separated from the organic phase and used for measurements with a cofactor concentration of 0.3 mM. Solutions that were monophasic were used without separation. Some substrates (*e.g.* cinnamaldehyde) had a large intrinsic absorbance at 340 nm, so measurements were taken at 365 nm. NADH was also tested in an analogous manner. Specific activity was calculated using the extinction coefficients from Larroy *et al.*[5] The reduction of pivaldehyde and benzaldehyde was confirmed by gas chromatography with the presence of *neo*-pentanol and benzyl alcohol, respectively.

To calculate kinetics for NADPH, measurements of reaction rate at varying concentrations of cofactor were made with saturated pivaldehyde solution. Kinetic parameters were calculated from Lineweaver–Burk plots made in triplicate with different enzyme preparations. Further corroboration of K_M measurements of NADPH was obtained by measuring fluorescence of the BFP and its variants compared to the fluorescence of pure NADPH in solution at varying concentrations. The apparent K_M value was calculated using a plot analogous to the Lineweaver–Burk plot using the reciprocal fluorescence intensity in place of the reciprocal reaction velocity.

The wild-type BFP is an active NADPH-dependent dehydrogenase enzyme with activity on many of the redox substrates examined. No activity was observed with NADH. Table 1 outlines the substrate specificity of the enzyme. In general, it has a strong preference for the reduction direction. For example, the specific

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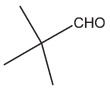
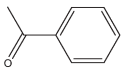
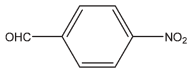
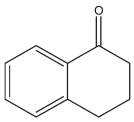
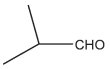
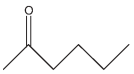
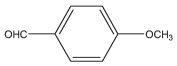
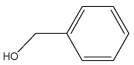
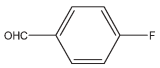
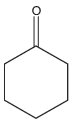
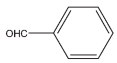
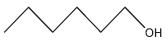
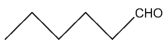
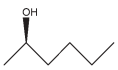
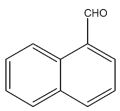
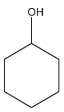
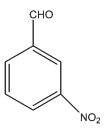
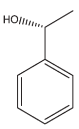
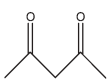
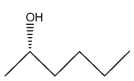
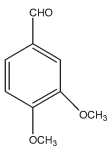
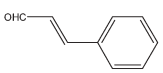
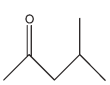
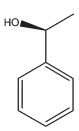
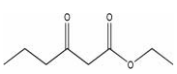
† Electronic supplementary information (ESI) available: Codon optimized DNA sequence of wild-type BFP, oligonucleotides used in synthesis, gene synthesis procedure, SDS-PAGE gel of purified proteins. See DOI: 10.1039/b616763b

Table 1 Maximum specific activity of wild-type BFP on various substrates

Substrate	Structure	Specific activity (U mg^{-1})	Substrate	Structure	Specific activity (U mg^{-1})
pivaldehyde		18.92 ± 0.48	acetophenone		0.21 ± 0.04
p-nitrobenzaldehyde[a]		10.01 ± 0.11	α-tetralone		0.16 ± 0.02
*iso*butyraldehyde		7.60 ± 0.84	2-hexanone		0.13 ± 0.05
p-anisaldehyde		7.59 ± 0.88	benzyl alcohol		0.12 ± 0.02
p-fluorobenzaldehyde		6.62 ± 0.11	cyclohexanone		0.10 ± 0.01
benzaldehyde		5.40 ± 0.44	1-hexanol		0.03 ± 0.004
hexanal		4.15 ± 0.67	(*S*)-2-hexanol		0.02 ± 0.0004
n-butyraldehyde		3.47 ± 0.20	1-butanol		0.01 ± 0.0004
1-naphthaldehyde[a]		3.44 ± 0.31	cyclohexanol		0.01 ± 0.001
m-nitrobenzaldehyde[a]		1.66 ± 0.03	(*R*)-phenylethanol		0.002 ± 0.0004
acetoacetone		1.10 ± 0.12	(*R*)-2-hexanol		0.001 ± 0.0005
3,4-dimethoxybenzaldehyde		0.60 ± 0.12	acetone		n.d.
cinnamaldehyde[a]		0.45 ± 0.05	methyl *iso*butyl ketone		n.d.
(*S*)-phenylethanol		0.26 ± 0.01	ethyl 3-oxohexanoate		n.d.

[a] Monitored at 365 nm. [b] n.d. no activity detected. [c] Standard assay conditions: 25 °C, 0.3 mM NADPH, 50 mM phosphate buffer pH 8.0, saturated organic solution.

activity for the reduction of benzaldehyde is almost 50-fold that for the oxidation of benzyl alcohol. Similarly, the activity for the reduction of hexanal is more than 100 times that for the oxidation of 1-hexanol. The one exception is the oxidation of (*S*)-phenylethanol which has a specific activity nearly equivalent to the reduction of the corresponding ketone, acetophenone.

BFP also favors aldehydes over ketones. For instance, the specific activity on hexanal is more than 33 times higher than on 2-hexanone. Additionally, many of the smaller ketones such as acetone and methyl *iso*butyl ketone were not active substrates for the enzyme. Larger ketones, such as acetophenone, cyclohexanone, and α-tetralone, had low levels of activity. Interestingly, despite not

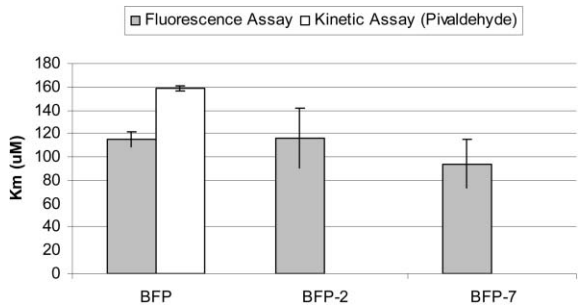


Fig. 1 Calculated K_M values for BFP and its variants. K_M values for BFP-2 and BFP-7 were measured solely *via* fluorescence due to their low reactivity.

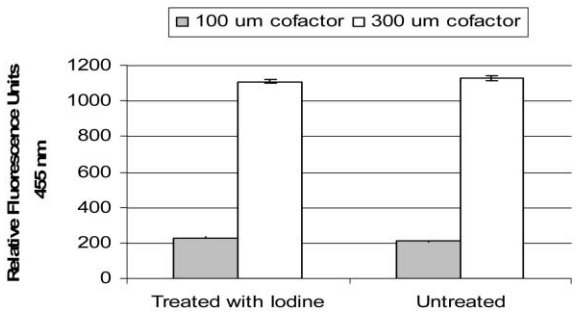


Fig. 2 Inactivation of the active site tyrosine retains fluorescence. The background fluorescence of NADPH (1250 RFU and 1500 RFU, respectively) has been corrected for.

accepting MIBK as a substrate, BFP has activity greater than 1 U mg^{-1} on acetoacetone, which is of a similar size and shape but is a more activated ketone.

Although the oxidation activity is weak, BFP favors the (*S*)-enantiomer over the (*R*)-enantiomer. The specific activity on (*S*)-2-hexanol is 17-fold that of (*R*)-2-hexanol and the activity on (*S*)-phenylethanol is more than 150 times that of (*R*)-phenylethanol.

The unusual substrate specificity of BFP is most likely due to a combination of sterics and electronics. Aldehydes are more activated than ketones (and consequently more easily reduced), but they are also less sterically hindered. The fact that BFP favors substrates that contain a phenyl ring can also be partially explained by their activated nature, but may also point to a hydrophobic cavity that forms favorable contacts with somewhat bulky substrates. However, the cavity size likely is limited: although cinnamaldehyde and benzaldehyde should have similar reactivity, owing to the vinylogous effect, BFP has more than 10-fold lower specific activity on cinnamaldehyde, indicating steric hindrance on the latter. Likewise, *meta*- and *para*-nitrobenzaldehyde are electronically very similar (σ = 0.71 and 0.78 for the *m*- and *p*-form, respectively)[6] but BFP has approximately 6-fold reduced activity on the *meta*-isomer. Overall, the specific activity data indicate that BFP has highest reactivity with activated substrates that are not sterically demanding, suggesting that BFP may have a partially impaired active site. The reactivities also suggest that the carbonyl group of the substrate binds in a head-first manner through a fairly hydrophobic pocket that stabilizes substrates of intermediate size *via* favorable interactions.

Neither BFP-2 nor BFP-7 had high levels of oxidoreductase activity. In the case of BFP-2 the activity on pivaldehyde (the best substrate for wild-type enzyme) was near the threshold of detection, almost 10000-fold lower than wild-type BFP. BFP-7 had no detectable level of activity. Increased fluorescence can come about by loss of catalytic activity (*i.e.*, NADPH is bound, but cannot turn over for reaction, and is retained) or by an increase in affinity for NADPH (*i.e.*, a lower K_M). Our activity data suggest that the former is the cause of improved fluorescence in this case. Indeed, many of the mutations that lead to increased fluorescence are in close proximity to residues known to be important for SDR function. In particular, G176 is the same glycine that is part of the PG-motif in the cofactor binding area, implicated in the hydride transfer reaction. In addition, V76, L77, and V83 are near the NNAG motif which is known to stabilize the central β-sheet of the fold. E179 is also next to a threonine residue which is thought to stabilize the nicotinamide binding.[7] Fig. 1 compares the K_M values for NADPH of three BFP variants. The K_M value for wild-type BFP as determined by the kinetic assay using pivaldehyde as the second substrate is very similar to the apparent $K_{M,app}$ value obtained by the fluorescence assay. Comparing $K_{M,app}$ for the wild-type and two variants from the fluorescence assay corroborates the conclusion that the increase in fluorescence is due to the impairment of catalytic activity as the three values are very similar, although there is a small possibility that the further improvement in fluorescence going from BFP-2 to BFP-7 comes from a slightly decreased K_M value.

Since BFP-2 and BFP-7 are inactive as redox enzymes, but still retain fluorescence, we believe that binding events and catalytic events can be separately monitored for BFP, making it useful for biophysical studies. To further corroborate the separate nature of binding and catalysis, the active site Y145 was inactivated by iodination[8] until no detectable reduction of benzaldehyde remained. The fluorescence was then tested at two concentrations of NADPH, at a protein concentration of 10 μg mL^{-1}. Fig. 2 clearly shows that the fluorescence intensity of the treated and untreated protein is the same. Thus, binding of cofactor is not affected by the ability of the active site to catalyze the reaction.

In summary, we have shown that the BFP from *Vibrio vulnificus* is a functional oxidoreductase with a preference for the reduction of aldehydic substrates. The binding (fluorescence) events are separate from the catalytic events (redox activity) making this protein useful for biophysical studies since the two can be monitored separately.

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