Biosensors and Bioelectronics 48 (2013) 251–257

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Fluorescent proteins as biosensors by quenching resonance energy transfer from endogenous tryptophan: Detection of nitroaromatic explosives

Alexa Gingras, Joseph Sarette, Evan Shawler, Taeyoung Lee, Steve Freund, Eric Holwitt, Barry W. Hicks *

Department of Chemistry, US Air Force Academy, USAFA, CO 80840, USA

ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form
18 March 2013
Accepted 23 March 2013
Available online 23 April 2013

Keywords:
Fluorescent protein
FRET
Explosive
TNT
RDX
Vapor

ABSTRACT

Ensuring domestic safety from terrorist attack is a daunting challenge because of the wide array of chemical agents that must be screened. A panel of purified fluorescent protein isoforms (FPs) was screened for the ability to detect various explosives, explosive simulants, and toxic agents. In addition to their commonly used visible excitation wavelengths, essentially all FPs can be excited by UV light at 280 nm. Ultraviolet illumination excites electrons in endogenous tryptophan (W) residues, which then relax by Förster Resonance Energy Transfer (FRET) to the chromophore of the FP, and thus the FPs emit with their typical visible spectra. Taking advantage of the fact that tryptophan excitation can be quenched by numerous agents, including nitroaromatics like TNT and nitramines like RDX, it is demonstrated that quenching of visible fluorescence from UV illumination of FPs can be used as the basis for detecting these explosives and explosive degradation products. This work provides the foundation for production of an array of genetically-modified FPs for *in vitro* biosensors capable of rapid, simultaneous, sensitive and selective detection of a wide range of explosive or toxic agents.

Published by Elsevier B.V.

1. Introduction

Accurate and rapid identification of explosives, toxins, and poisons is critical for the military, civilian security, first responders, and for humanitarian remediation work. Detection of TNT and other nitroorganic explosives is of particular concern. Fortunately, the past decade has seen a tremendous advancement in both sensitivity and selectivity for TNT detection methodologies. These detection techniques can be generally classified as physical, chemical or biological, depending upon the nature of the sensor utilized (Wang et al., 2012). Techniques amenable to TNT biosensing include colorometric analysis (Smith et al., 2008), fluorometric analysis (Bromage et al., 2007) including FRET (Medintz et al., 2005) and quenching (Abe et al., 2011), chemiluminescence (Maioloni et al., 2009), gravimetric analysis (Wang et al., 2012), electrochemical analysis (Caygill et al., 2013) and surface plasmon resonance (Shankaran et al., 2005) among others. Sensitivities for these methods vary widely, as do the times required to accurately identify and quantify TNT, but concentrations of TNT in the ppm–ppb range can commonly be detected with times ranging from seconds to minutes. Most of the biosensing techniques utilize monoclonal or polyclonal antibodies that recognize TNT (Charles et al., 2004), although TNT binding peptides (Jaworski, 2009), nucleic acid polymers (Ehrentreich-Foerster et al., 2008), and even live cell techniques (Burlage, 2009) have also been utilized. With improvements in antibody production and specificity using better haptens, these techniques should continue to improve detection limits and selectivity (Ramin and Weller, 2012). While antibody stability is acceptable, those from most species except camelids (Anderson and Goldman, 2008) are subject to thermal denaturation at relatively moderate temperatures (Zhang et al., 2012). Antibodies are also sensitive to proteases (Cho et al., 2006) and chemical denaturants, and relatively low concentrations of many organic solvents (Horacek and Skladal, 2000) alter binding. Finally when fluorescence-based biosensing with antibodies utilizes conventional organic dyes, they are subject to bleaching (Lesner, 2012).

In the past two decades, thousands of unique FP isoforms and fusion proteins have been produced with emission maxima in every portion of the visible spectrum (Shaner et al., 2005; Tsien, 2009; Miyawaki, 2011; Suetsugu et al. 2012; Vizcaino-Caston and Wyre, 2012), and the Protein Data Bank contains over 400 entries of various FPs. Of the hundreds of thousands of papers describing FPs and their applications, only a handful mention the UV excitation peak centered near 280 nm (Niwa et al. 1996; van Thor et al., 2002; Sniegowski et al., 2005; Gurskaya et al., 2001; Alvarez et al., 2010), and fewer still discuss or evaluate that peak in any detail

* Corresponding author. Tel.: +1 719 3336050, +719 333 2960; fax: +1 719 3332947.
E-mail address: barry.hicks@usafa.edu (B.W. Hicks).

0956-5663/$ - see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bios.2013.03.076

(van Thor et al., 1998; Visser et al., 2005; Budisa et al., 2004). Visser et al. (2005) unambiguously demonstrated that FRET from endogenous W residues to the chromophore is genuinely occurring within FPs and is responsible for the 280 nm excitation peaks. Because most applications for FPs have been for *in vivo* use, the UV excitation has been largely ignored as excessive UV illumination would almost certainly lead to cellular damage in living cells and complicate interpretation of results. However, UV excitation of FPs could be valuable for *in vitro* biosensing. Importantly, the FPs are superior to dye-labeled fluorescent probes in that they are remarkably stable to proteases and chemical agents (Mazzola et al., 2006), they can tolerate relatively high concentrations of many organic solvents (Samarkina et al., 2009), they are extremely resistant to bleaching (Yoo et al., 2012), and mutations providing hyperthermophilic stability have been identified (Kiss et al., 2009). Herein, we describe a panel of fluorescent proteins used for detection of explosives and toxic agents including TNT, RDX and paraoxon. This is the first report describing the use of endogenous W FRET within FPs for biosensing.

2. Materials and methods

Chemicals were obtained from Sigma-Aldrich or Fisher Scientific unless specified. TNT was produced in-house, and RDX was obtained from the US Army Criminal Investigation Laboratory.

2.1. Production and isolation of fluorescent proteins

Plasmid DNA encoding for seven FPs [ultraviolet-exciting green fluorescent protein (GFP_{UV}), Fluoroblue, modified teal fluorescent protein (mTFP0.7), enhanced cyan fluorescent protein (ECFP), enhanced consensus green protein (eCGP123), *Zoanthus* sp fluorescent protein (ZsYellow), and *Anemonia sulcata* red fluorescent protein (AsRed2)] with emission wavelengths covering the entire visible spectrum were obtained from the sources indicated in Table 1S. All plasmids were transformed into the KRX strain of *E. coli* (Promega) as recommended by the manufacturer and selected on LB plates using the appropriate antibiotics (Amp or Kan) at a concentration of 50 μg/mL and inducers (rhamnose or IPTG) at 0.5 mM. In addition to antibiotic selection, bacterial fluorescence was visualized either with UV illumination, or using a blue LED flashlight and yellow filtered glasses (NightSea). Single bright colonies were used to inoculate 10 mL of LB broth containing the appropriate antibiotic and grown overnight. The overnight cultures were diluted into 200 mL of LB broth containing antibiotic and grown for 4 h at 37 °C. Induction was begun by addition of either 1 mM rhamnose for plasmids containing the T7 promoter, or 1 mM IPTG for plasmids containing the lac promoter, and cultures were maintained for 12 h at 28 °C. Cells were harvested in 50-mL tubes by centrifugation at 4000 rpm using an SH3000 swinging bucket rotor in a Sorvall Evolution RC centrifuge and pelleted bacteria stored at −20 °C until needed.

Bacteria were lysed for 30 min at 37 °C in 10 mL of lysate buffer containing 10 μg/mL lysozyme, 1% deoxycholate, 0.1% Triton-X100, and 0.5 M urea essentially as described (Danilevich et al., 2008). Glass beads (0.1 mm), 0.3 × v/v, were added to the lysate and it was vigorously vortexed for about 3–5 min to shear genomic DNA. The resulting mixture was centrifuged at 4000 rpm and the supernatant decanted and used for either three phase partitioning with ammonium sulfate and t-butanol (Jain et al., 2004) or for organic extraction using NaCl, ammonium sulfate, ethanol and 1-butanol as described (Samarkina et al., 2009) to generate partially pure proteins. These proteins were dialyzed against 5 mM Tris buffer for 24–48 h. Proteins were then purified by column chromatography using anion exchange (DEAE sephacel), hydrophobic interaction (phenyl sepharose CL-4B), size exclusion (Sephadex G-100) or a combination of the above. The ZsYellow and AsRed2 proteins required 1% deoxycholate for removal from the phenyl sepharose columns, likely due to their hydrophobic aggregation (Zubova et al., 2005) and these were used without removal of the detergent to maintain stable dimeric/tetrameric structures in solution.

All purified proteins were quantified by absorbance at 280 nm against a bovine serum albumin standard in UV flat bottom 96-well plates (Thermo) using a Biotek μQuant plate-reading spectrophotometer. Purity was verified by SDS PAGE (Fig. 5S) using a BioRad Mini-Protean apparatus on 10% acrylamide Ready-Gels after staining with Coomassie Blue.

2.2. Spectrofluorometry

Fluorescence measurements were done in Costar black 96-well fluorescence plates using a Varian-Cary Eclipse Spectrofluorometer with a plate-reading accessory. Excitation spectra were generally obtained while monitoring emission at 20–40 nm beyond the maxima, and emission spectra were obtained while exciting at 20–40 nm below the maxima, or by exciting at 254–280 nm. Instrument parameters were optimized for each protein and included adjustment of excitation and emission slit widths (2.5–20 nm), filters, and PMT tube voltage (400–800 V).

2.3. Quenching assays in solution

Quenching assays were done in Costar black 96-well fluorescence plates. Stock solutions of quenching agents were made in either 100% DMSO or 80% DMSO for DNT, TNT, and RDX (which contained 20% of either ethyl acetate or acetonitrile). The FPs were used at between 4 and 20 μg/mL (~33 nM) in 5 mM Tris with maximum DMSO concentrations of about 8% (it was shown that DMSO up to 25% had little effect on FP emission, with a slight stimulation of emission being observed at about 5% for most FPs). For each FP, and for each agent, 380 μL of sample were prepared with 10 different agent concentrations (including blanks) ranging from 5 to 1500 μM. Then 360 μL of sample was transferred to the wells of the 96-well plates for analysis and read immediately (prolonged incubation had no significant effect). Spectrofluorometer parameters were adjusted so that controls containing only DMSO, but no agent, gave nearly maximal emission spectra, and then full data sets were collected using those parameters. Unless indicated, assays were performed in triplicate or greater. The mean integrated areas of the visible emission spectra at each quenching agent concentration were used to determine unquenched (F_o) and quenched (F) values, and plotted as Stern–Volmer plots in Excel with standard deviations indicated by error bars. Correlation coefficients were generally above 0.97, but for YFP and RFP (which required addition of 1% deoxycholate for elution from phenyl sepharose column), the values were sometimes as low as 0.9 (due to scatter, not due to non-linearity). Data for all Stern–Volmer plots were collected with comparable K_{SV} values generally agreeing to within 5–10%.

For determining minimal detection limits, more dilute eCGFP123, GFP_{UV} or ECFP solutions were used (typically 10- to 50-fold lower than used for the rest of the work described above) that pushed the spectrofluorometer parameters to maximal values, and FPs were titrated with nanomolar concentrations of DNT or TNT. When quenching could be detected, it was verified at the lowest concentration in triplicate or quintuplicate.

When using bacterial lysates or live bacteria all assays were performed similarly except that pellets of the live bacteria expressing GFP_{UV} were resuspended in PBS prior to testing, and 5 mM Tris containing 150 mM NaCl was used to maintain osmolality for