Analytical Biochemistry 525 (2017) 60–66

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Engineering a switch-based biosensor for arginine using a *Thermotoga maritima* periplasmic binding protein

Teraya Donaldson [a, 1], Luisa Iozzino [a, b, 1], Lindsay J. Deacon [a], Hilbert Billones [a], Alessio Ausili [b], Sabato D'Auria [b], Jonathan D. Dattelbaum [a, *]

[a] Department of Chemistry, University of Richmond, Richmond, VA, 23173, USA
[b] Laboratory for Molecular Sensing, ISA-CNR, Via Roma 64, 83100 Avellino, Italy

ARTICLE INFO

Article history:
Received 2 December 2016
Received in revised form
26 February 2017
Accepted 28 February 2017
Available online 1 March 2017

Keywords:
Periplasmic binding protein
Fluorescence
Switch-based biosensor
Hyperthermophilic bacteria
Photo-induced electron transfer

ABSTRACT

The *Thermotoga maritima* arginine-binding protein (*Tm*ArgBP) has been modified to create a reagentless fluorescent protein biosensor. Two design methods for biosensor construction are compared: 1) solvent accessibility of environmentally-sensitive probes and 2) fluorescence deactivation due to photo-induced electron transfer (PET). Nine single cysteine *Tm*ArgBP mutants were created and labeled with three different environmentally sensitive fluorescent probes. These mutants demonstrated limited changes in fluorescence emission upon the addition of arginine. In contrast, the PET-based biosensor provides significant enhancements over the traditional approach and provides a fluorescence quenching mechanism that was capable of providing quantitative detection of arginine. Site-directed mutagenesis of *Tm*ArgBP was used to create attachment points for the fluorescent probe (K145C) and for an internal aromatic residue (D18X) to serve as the PET quencher. Both tyrosine and tryptophan, but not phenylalanine, were able to quench the emission of the fluorescent probe by more than 80% upon the addition of arginine. The dissociation constant for arginine ranged from 0.87 to 1.5 μM across the different sensors. This PET-based strategy provides a simple and broadly applicable approach for the analytical detection of small molecules that may be applied to any protein that exhibits conformational switching in a ligand dependent manner.

1. Introduction

Argininemia (omim.org – 207800) is a rare autosomal recessive genetic disorder caused by an arginase enzyme deficiency that affects the last step of the urea cycle in mammals [1,2]. Elevated plasma levels of L-arginine (L-Arg) clinically present later in childhood in a variety of neurological manifestations, including developmental regression, seizures, and progressive spasticity [2]. Treatment usually consists of nutrient restriction combined with phenylbutyrate or benzoate as an ammonia scavenging agent [3]. Detection of L-Arg levels in plasma and tissue is typically performed using HPLC and requires a derivatization step [4]. Here, we describe a reagentless fluorescent biosensor for the detection of L-Arg using a rationally redesigned protein.

Engineered proteins have been utilized for the detection of biochemically interesting small molecules [5,6]. Bacterial periplasmic binding proteins (PBPs) are part of the toolkit being established for measuring natural and synthetic analytes [7–11]. PBPs are a class of soluble, microbial receptors associated with two component transport systems that couple ATP hydrolysis to the passage of extracellular metabolites into the cell [12,13]. Importantly for biosensor design, PBPs exhibit high selectivity for a variety of ligands essential for metabolism, chemotaxis, and signal transduction [14]. Ligand binding commonly produces a global conformational switch from open to closed state that has been monitored by a variety of fluorescence-based tagging strategies. Coupling of the conformational switch with a change in fluorescence intensity has been utilized for the analytical detection of many sugars, anions, and amino acids [5,7–9,11,15].

To advance the engineering opportunities of PBP-based sensors, some research groups have turned to genome mining of thermophilic microorganisms for the discovery of new protein scaffolds that may offer additional physicochemical stability. Our work has

* Corresponding author. Department of Chemistry, 28 Westhampton Way, Richmond, VA 23173, USA.
E-mail address: jdattelb@richmond.edu (J.D. Dattelbaum).
[1] These authors contributed equally to this project.

http://dx.doi.org/10.1016/j.ab.2017.02.021
0003-2697/

Abbreviations

PBPs	Periplasmic binding proteins
*Tm*ArgBP	*Thermotoga maritima* arginine-binding protein
PET	photo-induced electron transfer
ABD	4-fluoro-7-aminosulfonylbenzofurazan
NBD	N-(((2-iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD amide)
K_{sv}	Stern-Volmer quenching constant
k_q	bimolecular quenching constant

focused on the characterization of a new arginine-binding protein from *Thermotoga maritima* (*Tm*ArgBP) [16–18]. This thermostable protein may be a good candidate for development as an arginine biosensor due to its structural resilience even under strong denaturing conditions [16,19]. Our structural determination of *Tm*ArgBP, as well as an initial screen of several single tryptophan mutants of *Tm*ArgBP, allowed us to begin to identify sites on the protein that may be exploited for the analytical detection of arginine.

This work investigates a rational approach to PBP biosensor design using the *Tm*ArgBP conformational switch to modulate fluorescence emission in an arginine-dependent manner. We first employ a traditional approach to ligand detection through inserting single cysteine residues at selected locations around the protein. Environmentally sensitive fluorophores are then covalently attached via thiol-reactive linkages and used to report changes in their protein environment through short-range or long-range dipole-dipole interactions [20]. While this traditional approach to biosensor design appears straightforward, a theoretical framework that accurately matches location and fluorophore to yield a satisfactory ligand-dependent change in fluorescence intensity has been elusive. Alternative methods including the use of FRET, fluorescence proteins, and excitonic interactions have been applied to the creation of new protein biosensors [15,21–24]. Yet these methods can result in low signal changes and/or require complex purification protocols.

Here, we adapted photo-induced electron transfer (PET) as a reporter mechanism to directly link the PBP conformational dynamics to fluorescence intensity changes. In this study, we found that a significantly better fluorescence response may be achieved by inserting individual aromatic residues on the opposite lobe of the protein from the reporter molecule to serve as a quencher of fluorescence intensity upon ligand binding. In this way, we couple arginine-dependent conformational flexibility with changes in the fluorescence intensity. We demonstrate that PET is a beneficial tool in the development of a reagentless bioassay for arginine. Further, this PET-based strategy provides a simple and broadly applicable approach for the analytical detection of small molecules using any protein that exhibits conformational switching in a ligand dependent manner.

2. Experimental section

2.1. Preparation of TmArgBP mutants

Primers were designed for the *T. maritima* TM0593 gene encoding the arginine-binding protein and used for cloning into the *Nde*I-*Bam*HI restriction sites of pET21a (Novagen, Madison, WI) as previously described [18]. The N-terminal signal sequence and stop codons were removed to create a C-terminal translational fusion with a His_6 tag under the control of an IPTG-inducible promoter system. Point mutations were introduced into the construct by inverse PCR amplification of the template, as previously described [25,26]. Mutations were confirmed by DNA sequencing (VCU Nucleic Acid Research Facility, Richmond, VA). *Tm*ArgBP mutants were expressed in *E. coli* Origami Rosetta, a BL-21 derived strain, and initial purification was performed using thermos-precipitation at 65 °C and centrifugation (13,000 rpm, 25 min) to remove debris. The supernatant was further purified using Ni-NTA affinity chromatography. The yield of *Tm*ArgBP was ~400 μM per liter of culture. Protein purity was checked using SDS-PAGE and molecular weight was verified by MALDI mass spectrometry. Purified *Tm*ArgBP mutants were dialyzed (molecular weight cutoff, 10,000 Da) for 5 days at 4 °C to remove bound arginine and to equilibrate the samples in 5 mM phosphate buffer (pH 7.0). Model visualization of the *Tm*ArgBP structure was performed using PyMOL [27].

2.2. Preparation of fluorophore-bound TmArgBP single cysteine mutants

Protein at a concentration of 20 μM in 5 mM phosphate buffer (pH 7.0) was incubated overnight with a four-fold molar excess of thiol-reactive fluorophore at 4 °C in the dark. The environmentally sensitive probes HiLyte, DyLight, acrylodan, NBD, and ABD, were used to covalently modify *Tm*ArgBP mutants. Unreacted fluorophore was separated from the labeled protein using Econo-Pac 10DG (BioRad, Hercules, CA) columns pre-equilibrated with 5 mM phosphate (pH 7.0). Acrylodan and NBD were purchased from Invitrogen/Molecular Probes (Carlsbad, CA). ABD and HiLyte Fluor were purchased from Anaspec, Inc (Fremont, CA). DyLight Fluor was purchased from Thermo Scientific (Waltham, MA). Fluorophores were first dissolved in dry DMSO (20 mM) and used for the labeling experiments without further purification.

2.3. Steady-state fluorescence

Ligand-induced changes in fluorescence intensity were measured on a Varian Cary Eclipse spectrofluorometer in the absence and in the presence of arginine. Titrations of conjugated protein (1 μM) in 5 mM phosphate buffer, pH 7.0, were performed using a quartz cuvette with an arginine concentration range from 0 to 130 μM. Excitation wavelengths were 415 nm for ABD, 478 nm for NBD, and 426 nm for acrylodan. A minimum of three experimental trials were averaged for the calculation of dissociation constants. All data analysis was performed using the Origin 7.0 software package (OriginLab Corporation, Northampton, MA).

2.4. Time-resolved fluorescence spectroscopy

A Chronos time-domain spectrofluorometer (ISS, Champaign, Illinois) was used to measure time-resolved data for the *Tm*ArgBP mutants. Excitation of the ABD fluorophore was performed using a diode laser at 415 nm with pulse width of 70 picoseconds. Emission was detected through a magic angle polarizer (54.7°) and appropriate long-pass filters. All measurements were taken at 22 °C. Data collection and analysis was performed via the Vinci Multidimensional Fluorescence Spectroscopy software supplied with the instrument.

3. Results and discussion

3.1. Environmentally-sensitive probes produce limited fluorescence response for Arg binding

While there is limited sequence identity among PBPs, this superfamily is characterized by a common overall structural fold

consisting of two globular domains linked by a hinge region that facilitates large bending and twisting motions of the two domains upon ligand binding [28–31]. Consistent with this model, we found that the structure of *Tm*ArgBP shifts from a ligand-free, open conformation, to a closed, ligand-bound conformation, upon the addition of L-arginine (Fig. 1) [32]. This switch between the open and closed conformations has been utilized by a number of research groups for the construction of fluorescent protein sensing systems. A common procedure used to measure ligand binding to a protein is to select amino acids that are located within or around the ligand binding pocket for placement of a fluorescent reporter group. Alternatively, areas away from the binding pocket (allosteric sites) that are predicted to undergo significant changes in solvent accessibility following ligand binding have been successfully utilized as well. Wild-type *Tm*ArgBP does not contain any Cys residues, and single Cys mutations were introduced by site-directed mutagenesis to serve as attachment points for thiol-reactive environmentally sensitive fluorophores. We selected 5 locations based on a previous biophysical study of this protein using limited sequence homology to the *Escherichia coli* glutamine binding protein [33]. The remaining 4 locations were chosen based on an analysis of the x-ray crystallographic structural data recently reported for *Tm*ArgBP [32].

Three environmentally-sensitive fluorophores, ABD, NBD, and acrylodan, were investigated for their ability to signal arginine binding to *Tm*ArgBP (Fig. 2). Nine amino acids were chosen from solvent-exposed regions of *Tm*ArgBP and were individually mutated to determine the optimal position for reporting on arginine binding. Four of the mutations (G75C, M76C, Q97C, V98C) were peristerically located inside the arginine-binding pocket. Modest changes (<30%) in fluorescence intensity upon ligand binding were measured for the acrylodan modified proteins, while almost no change in emission intensity was observed at these sites for the NBD and ABD probes. The remaining mutations are located outside the pocket and showed little change in fluorescence intensity with any of the fluorescent probes. Consequently, this limited series of mutations did not provide a satisfactory change in emission intensity for the detection of arginine binding to *Tm*ArgBP.

While additional sites could have been selected for further mutagenesis studies, a confounding factor is represented by our observations for *Tm*ArgBP:T127C. Interestingly, T127C-NBD demonstrated an increase in fluorescence (+27%) upon the addition of arginine, whereas an average decrease in emission intensity (−20%) was observed for the same mutant bound to a different environmentally-sensitive fluorophore (T127C-acrylodan). This result demonstrates the complicated empirical nature of selecting both the optimal site of probe attachment and the specific identity of the fluorophore when evaluating the potential efficacy of such a biosening system. Consequently, a better-defined theoretical

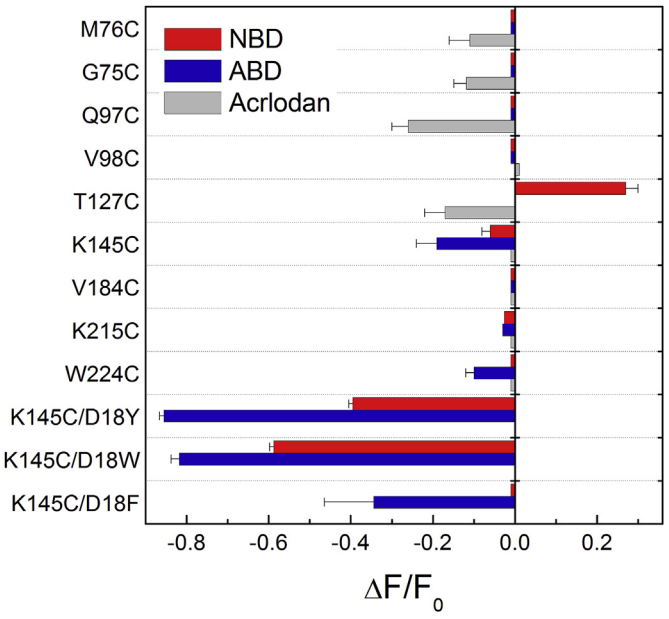

Fig. 2. Changes in fluorescence intensity of single and double mutants of *Tm*ArgBP. *Tm*ArgBP mutants (1 μM) were covalently modified with the environmentally-sensitive probes: NBD, ABD, or acrylodan. Fluorescence emission intensity of labeled protein was measured in the absence and in the presence of 100 μM arginine. The normalized change in fluorescence after binding arginine was calculated as $\Delta F/F_o = I_{sat} - I_{apo}/I_{sat}$.

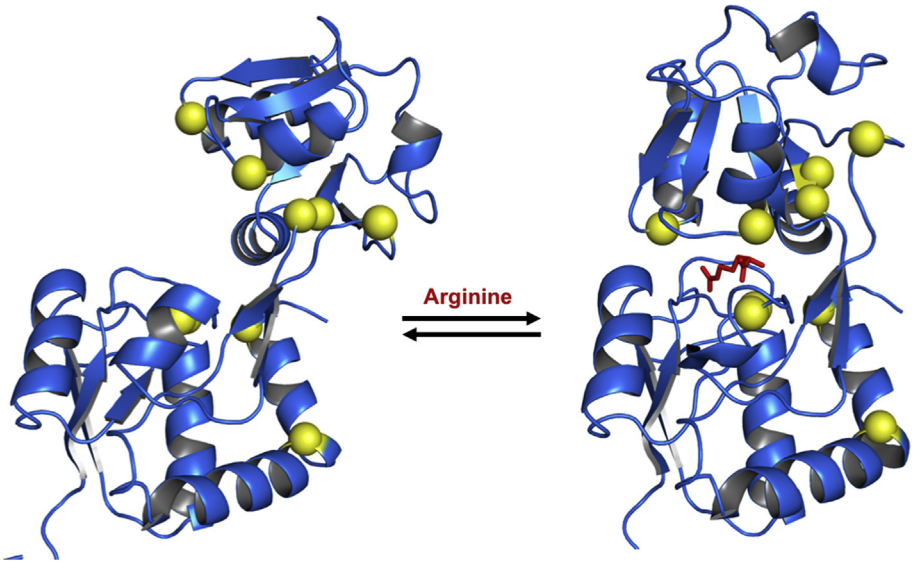

Fig. 1. *Tm*ArgBP conformational changes in the absence (PDB ID: 4PRS) and presence (4PSH) of arginine. The α-carbons of amino acids used for the covalent ligation of ABD, NBD, or acrylodan are depicted as yellow spheres. Figure generated by PyMOL [27]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

framework for more efficient construction of fluorescent protein biosensors is needed.

3.2. Fluorescence quenching using photo-induced electron transfer (PET) to report on ligand binding

One method of reporting protein conformational dynamics is through photo-induced electron transfer (PET) [34,35]. Electron donors, such as the aromatic amino acids, can participate in short-range interactions via π-stacking, hydrogen bonding, and van der Waals dynamics with a fluorophore. The ability to undergo electron transfer is a function of the oxidation and reduction potentials of the electron donor and acceptor [20,34]. In the arginine biosensing system described here (Fig. 3), the open conformation of the *Tm*ArgBP prevents the electron donor-acceptor bridge from forming and fluorescence emission is allowed to occur normally (on-state). Upon the addition of arginine, electron transfer is predicted to occur from the aromatic amino acid to fluorophore when the two moieties are in close proximity which results in quenching of the fluorescence emission (off-state). PET contrasts with another distance-dependent mechanism, Förster resonance energy transfer (FRET). With FRET the distance dependence for energy transfer from donor to acceptor is typically between 1 and 10 nm [36]; whereas in PET, donors and acceptors are brought into sub-nanometer proximity facilitating electron transfer that results in a decrease in fluorophore quantum yield (i.e., fluorescence emission quenching) [34].

3.3. PET quenching of TmArgBP K145C coupled to ABD or NBD

In the present study we took advantage of the large conformational change exhibited by *Tm*ArgBP that occurs following ligand binding. We determined the change in distance between all α-carbons in the open and closed states (i.e., before and after binding arginine). We found that in the open conformation the separation between α-carbons for K145 and D18 was 18.6 Å, while they are only 3.9 Å apart in the closed conformation. Based on this structural analysis, we inserted a Cys residue at K145 and a series of aromatic amino acids at D18 in the protein structure. We predicted that the aromatic amino acid will donate an electron to the excited fluorophore as the two groups come into close proximity following arginine binding (Fig. 3). Based on the known amino acid reduction potentials, Trp and Tyr were expected to be more efficient PET electron donors than Phe [20]. Previous work has shown that aromatic amino acids are able to quench the fluorescence emission of several different types of fluorescent moieties via a PET-based mechanism [34,37,38]. In particular, a PET mechanism has been extensively characterized for quenching of NBD-based chemosensors [39–42]. The theoretical basis for PET between NBD and Trp has been established previously [42]. The rate of the PET reaction is governed by the ground and excited state energies of the redox partners and may be estimated using the Rehm–Weller equation [43]. Oxidation and reduction potentials were used to estimate the ΔG for PET between −13 and −15 kcal mol^{-1}, which indicates that electron transfer between NBD and Trp is a thermodynamically allowable process [42]. Because the literature values for redox potentials of Tyr and Trp are similar, a similar estimation demonstrating the thermodynamic feasibility for PET between Tyr and NBD may be performed.

We first established a baseline change in fluorescence emission for either ABD or NBD covalently attached to *Tm*Arg containing a single point mutation at K145C. Upon the addition of arginine, ABD and NBD emission intensity decreased by a modest 19% and 6%, respectively (Fig. 2). Next, we constructed a double *Tm*ArgBP containing K145C and a second mutation at D18X, where X was either Trp, Tyr, or Phe. Our models of this system showed that this change in distance should provide adequate spacing for PET quenching even with the additional atoms from the side chains.

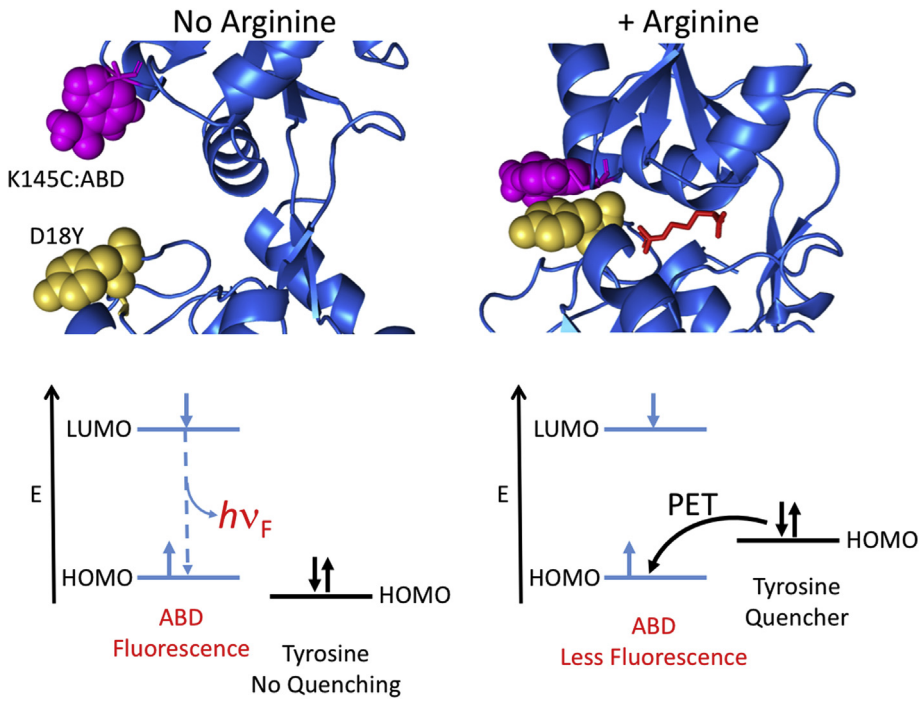

Fig. 3. Residues involved in PET interactions. (Top) *Tm*ArgBP residues at positions 18 and 145 are shown in the open and closed (ligand-binding) conformations as yellow space filling model, left and right, respectively. Arginine is depicted as a red stick structure in the *Tm*ArgBP closed binding pocket on the right. (Bottom) PET sensing scheme based on the conformational changes exhibited by *Tm*ArgBP in the absence and in the presence of arginine. Figure generated by PyMOL [27]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The *Tm*ArgBP double mutants were labeled with either NBD or ABD and the change in fluorescence measured in the absence and in the presence of arginine (Fig. 2). Since both measurements are made on the same sample, small differences in the fluorophore labeling efficiency should not impact the relative fluorescence measurements and analysis. K145C:D18Y covalently bound to ABD displayed an 85% decrease in fluorescence emission upon addition of 100 μM arginine, and a 39% decrease with NBD attached. This means that insertion of the Tyr residue caused a 4.5-fold change in the observed $\Delta F/F_0$ compared to the single K145C-ABD mutant. Although the magnitude of the NBD emission intensity is lower, these data indicated a 6.5-fold change for the protein modified with NBD. Similarly, *Tm*ArgBP K145C:D18W modified with ABD demonstrated an 81% decrease upon the addition of arginine, and a 58% decrease with NBD as the probe. In contrast, the K145C:D18F bound with ABD showed only a 34% decrease in emission intensity with the addition of arginine, and no measurable decrease with NBD. Since the reduction potential of Phe is known to be lower in comparison to tryptophan and tyrosine, the decrease arginine-dependent quenching of fluorescence emission was expected [34].

Due to the short distances between the fluorophore and aromatic moieties required to produce efficient quenching of the emission intensity, the data suggest that the PET mechanism is responsible for the arginine-dependent decrease observed. Because we employed the environmentally-sensitive probes NBD and ABD in these measurements, we cannot completely rule out the impact that local polarity effects had on the observed changes in the fluorescence signal following the insertion of an aromatic residue. However, based on the measurements with NBD attached to the double mutants, a PET quenching mechanism better explains the data. Based on the reduction potentials of Trp and Tyr, we predicted that these aromatic amino acids would show larger PET-based quenching of the fluorescence emission. The absence of any measureable change in fluorescence with the insertion of a Phe residue, which has a much lower reduction potential while still being a nonpolar, aromatic amino acid, suggests that a PET mechanism rather than micro-environmental changes in polarity is being observed.

Having established that the aromatic amino acids Tyr and Trp produced very high levels of fluorescence quenching, the emission spectra of the K145C double mutants were measured and the dissociation constants were determined upon titration with arginine (Fig. 4). From these data, we calculated a dissociation constant for arginine of 0.89 ± 0.13 μM and 0.87 ± 0.21 μM for the K145C:D18W-ABD and K145C:D18Y-ABD protein sensors, respectively. A slightly higher dissociation constant was measured for the sensors conjugated with NBD of 1.4 ± 0.4 μM and 1.5 ± 0.3 μM, respectively. Based on the binding constant determined for arginine of the PET-based sensor described here, serial dilution of a sample may be necessary to accurately estimate arginine levels, since the concentration of arginine in human plasma is approximately 40 μM [44]. In contrast, no change in fluorescence emission was detected upon the addition of structurally similar amino acids L-glutamine, L-lysine, or L-histidine (data not shown), which demonstrates the specificity of the *Tm*ArgBP.

Previous work has shown that fluorescent probes containing a xanthene group may also produce PET-based quenching. Hilyte (AnaSpec) and Dylite (Thermo Scientific) fluorophores contain a xanthene ring system and thiol-reactive versions of these probes were covalently attached to *Tm*ArgBP K145C:D18X. However, we did not detect any changes in fluorescence intensity upon binding arginine in combination with any of the aromatic resides at position D18. This finding was unexpected, and may potentially be attributed to an undetermined steric influence exerted by the protein in the closed conformation that does not allow proper contact between the bulkier xanthene ring structures of these fluorescent probes and the aromatic quenchers.

3.4. Time-resolved fluorescence intensity decay

Time-resolved fluorescence intensity measurements were performed for K145C:D18Y and K145C:D18W double mutants covalently modified with ABD because these mutants produced the largest decrease in emission intensity (Table 1). Both proteins with and without arginine were best fit by a bi-exponential model, although the second fractional lifetime component (τ_2) accounted for >93% of the emission intensity decay in all cases. In the absence of arginine, the fluorescence emission lifetime of ABD bound to both mutants was approximately 6 ns? When attached to the *Tm*ArgBP K145C:D18Y mutant, the lifetime decreased as expected upon the addition of arginine. In contrast, an increase in the ADB lifetime was observed for *Tm*ArgBP K145C:D18W mutant upon the addition of arginine. The limited lifetime data presented in Table 1 may be combined with the data from Fig. 2 showing the decrease in emission intensity in order to begin to understand the nature of the PET quenching processes being observed. If the ratio of average lifetime in the absence and in the presence of arginine is similar to the ratio of the fluorescence emission (as a proxy of quantum yield) measured with and without arginine, then a dynamic quenching may be dominant. Alternatively, if the ratio of average lifetimes is near unity, while the fluorescence emission ratio is much larger, then static quenching may better describe the PET mechanism that results from the closer proximity of the fluorophore and aromatic quencher upon the addition of arginine [36]. Using the data from Table 1, the ratio of average ABD lifetimes in the absence and in the presence of arginine is 1.20 and 0.84 for the Tyr and Trp *Tm*ArgBP mutants, respectively. Whereas, using the data from Fig. 2 (right panels), the ratio of fluorescence emission in the absence and in the presence of arginine is approximately 6 for both systems. This indicates that static quenching is likely the dominate mechanism responsible for the emission decreases observed for both systems. This kind of quenching analysis has been observed following for the close interactions of other donor-quencher pairs as well [45]. An interesting and unexpected result from this data is that the average lifetime for the *Tm*ArgBP K145C:D18W increases upon the addition of arginine, which is in contrast to the large decrease in fluorescence emission. More investigation with additional concentrations of arginine are needed to understand if this effect persits. We may speculate that in the open conformation, the ABD probe experiences a microenvironment that provides a deactivation pathway that is no longer accessible following the *Tm*ArgBP conformational change in the presence of arginine; however the emission is quenched to a larger extent upon close contact with the Trp quencher via PET. These data may suggest that the mechanism of action for the interaction between Trp and ABD may be different than that for Tyr and ABD in these systems, although a more thorough photodynamic analysis is needed.

4. Conclusion

We describe the construction of a fluorescent protein biosensor for the determination of L-arginine in solution. Many successful examples of using microbial periplasmic proteins as scaffolds for biosensor design have been described in the literature (e.g., [7–11]). Typically, this protein biosensor design approach includes the production of single cysteine point mutants that are covalently modified with environmentally-sensitive fluorophores. The locations for cysteine insertion are traditionally chosen based on predicted changes in solvent accessibility for specific residues in the open and closed conformations. Based on this approach, we

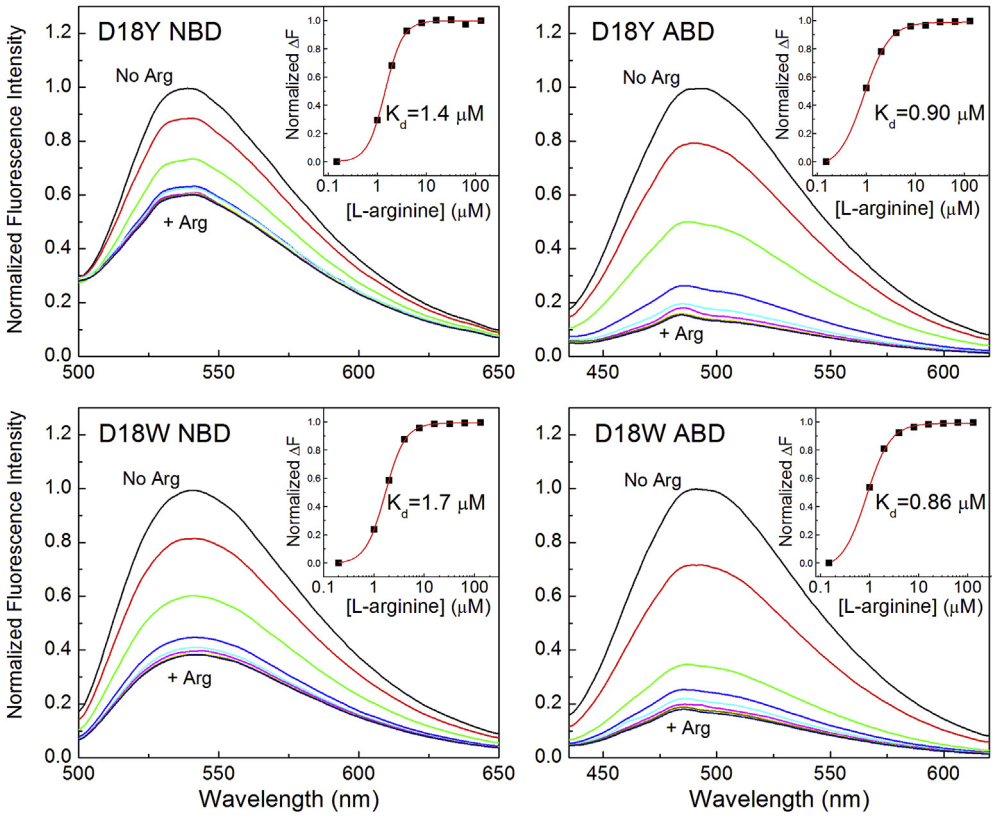

Fig. 4. Representative data from a single experimental trial of normalized emission spectra for K145C double mutants titrated with arginine. Panels show changes in fluorescence intensities for a) K145C:D18Y NBD (top left), b) K145C:D18Y ABD (top right), c) K145C:D18W NBD (bottom left), and d) K145C:D18W ABD (bottom right). Inserts are dissociation constants calculated from each experiment in the presence of arginine concentrations from 0 to 130 μM.

Table 1
Fluorescence intensity decay data for ABD covalently attached to either *Tm*ArgBP K145C:D18Y or K145C:D18W.

*Tm*ArgBP Mutant	τ_1 (ns)	τ_2 (ns)	f_1	f_2	$\overline{\tau}$ (ns)[a]	χ^2
K145C:D18**Y**						
0 μM Arg	0.08	5.92	0.01	0.99	5.9	1.48
130 μM Arg	0.38	5.16	0.04	0.95	4.9	1.67
K145C:D18**W**						
0 μM Arg	0.06	6.00	0.02	0.98	5.9	1.26
130 μM Arg	0.61	7.48	0.07	0.93	7.0	1.48

[a] $\overline{\tau} = \Sigma f_i \tau_i$.

attempted to use the conformational changes exhibited by *T. maritima* arginine binding protein to design an arginine biosensor. A matrix of 9 single cysteine attachment sites and 3 fluorescent probes was constructed using the *Tm*ArgBP as the scaffold that provides ligand specificity. However, only small changes in fluorescence emission upon introduction of the arginine were observed (Fig. 2). While this brute force method is experimentally straightforward, it offers a limited theoretical framework for successful biosensor design.

To overcome this challenge, we predicted that ligand-dependent conformational changes in *Tm*ArgBP could be measured by photo-induced electron transfer (PET) in a quantitative manner. As a proof of concept, we used the *Tm*ArgBP structural data to select a single pair of sites (D18 and K145) that were predicted to provide the spatial requirements of PET-based emission quenching coupled to the protein conformational changes upon binding arginine. Based on the available redox properties, aromatic residues were site-specifically introduced into *Tm*ArgBP to serve as intrinsic PET quenchers of a covalently attached fluorescent probe. Quenching efficiency is dictated by the orientation of fluorophore and the quenching group and is distance-dependent [20,34]. Of the aromatic residues tested, electron transfer between tyrosine and covalently bound ABD appears to be the most efficient. Limited changes in emission intensity were observed in the absence of the aromatic group or in the presence of a Phe residue which does not have a reduction potential that is predicted to support PET in this case. Taken together this data provides support for a PET-based quenching mechanism.

*Tm*ArgBP is uniquely suited for utilization as a potential fluorescent protein biosensor due to its physicochemical stability [17,19]. The incorporation of an internal PET quencher allows us to take advantage of known structural changes rather than predicted solvent accessibility and simplifies the construction of protein biosensors. Future work may attempt to incorporate the ABD probe into a growing organism to produce a genetically encodable fluorescence biosensor using an orthogonal tRNA synthetase system [46,47]. The benefit of this design strategy, which utilizes a specifically engineered fluorescence quenching interaction, is that it may be broadly utilized by any binding protein that exhibits a ligand-dependent conformational switch.

Conflict of interest disclosure

The authors declare no completing financial interests.

Acknowledgements

The authors would like to thank the University of Richmond

School of Arts and Sciences and the project Cluster CLAN “Safe-&Smart” for funding research materials and student summer stipends to support this work. Additionally, funding for the time-resolved fluorometer was provided by a NSF MRI (0722600) grant.

References

[1] K. Vaidyanathan, Molecular diagnosis of urea cycle disorders: current global scenario, Indian J. Biochem. Biophysics 50 (2013) 357–362.

[2] D.R. Carvalho, J.M. Brum, C.E. Speck-Martins, F.D. Ventura, M.M. Navarro, K.E. Coelho, D. Portugal, R. Pratesi, Clinical features and neurologic progression of hyperargininemia, Pediatr. Neurol. 46 (2012) 369–374.

[3] S.A. Berry, U. Lichter-Konecki, G. Diaz, S.E. McCandless, W. Rhead, W. Smith, C. LeMons, S.C.S. Nagamani, D.F. Coakley, M. Mokhtarani, B.F. Scharschmidt, B. Lee, Glycerol phenylbutyrate treatment in cheldren with urea cycle disorders: pooled analysis of short and long-term ammonia control and outcomes, Mol. Genet. Metabolism 112 (2014) 17–24.

[4] Y.Y. Sin, L.L. Ballantyne, K. Mukherjee, T. St Amand, L. Kyriakopoulou, A. Schulze, C.D. Funk, Inducible arginase 1 deficiency in mice leads to hyperargininemia and altered amino acid metabolism, PLOS One 8 (2013) e80001.

[5] L. Tolosa, On the design of low-cost fluorescent protein biosensors, Adv. Biochem. Eng. Biotechnol. 116 (2010) 143–157.

[6] S. Vigneshvar, C.C. Sudhkumari, B. Senthikumaran, H. Prakash, Recent advances in biosensor technology for potential applications - an overview, Front. Bioeng. Biotechnol. 4 (2016) 11.

[7] J.D. Dattelbaum, J.R. Lakowicz, Optical determination of glutamine using a genetically engineered protein, Anal. Biochem. 291 (2001) 89–95.

[8] R.M. deLorimier, J.J. Smith, M.A. Dwyer, L.L. Looger, K.M. Sali, C.D. Paavola, S.S. Rizk, S. Sadigov, D.W. Conrad, L. Loew, H.W. Hellinga, Construction of a fluorescent biosensor family, Protein Sci. 11 (2002) 2655–2675.

[9] L.L. Salins, S.K. Deo, S. Daunert, Phosphate binding protein as the bio-recognition element in a biosensor for phosphate, Sensors Actuators B-Chemical 97 (2004) 81–89.

[10] M.A. Dwyer, H.W. Hellinga, Periplasmic binding proteins: a versatile superfamily for protein engineering, Curr. Opin. Struct. Biol. 14 (2004) 495–504.

[11] J. Siegrist, T. Kazarian, C.M. Ensor, J. Smita, M. Madou, P. Wang, S. Daunert, Continuous glucose sensor using novel genetically engineered binding polypeptides towards in vivo applications, Sensors Actuators B-Chemical 149 (2010) 51–58.

[12] A.S. Ethayathulla, Y. Bessho, A. Shinkai, B. Padmanabhan, T.P. Singh, P. Kaur, S. Yokoyama, Purification, crystallization and preliminary x-ray diffraction analysis of the putative abc transporter atp-binding protein from thermotoga maritima, Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun. 64 (2008) 498–500.

[13] C.F. Higgins, Abc transporters: from microorganisms to man, Annu. Rev. Cell Biol. 8 (1992) 67–113.

[14] G.F. Ames, Bacterial periplasmic transport systems: structure, mechanism, and evolution, Annu. Rev. Biochem. 55 (1986) 397–425.

[15] B.S. Der, J.D. Dattelbaum, Construction of a reagentless glucose biosensor using molecular exciton luminescence, Anal. Biochem. 375 (2008) 132–140.

[16] A. Ruggiero, J.D. Dattelbaum, A. Pennacchio, L. Iozzino, M. Staiano, M.S. Luchansky, B.S. Der, R. Berisio, S. D'Auria, L. Vitagliano, Crystallization and preliminary x-ray crystallographic analysis of ligand-free and arginine-bound forms of *thermotoga maritima* arginine-binding protein, Acta Crystallogr. F67 (2011) 1462–1465.

[17] A. Scire, A. Marabotti, M. Staiano, L. Iozzino, M.S. Luchansky, B.S. Der, J.D. Dattelbaum, F. Tanfani, S. D'Auria, Amino acid transport in thermophiles: characterization of an arginine-binding protein in *thermotoga maritima*. 2. Molecular organization and structural stability, Mol. Biosyst. 6 (2010) 687–698.

[18] M.S. Luchansky, B.S. Der, S. D'Auria, G. Pocsfalvi, L. Iozzino, D. Marasco, J.D. Dattelbaum, Amino acid transport in thermophiles: characterization of an arginine-binding protein in *thermotoga maritima*, Mol. Biosyst. 6 (2010) 132–141.

[19] A. Ausili, A. Pennacchio, M. Staiano, J.D. Dattelbaum, D. Fessas, A. Sciraldi, S. D'Auria, Amino acid transport in thermophiles: charaterization of an arginine-binding protein from *thermotoga maritima*. 3. Conformational dynamics and stability, J. Photochem. Photobiol. B 118 (2013) 66–73.

[20] N. Marme, J.-P. NKnemeyer, S. Markus, J. Wolfrum, Inter- and intramolecular fluorescence quenching of organic dyes by tryptophan, Bioconjugate Chem. 14 (2003) 1133–1139.

[21] L.L. Looger, S. Lalonde, W.B. Frommer, Genetically encoded fret sensors for visualizing metabolites with subcellular resolution in living cells, Plant Physiol. 138 (2005) 555–557.

[22] S.B. VanEngelenburg, A.E. Palmer, Fluorescent biosensors of protein function, Curr. Opin. Chem. Biol. 12 (2008) 60–65.

[23] J. Lippincott-Schwartz, Emerging in vivo analyses of cell function using fluorescence imaging, Annu. Rev. Biochem. 80 (2011) 327–332.

[24] J.S. Marvin, B.G. Borghuis, L. Tian, J. Cichon, T. Harnett, J. Akerboom, A. Gordus, S.L. Renninger, T. Che, C.I. Bargmann, M.B. Orger, E.R. Schreiter, J.B. Demb, W. Gan, S.A. Hires, L.L. Looger, An optimized fluorescent probe for visualizing glutamate neurotransmission, Nat. Methods 10 (2013) 162–170.

[25] J. Chiu, P.E. March, R. Lee, D. Tillett, Site-directed, ligase-independent mutagenesis (slim): a single-tube methodology approaching 100% efficiency in 4 h, Nucleic Acids Res. 32 (2004) e174.

[26] J. Chiu, D. Tillett, I.W. Dawes, P.E. March, Site-directed, ligase-independent mutagenesis (slim) for highly efficient mutagenesis of plasmids greater than 8kb, J. Microbiol. Methods 73 (2008) 195–198.

[27] W.L. DeLano, The Pymol Molecular Graphics System, Delano Scientific, San Carlos, CA, 2002.

[28] J.S. Marvin, H.W. Hellinga, Engineering biosensors by introducing fluorescent allosteric signal transducers: construction of a novel glucose sensor, J. Am. Chem. Soc. 120 (1998) 7–11.

[29] M.J. Borrok, L.L. Kiessling, K.T. Forest, Conformational changes of glucose/galactose-binding protein illuminated by open, unliganded, and ultra-high-resolution ligand-bound structures, Protein Sci. 16 (2007) 1032–1041.

[30] F.A. Quiocho, P.S. Ledvina, Atomic structure and specificity of bacterial periplasmic receptors for active transport and chemotaxis: variation of common themes, Mol. Microbiol. 20 (1996) 17–25.

[31] A.J. Bjorkman, R.A. Binnie, H. Zhang, L.B. Cole, M.A. Hermodson, S.L. Mowbray, Probing protein-protein interactions. The ribose-binding protein in bacterial transport and chemotaxis, J. Biol. Chem. 269 (1994) 30206–30211.

[32] A. Ruggiero, J.D. Dattelbaum, M. Staiano, R. Berisio, S. D'Auria, L. Vitagliano, A loose domain swapping organization confers a remarkable stability to the dimeric structure of teh arginine binding protein from thermotoga maritima, PLOS One 9 (2014) e96560.

[33] L.J. Deacon, H. Billones, A.A. Galyean, T. Donaldson, A. Pennacchio, L. Iozzino, S. D'Auria, J.D. Dattelbaum, Tryptophan-scanning mutagenesis of the ligand binding pocket in *thermotoga maritima* arginine-binding protein, Biochimie 99 (2014) 208–214.

[34] S. Doose, H. Neuweiler, M. Sauer, Fluorescence quenching by photinduced electron transfer: a reporter for conformational dynamics of macromolecules, ChemPhysChem 10 (2009) 1389–1398.

[35] A.J.M. Huxley, M. Schroeder, N. Gunaratne, A.P. de Silva, Modification of fluorescent photoinduced electrong transfer (pet) sensors/switches to produce molecular phot-ionic action, Angew. Chem. 53 (2014) 3622–3625.

[36] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Springer, New York, 2006.

[37] H. Chen, S.S. Ahsan, M.B. Santigao-Berrios, H.D. Abruna, W.W. Webb, Mechanisms of quenching of alexa fluorophores by natural amino acids, J. Am. Chem. Soc. 132 (2010) 7244–7245.

[38] A.M. Jones Brunette, D.L. Farrens, Distance mapping in proteins using fluorescence spectroscopy: tyrosine, like tryptophan, quenches bimane fluorescence in a distance-dependent manner, Biochemistry 53 (2014) 6290–6301.

[39] Y. Chen, X. Zhang, X. Huang, S. Wen, Y. Deng, Y. Zhang, C. CZhang, J. Jin, H. Li, S. Yao, A simple and reversible fluorescent probe based on nbd for rapid detection of hypochlorite and its application for bioimaging, RCS Adv. 5 (2015) 79519–79524.

[40] Y. Shen, Y. Zhang, X. Zhang, C. Zhang, L. Zhang, J. Jin, H. Li, S. Yao, A new turn-on fluorescent sensor based on nbd for highly selective detection for hg^{2+} in aqueous media and imaging in live cells, Anal. Methods 6 (2014) 4797–4802.

[41] W. Jiang, Q. Fu, H. Fan, W. Wang, An nbd fluorophore-based sensitive and selective fluorescent probe for zinc ion, Chem. Commun. (2008) 259–261.

[42] M.C. Cuquerella, J. Rohacova, M.L. Marin, M.A. Miranda, Stereodifferentiation in fluorescence quenching within cholic acid aggregates, Chem. Commun. 46 (2010) 4965–4967.

[43] D. Rehm, A. Weller, Kinetics of fluorescence quenching by electron and h-atom transfer, Israel J. Chem. 8 (1970) 259–271.

[44] N. Luneburg, V. Xanthankis, E. Schwedhelm, L.M. Sullivan, R. Maas, M. Anderssohn, U. Riederer, N.L. Glazer, R.S. Vasan, R.H. Boger, Reference intervals for plasma l-arginine and the l-arginine:Asymmetric dimthylarginine ratio in the framingham offspring cohort, J. Nutr. 14 (2011) 2186–2190.

[45] M.K. Johansson, H. Fidder, D. Dick, R.M. Cook, Intramolecular dimers: a new strategy to fluroescence quenching in dual-labeled oligonucleotide probes, Jouranl Am. Chem. Soc. 124 (2002) 6950–6956.

[46] H.S. Lee, J. Guo, E.A. Lemke, R.D. Dimia, P.G. Schultz, Genetic incorporation of a small, environmentally sensitive, fluorescent probe into proteins in *sacchromyces cerevisiae*, Jouranl Am. Chem. Soc. 131 (2009) 12921–12923.

[47] T.L. Hendrickson, V. de Crecy-Lagard, P. Schimmel, Incorporation of nonnatural amino acids into proteins, Annu. Rev. Biochem. 73 (2004) 147–176.