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Interactions of AsCy3 with Cysteine-Rich Peptides

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Supporting Information

ABSTRACT: There is great interest in fluorogenic compounds that tag biomolecules within cells. Biarsenicals are fluorogenic compounds that become fluorescent upon binding four proximal Cys thiols, a tetracysteine (Cys4) motif. This work details interactions between the biarsenical AsCy3 and Cys4 peptides. Maximal affinity was observed when two Cys-Cys pairs were separated by at least 8 amino acids; the highest affinity ligand bound in the nanomolar concentration range (K_{app} = 43 nM) and with a significant (3.2-fold) fluorescence enhancement.

There is great interest in the identification of fluorogenic compounds that tag biomolecules within cells. Molecules with these properties, especially those that are bright, specific, and nontoxic, can often extract high-resolution information from within a complex, heterogeneous environment.1 When the biomolecule is a protein, fluorogenic compounds can define intracellular location, monitor protein–protein interactions, discriminate conformations, and quantify protein activity. Biarsenical dyes,2 exemplified by FlAsH3 and ReAsH,4 represent one such class of fluorogenic compound. These compounds are distinguished by a fluorescence enhancement that occurs upon binding to proteins containing four proximal Cys thiols, a tetracysteine (Cys4) motif. Over the past dozen years, fluorogenic biarsenicals have been used to label and visualize β-tubulin,5 monitor amyloid formation,6 localize viruses,7 probe transmembrane α-helix interactions and orientations,8 and evaluate conformational changes in the β2-9 and δ2-9-arenergic receptors,10 among other applications.11

These important discoveries notwithstanding, the application of FlAsH and ReAsH to discover new biology (especially within the cell) is limited by strong background labeling and relatively weak fluorescence.2,4 Background labeling results from the interaction of FlAsH and ReAsH with nonspecific thiols as well as membranes and hydrophobic protein pockets.5 Even with an improved binding sequence,12 ReAsH is still less bright than common fluorophores such as Alexafluor-488 and BODIPY FL. Moreover, despite the differences in their emission maxima (528 and 608 nm, respectively) the similarity of the FlAsH and ReAsH structures prohibits their use in simultaneous two-color labeling experiments.4

Recently we applied the biarsenical ReAsH in a bipartite mode13 along with total internal reflectance microscopy (TIR-FM) to detect, characterize, and differentiate ligand-induced conformational changes within the epidermal growth factor receptor (EGFR) on the mammalian cell surface.13,14 Through the design of EGFR variants with Cys-Cys pairs within the cytosolic juxtamembrane (JM) segment, we discovered that the binding of the growth factor EGF induced the formation of an anti-parallel coiled coil within the JM that was functionally linked to kinase activation. Other growth factors, most notably TGF-α, induced a different structure.13,14 Our ability to probe and differentiate structures within the juxtamembrane segment would be greatly enhanced by an alternative to FlAsH and ReAsH, especially one that was bright and photostable and could detect and report on alternative Cys4 motifs.

One molecule with some potential in this regard, AsCy3 (Figure 1A), was reported in 2007.15 In AsCy3 the biarsenical motif is displayed on a Cy3 scaffold and was reported to bind the alternative Cys4 motif Cys-Cys-Lys-Ala-Ala-Ala-Cys-Cys with a brightness comparable to that of ReAsH (5.0 × 10^4 M^{-1} cm^{-1})4,10 and significantly greater (>30-fold) photo-stability.15 Since 2007, AsCy3 has been transformed into a super-resolution probe16 and a membrane-permeable dye via substitution of the anionic sulfonate side chains for methyl esters,17 and the monoarsenic variant has explored dithiol oxidation in bacteria.18 Here we report that the initially described Cys4 motif binds AsCy3 with only modest affinity and fluorescent enhancement, but that higher affinity (100-fold) and brightness (>3-fold) is seen with the expanded Cys4 motif Cys-Cys-Lys-Ala-Glu-Ala-Ala-Lys-Ala-Glu-Ala-Lys-Cys. We hope that this information will aid researchers as they apply AsCy3 to characterize protein interactions on the cell surface and ultimately within the cytosol.

AsCy3 was synthesized following a modified procedure (Scheme S1 in Supporting Information), and its identity was confirmed with 2H and 31C NMR and high-resolution mass spectrometry. When dissolved at 10 μM in 50 mM HEPES (pH 7.5) containing 10% DMSO, the parent Cy3 displayed an absorbance maximum at 546 nm (ε_{546} = 126,000 M^{-1} cm^{-1})
These values di
emission at 575 nm (Figure S1 in Supporting Information). ε
(absorbance, (emission).
changes in both
repeated the titration of AsCy3 with CyTag, monitoring
the hypothetical complex between AsCy3 and CyTag. (A) Sequence of
were also studied by characterizing the changes in the fluorescence
AsCy3 bound to two ethanediithiol (EDT) ligands (calculated using Gaussian 0921 and the internal molecular mechanics UFF package) was characterized by an interatomic As—As distance of 15.83 Å, a value longer than that between the α-carbons of residues i and i + 7 on a canonical α-helix (10.8 Å), the proposed target site for AsCy3.15 Calculation of the ground state geometry of AsCy3 bound to Cys-Cys-Lys-Ala-Glu-Ala-Ala-Cys-Cys suggested a compressed interatomic As—As distance (13.44 Å) and a non-α-helical peptide backbone (Figure 1D). Furthermore, in the calculated complex, AsCy3 was nonplanar, with a > 100° angle between the normal vectors to the two indole ring planes (Figure S2 in Supporting Information). Cy3 fluorophores demand a planar, conjugated π system to achieve significant quantum yields.22 These calculations imply a mismatch between the structure of AsCy3 and the most favorable disposition(s) of Cys-Cys ligands on CyTag. They also suggest two factors that could contribute to the low AsCy3—CyTag affinity: (1) interaction of AsCy3 with only one Cys-Cys motif (not two) and (2) strain energy associated with forming the Cys4-coordinate complex. More importantly, the calculations suggest that target sites with longer intervening sequences would better match the AsCy3 structure and permit the formation of a more planar, higher-affinity, and more fluorogenic complex.

To better explore the AsCy3—CyTag binding mode, we synthesized a pair of CyTag variants in which one (TagΔ2) or both (TagΔ4) Cys-Cys motifs were replaced by Ala-Ala (Figure 2A). The interactions of TagΔ2 and TagΔ4 with AsCy3 were evaluated by monitoring changes in both fluorescence intensity (Figure 2B) and fluorescence polarization (Figure 2C) as a function of peptide concentration. Only TagΔ2 showed evidence of an interaction with AsCy3 (Figure 2B). As was true for the CyTag interaction, the data could be fit to a 1:1 binding isotherm, yielding a K_{app} value of 960 ± 150 nM based on fluorescence intensity changes and K_{app} = 410 ± 92 nM based on changes in fluorescence polarization. These K_{app} values equal or exceed those determined for CyTag itself, depending on the method (FI, 970 ± 140 nM; FP, 2.3 ± 0.6

and a emission maximum at 557 nm (Figure S1 in Supporting Information). These values compare well with those in methanol, where an absorbance maximum at 549 nm (ε_{549} = 120,000 M^{-1} cm^{-1}) was reported.19 Both the absorbance and emission maxima of AsCy3 are red-shifted relative to Cy3; under identical conditions AsCy3 exhibited maximal absorbance at 564 nm (ε_{564} = 103,000 M^{-1} cm^{-1}) and maximal emission at 575 nm (Figure S1 in Supporting Information). These values differ slightly from those reported: λ_{max} = 560 nm (absorbance, ε_{560} = 180,000 M^{-1} cm^{-1}); λ_{max} = 568 nm (emission).

The initial AsCy3 report described interactions with Cy3Tag, a 34-aa peptide containing two Cys-Cys motifs separated by the sequence Lys-Ala-Glu-Ala-Ala (Figure 1A). As reported, the complex formed with an equilibrium dissociation constant (K_{app}) of 80 ± 10 nM, calculated on the basis of CyTag-dependent changes in fluorescence intensity at 576 nm. We repeated the titration of AsCy3 with CyTag, monitoring changes in both fluorescence polarization (FP, which measures binding directly) and fluorescence intensity (FI, which does not). Experiments were performed initially under the conditions reported: HEPES buffer containing 10% DMSO, 140 mM KCl, 1 mM TCEP, 100 μM EDT, and 1 mM BME. No interaction between AsCy3 and Cy3Tag was observed under these conditions whether the association was monitored by changes in FI or FP at Cy3Tag concentrations as high as 15 μM (Figure 1B). However, in a buffer lacking the competitive inhibitors EDT and BME, concentration-dependent changes in both FP and FI were observed. In each case, the data fit a simple 1:1 equilibrium-binding isotherm to provide K_{app} values of 970 ± 140 nM (FI) and 2.4 ± 0.6 μM (FP). These values are at least an order of magnitude higher than those reported by Cao et al.15 and were obtained only in the absence of thiol competitors. Competition of the AsCy3—CyTag complex with EDT yielded an inhibition constant (K_i) of 5.6 ± 0.9 μM, a value only slightly higher than the K_{app} values determined for the CyTag complex, providing additional evidence for a low-affinity AsCy3—CyTag interaction. Although cyanine dyes such as AsCy3 can aggregate,20 our experimental setup minimizes the effect of [AsCy3] on the calculated K_{app} (see Supporting Information).

The interactions between AsCy3 and CyTag were also studied by characterizing the changes in the fluorescence spectrum of AsCy3 in the presence of Cy3Tag (Figure S1C in Supporting Information). Upon incubation of 100 nM AsCy3 (in 50 mM HEPES pH 7.5, 140 mM KCl, and 1 mM TCEP) with 10 μM Cy3Tag, the emission maximum was maintained at 575 nm as expected,15 but fluorescence emission was increased by only 2.5-fold, significantly less than the reported 6-fold increase under similar conditions.15

These results prompted us to evaluate the structure of AsCy3 and its complex with CyTag. The ground state geometry of AsCy3 bound to two ethanedithiol (EDT) ligands (calculated using Gaussian 0921 and the internal molecular mechanics UFF package) was characterized by an interatomic As—As distance of 15.83 Å, a value longer than that between the α-carbons of residues i and i + 7 on a canonical α-helix (10.8 Å), the proposed target site for AsCy3.15 Calculation of the ground state geometry of AsCy3 bound to Cys-Cys-Lys-Ala-Glu-Ala-Ala-Cys-Cys suggested a compressed interatomic As—As distance (13.44 Å) and a non-α-helical peptide backbone (Figure 1D). Furthermore, in the calculated complex, AsCy3 was nonplanar, with a > 100° angle between the normal vectors to the two indole ring planes (Figure S2 in Supporting Information). Cy3 fluorophores demand a planar, conjugated π system to achieve significant quantum yields.22 These calculations imply a mismatch between the structure of AsCy3 and the most favorable disposition(s) of Cys-Cys ligands on CyTag. They also suggest two factors that could contribute to the low AsCy3—CyTag affinity: (1) interaction of AsCy3 with only one Cys-Cys motif (not two) and (2) strain energy associated with forming the Cys4-coordinate complex. More importantly, the calculations suggest that target sites with longer intervening sequences would better match the AsCy3 structure and permit the formation of a more planar, higher-affinity, and more fluorogenic complex.

To better explore the AsCy3—CyTag binding mode, we synthesized a pair of CyTag variants in which one (TagΔ2) or both (TagΔ4) Cys-Cys motifs were replaced by Ala-Ala (Figure 2A). The interactions of TagΔ2 and TagΔ4 with AsCy3 were evaluated by monitoring changes in both fluorescence intensity (Figure 2B) and fluorescence polarization (Figure 2C) as a function of peptide concentration. Only TagΔ2 showed evidence of an interaction with AsCy3 (Figure 2B). As was true for the CyTag interaction, the data could be fit to a 1:1 binding isotherm, yielding a K_{app} value of 960 ± 150 nM based on fluorescence intensity changes and K_{app} = 410 ± 92 nM based on changes in fluorescence polarization. These K_{app} values equal or exceed those determined for CyTag itself, depending on the method (FI, 970 ± 140 nM; FP, 2.3 ± 0.6

Figure 1. AsCy3 and its interactions with Cy3Tag. (A) Sequence of Cy3Tag (left) and structure of AsCy3 (right). (B) Plots illustrating the changes in FI (left) and FP (right) of 0.1 μM AsCy3 after incubation with the [Cy3Tag] indicated in the presence (closed) and absence (open) of 100 μM EDT and 1 mM BME. (C) Competition between 10 μM Cy3Tag and [EDT] for 0.1 μM AsCy3, measured by changes in FI. Errors show standard error. (D) Minimized structure (Gaussian) of the hypothetical complex between AsCy3 and Cy3Tag.
The observation that AsCy3 interacts comparably with peptides containing one or two Cys-Cys motifs suggests that only one Cys-Cys pair in the Cy3Tag sequence contributes to complex stability. Indeed, the change in AsCy3 fluorescence emission (100 nM) in the presence of TagΔ2 (10 μM) is >60% of the enhancement observed with CyTag. This observation indicates that the second Cys-Cys pair contributes minimally, if at all, to AsCy3 fluorogenicity, and the dye may only be partially bound to all four cysteines (Figure S1C in Supporting Information).

Next, we synthesized a second set of potential AsCy3 ligands containing progressively longer intervening sequences and evaluated their interactions with AsCy3 using fluorescence intensity and polarization assays (Figure 3). These potential AsCy3 ligands contained from 6 to 13 amino acids interposed between the two Cys-Cys motifs and were largely unordered at 30 μM in the absence of AsCy3, as judged by circular dichroism (CD) spectroscopy (5 mM phosphate (pH 7.5), 140 mM KCl, and 5 mM DTT) (Figure S3 in Supporting Information). All of the second-generation peptides evaluated formed complexes with AsCy3, exhibiting $K_{app}$ values between 49 nM and 1.3 μM in the absence of EDT and BME. With one exception (Tag+2), the values determined using FI and FP agreed to within their 95% confidence intervals (Table S1 in Supporting Information). Notably, the fitted value of $K_{app}$ decreased as the number of residues between the two Cys-Cys motifs increased from 5 to 9, with the largest increase between Tag+3 and Tag+4 (Figure S4 in Supporting Information). The highest affinity ligand was Tag+6, whose AsCy3 complex was characterized by a $K_{app}$ value of 94 ± 16 nM (FI); $K_{app} = 49 ± 13$ nM by fluorescence polarization. Titration of AsCy3 (100 nM) and Tag+6 (30 μM) with between 5 nM and 10 μM EDT led to a systematic decrease in fluorescence emission at 580 nm. This decrease could be fit to yield an inhibition constant ($K_i$) of 9.3 ± 4.6 μM (Figure S5 in Supporting Information), in agreement with the value determined on the basis of competition with CyTag ($K_i = 5.6 ± 0.9$ μM). Thus, Tag+6 binds AsCy3 more than 100 times more favorably than EDT or CyTag.

A final set of experiments was performed to provide additional resolution of the binding mode. Substitution of one Cys-Cys motif within Tag+6 to generate Tag+6Δ2 led to a 50-fold loss in equilibrium binding affinity, in contrast to the minimal changes observed upon removal of a single Cys-Cys motif from CyTag. The Tag+6Δ2-AsCy3 complex is characterized by a $K_{app}$ value of 1.4 ± 0.36 μM by FP (FI, 860 ± 160 nM) (Figure 4), values very similar to those of Cy3Tag itself, providing additional evidence that the Cy3Tag interacts minimally with the second Cys-Cys motif in CyTag. Incubation of Tag+6 with AsCy3 led to an overall 3.2-fold increase in fluorescence, compared to only a 1.5-fold increase in Cy3Tag. The Tag+6Δ2-AsCy3 complex characterized by a $K_{app}$ value of 94 ± 16 nM (FI) and 49 ± 13 nM (FP), values very similar to those of

![Figure 2](Image)

**Figure 2.** Interactions of AsCy3 with Cy3Tag and variants. (A) Sequence of Cy3Tag, TagΔ2, and TagΔ4 with $K_{app}$ values determined by FP. (B) Plot of the FI of 100 nM AsCy3 after incubation with the Cy3Tag, TagΔ2, and TagΔ4. (C) Plot of the FP under identical conditions. Error bars show standard error.

![Figure 3](Image)

**Figure 3.** Interactions of AsCy3 with Tag+n sequences. (A) Sequences of potential AsCy3 ligands and $K_{app}$ values determined by FP. (B) Plot illustrating relationship between $K_{app}$ and the number of amino acids separating the Cys-Cys motifs (n).

![Figure 4](Image)

**Figure 4.** Interactions of AsCy3 with Tag+6 and Tag+6Δ2. (A) Sequences of Tag+6 and Tag+6Δ2 with $K_{app}$ values measured by FP. (B) Plot of the FI of 100 nM AsCy3 after incubation with the indicated [Tag+6] and [Tag+6Δ2]. (C) Plot of the FP under identical conditions. Error bars represent the standard error.
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