New Biarsenical Ligands and Tetracysteine Motifs for Protein Labeling in Vitro and in Vivo: Synthesis and Biological Applications

Stephen R. Adams,† Robert E. Campbell,† Larry A. Gross,‡§ Brent R. Martin,‡||
Grant K. Walkup,‡|| Yong Yao,†,Ⅴ Juan Llopis,†,○ and Roger Y. Tsien*†,‡,§

 Contribution from the Department of Pharmacology, Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, and Biomedical Sciences Program, University of California, San Diego, La Jolla, California 92093-0647

Received December 5, 2001

Abstract: We recently introduced a method (Griffin, B. A.; Adams, S. R.; Tsien, R. Y. Science 1998, 281, 269–272 and Griffin, B. A.; Adams, S. R.; Jones, J.; Tsien, R. Y. Methods Enzymol. 2000, 327, 565–578) for site-specific fluorescent labeling of recombinant proteins in living cells. The sequence Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa is an noncysteine amino acid, is genetically fused to or inserted within the protein, where it can be specifically recognized by a membrane-permeant fluorescein derivative with two As(III) substituents, FIAsh, which fluoresces only after the arsenics bind to the cysteine thiol groups. We now report kinetics and dissociation constants (~10⁻¹¹ M) for FIAsh binding to model tetracysteine peptides. Affinities in vitro and detection limits in living cells are optimized with Xaa-Xaa = Pro-Gly, suggesting that the preferred peptide conformation is a hairpin rather than the previously proposed α-helix. Many analogues of FIAsh have been synthesized, including ReAsH, a resorufin derivative excitable at 590 nm and fluorescing in the red. Analogous biarsenicals enable affinity chromatography, fluorescence anisotropy measurements, and electron-microscopic localization of tetracysteine-tagged proteins.

Introduction

Modification of proteins with reporter probes has permitted a great many in vitro biophysical and biochemical studies of protein structure, dynamics, enzyme activity, and protein–protein interactions.1–3 However, studies with labeled proteins inside living cells have been more difficult and limited because the protein generally has to be purified in vitro, labeled, repurified, and then reintroduced into cells by such invasive techniques as microinjection or electroporation. Recently, genetic fusion of proteins to fluorescent proteins such as the jellyfish green fluorescent protein (GFP) has been highly valuable in understanding protein localization and dynamics in cell biology.4,5 However, GFP is a full-sized protein of 238 amino acids, whose folding is far from instantaneous and whose concatenation often perturbs the protein of interest. Furthermore, the only spectroscopic readout is fluorescence at emission maxima of up to 529 nm. Although red-emitting fluorescent proteins are now known, their slow maturation and obligate tetramerization have greatly restricted their utility as fusion partners.6

We recently introduced the first solution to the general problem of site-specifically attaching a small organic molecule to a short (6–8 residue) motif of natural, genetically encodable amino acids within recombinant proteins in living cells.1 The motif consists of four cysteines in the sequence Cys-Cys-Xaa-Xaa-Cys (where Xaa is any amino acid except cysteine), and the first complementary membrane-permeant small molecule is fluorescein with As(III) substituents on the 4′- and 5′-positions, “FIAsh”. The interaction of a single arsenic with a pair of thiol groups is well-known. The rigid spacing of the two arsenics in FlAsH enables it to bind with considerable affinity and specificity to the tetracysteine motif transplanted into a variety of proteins. Binding to endogenous cysteine pairs or lipoamide cofactors, which would cause toxicity and nonspecific labeling, is minimized by addition of micromolar levels of 1,2-dithiol antidotes such as 1,2-ethanedithiol (EDT) or 2,3-

dimercaptopropanol (BAL),\(^9\) which outcompete endogenous pairs of thiols for FLAsH binding. Millimolar concentrations of these antidotes can outcompete the tetracysteine motifs as well and thereby strip FLAsH off the target proteins if desired.

FLAsH is conveniently synthesized in one step by palladium-catalyzed transmetallation of fluorescein mercuric acetate with arsenic trichloride and is best isolated as the adduct with two EDT molecules already bound. That adduct, FLAsH–EDT\(_2\), is practically nonfluorescent but becomes more than 50 000 times more fluorescent (quantum yield \(\approx 0.5\)) upon exchanging the ETDs for a tetracysteine-containing peptide. Presumably FLAsH–EDT\(_2\) is quenched by vibrational deactivation or photoinduced electron-transfer mechanisms, which are hindered by the more rigid and constrained peptide complex. This enhancement is very valuable because it reduces the need to remove excess FLAsH–EDT\(_2\) rigorously. The first two examples of recombinant proteins labeled with FLAsH–EDT\(_2\) in living cells were a GFP mutant that adduct, FLAsH–EDT\(_2\), is quenched by vibrational deactivation or photoinduced electron-transfer mechanisms, which are hindered by the more rigid and constrained peptide complex. This enhancement is very valuable because it reduces the need to remove excess FLAsH–EDT\(_2\) rigorously. The first two examples of recombinant proteins labeled with FLAsH–EDT\(_2\) in living cells were a GFP mutant with a tetracysteine appended to its carboxy terminus, and calmodulin containing a tetracysteine motif inserted within an \(\alpha\)-helical region near the amino terminus.

To extend the understanding and utility of this promising system, we now describe analogues of FLAsH with other useful properties such as red or blue excitation and emission wavelengths, localizability by electron microscopy, or nonpermeability through membranes to confine labeling to surface-exposed tetracysteines. We quantify the affinity of FLAsH for a variety of tetracysteine motifs by measuring association and dissociative exchange rates. Surprisingly, the tightest binding is found with Pro-Gly between the cysteine pairs, suggesting that the preferred conformation is a hairpin rather than an \(\alpha\)-helix as previously conjectured. Fluorescent anisotropy measurements reveal that FLAsH is rotationally locked to the host protein, making it a useful probe for protein dynamics. The tight, specific, yet reversible binding of FLAsH for tetracysteine sites shows promise for affinity purification of recombinant proteins.

**Results and Discussion**

**Red- and Blue-Fluorescing Biarsenical Dyes.** Red-emitting fluorophores are particularly attractive for fluorescence microscopy because cellular absorbance, scattering, and autofluorescence decrease greatly at longer wavelengths. Red fluorophores would also be useful acceptors of fluorescein resonance energy transfer (FRET) from GFP. We therefore tried several strategies to obtain red analogues of FLAsH. Several rhodamine analogues of FLAsH were prepared by the same transmetallation strategy as used for FLAsH. The rhodamine free bases were mercurated under conditions used for analogous triphenylmethane dyes to give the appropriate 4',5'-substitution.\(^{10}\) Transmetallation with AsCl\(_3\) catalyzed by palladium acetate followed by addition of EDT gave the desired products in low yield (Scheme 1 in Supporting Information). Reaction with a model tetracysteine peptide gave complexes of the expected molecular weights but no increase in fluorescence. The absorbance spectra of the isolated complex lacked the strong visible peak typical of rhodamines, suggesting that the fluorophore was in the colorless lactone tautomer. Steric collisions between the peptide-bound 4',5'-arsenic and 3',6'-alkylamino groups probably twist the latter out of plane with the xanthene ring, destabilizing the quinonoid tautomer. To avoid such steric interactions, we turned to resorufin, the phenoxazine analogue of fluorescein. Mercuration by mercuric acetate gave a mixture of polysubstituted products as an intractable solid, which on treatment with arsenic (III) trichloride followed by EDT gave the desired product in low and variable yield. Mercuric trifluoroacetate gave exclusive dissubstitution that upon transmetallation gave modest yields of ReAsH–EDT\(_2\) (Scheme 1). A related red biarsenical of Nile Red has recently been reported as an environmentally sensitive fluorophore targetable to tetracysteine motifs.\(^{11}\)

ReAsH–EDT\(_2\) was nonfluorescent like FLAsH–EDT\(_2\) but rapidly formed a fluorescent complex with a tetracysteine-containing peptide. The complex had excitation and emission maxima of 593 and 608 nm, respectively (Figure 1A), slightly red-shifted from resorufin in the same way that FLAsH is bathochromically shifted from fluorescein. Addition of millimolar concentrations of EDT reversed the complex formation but did not reduce or add to the phenoxazine, contrary to the parent fluorophore, resorufin. To demonstrate specific labeling of a protein containing a tetracysteine motif by ReAsH in living cells, HeLa cells were transiently transfected with a GFP or yellow fluorescent protein (YFP) containing a C-terminal tetracysteine site. Exposure of the cells to 5 \(\mu\)M ReAsH–EDT\(_2\) with 10 \(\mu\)M EDT quenched the donor GFP or YFP emission by >80% and caused sensitized emission measured at 635 nm characteristic of FRET (Figure 1B). The completeness of the donor quenching indicated that at least 80% of the proteins were labeled, assuming 100% FRET efficiency in the complex. Alternatively, if the FRET efficiency were only 80%, then 100% of the protein molecules would have had to undergo labeling. The fluorescence staining and FRET could be completely reversed by 5 mM BAL. EYFP emission was quenched more completely than EGFP as expected because of more favorable spectral overlap of EYFP with the absorbance spectrum of ReAsH (data not shown).

Blue-fluorescing bisarsenicals (HoXAsH–EDT\(_2\) and ChoX-AsH–EDT\(_2\)) were generated by the usual mercuration and


\(^{10}\) Chalkley, L. J. Am. Chem. Soc. 1941, 63, 981–987.

Figure 1. (A) Fluorescence spectra of ReAsH and CHoXAsH bound to the model tetracysteine peptide, Ac-WEEAAPRECCCARE-NH2. The spectra of the FlAsH complex are also shown for comparison (dotted line). The excitation spectrum of HPLC-purified complex, in 100 mM KCl 10 mM K-MOPS pH 7.2, was collected with emission at 632 nm and the emission spectrum with excitation at 590 nm for ReAsH or with excitation at 388 nm and emission at 433 nm for CHoXAsH. Bandwidths for spectra were 2 nm. (B) Specific labeling of ReAsH with a tetracysteine peptide fused to GFP in living cells. Reversibly intracellular labeling of the peptide AEAAARECCCRECARA fused to the C terminus of GFP, transiently transfected in Hela cells. Fluorescent images were taken at the times indicated with an excitation of 480 nm (30 nm bandwidth at half-height) and emission at 535 nm (25 nm bandwidth) or 635 nm (45 nm bandwidth). The average fluorescence intensity of a region over individual transfected or nontransfected cells for each emission wavelength is shown. At the start of the experiment, the emission at 535 nm from the GFP–peptide fusion construct was high but was rapidly quenched upon addition and subsequent fast specific binding of ReAsH–EDT2 in the presence of a low concentration of EDT, FRET from the GFP to bound ReAsH now gave emission at 636 nm. Washing out the free ReAsH–EDT2 had little effect on the fluorescence, indicating tight binding to the peptide. Millimolar amounts of the diiodo BAL rapidly reversed the labeling, regenerating initial conditions. Nontransfected cells show minimal changes in fluorescence at these wavelengths, despite some nonspecific binding of ReAsH–EDT2, as it is poorly excited at 480 nm.

subsequent transmetalation reactions upon 3,6-dihydroxyxanthone and its 2,7-dichloro derivative respectively (Scheme 2 in Supporting Information). These compounds also gave a substantial enhancement of fluorescence (over 20-fold) upon binding with a tetracysteine peptide to form isolatable complexes of the expected molecular weights with excitation and emission maxima of about 380 and 430 nm, respectively (Scheme 1, Figure 1A). CHoXAsH showed more favorable properties as its peptide complex was brighter (quantum yield of 0.35) and showed no pH sensitivity within the physiological range (pKα < 5). HoXAsH–EDT2 and CHoXAsH–EDT2 both gave specific labeling of gap junctions in Hela cells transfected with tetracysteine-tagged connexin 43 although the contrast, brightness, and photostability of the staining were less than that achievable with FlAsH or ReAsH (data not shown). CHoXAsH may be useful as a FRET donor for GFP or YFP and for multicolor labeling with other bisarsenicals or autofluorescent proteins.

FlAsH Derivatives That Photosensitize the Polymerization of Diaminobenzidine. Fluorescent staining of intracellular components with appropriate dyes may also be visualized at much higher resolution by electron microscopy using “photoconversion” of diaminobenzidine. In this process, strong illumination of the dye generates singlet oxygen, which polymerizes diaminobenzidine into a highly localized precipitate that can be stained with osmium tetroxide to give an electron-dense marker. Previously, eosin-labeled antibodies have been used to localize antigens and epitopes, but access of the antibodies to protein epitopes is often hindered by the fixation required for optimal sample integrity. A means of genetically targeting singlet-oxygen-generating chromophores to recombinant proteins within living cells would therefore be a valuable complement. Unfortunately, GFP does not undergo photoconversion, presumably because the protein shell around the chromophore prevents the necessary ingress of triplet oxygen and egress of singlet oxygen. We were therefore interested to test whether bisarsenical-labeled proteins could undergo photoconversion to provide ultrastructural localization of any recombinant protein containing the tetracysteine motif.

Initial experiments with FlAsH stained connexin43–tetracysteine construct, expressed in HeLa cells where it forms distinctive gap junctions, indicated poor photoconversion of diaminobenzidine, as expected, because fluorescein-labeled antibodies are similarly inefficient. Additional heavy atoms such as the four bromines in eosin increase intersystem crossing, antibodies are similarly inefficient. Additional heavy atoms such as the four bromines in eosin increase intersystem crossing, resistance to photobleaching, and photoconversion efficiency, and thus we prepared several FlAsH derivatives incorporating heavy atoms such as bromine or sulfur (Table 1). As expected, photoconversion efficiency was correlated with the brightness of the fluorescent staining with each derivative, with the best derivatives scoring highly with both of these properties. Analogues in which the xanthene ring oxygen is replaced by sulfur (tFlAsH–EDT2 and ThAsH–EDT2) were too dimly fluorescent to locate labeled gap junctions to determine their photoconversion efficiency, although controls indicated binding of tFlAsH to a cyan fluorescent protein (CFP)–tetracysteine construct by measuring the quenching of the CFP fluorescence. Addition of bromine substituents to FlAsH or ReAsH to form BrAsH, Br2AsH, and Br2ReAsH increased the photoconversion efficiency modestly while retaining sufficient fluorescent staining, but prolonged illumination increased fluorescence probably by photodebromination, thereby decreasing the total output of singlet oxygen. Surprisingly, ReAsH gave the best photoconversion and, with its bright fluorescent staining, gave the optimal results for both requirements. The trafficking of this con-

the extracellular tetracysteines were initially oxidized but full extracellular GFP. These results indicate that most, if not all, unlabeled cells that were rapidly acidified to pH 5.2 to quench FRET. This percentage quench of the GFP was similar to high affinity of biarsenical/tetracysteine complexes suggested - quenched approximately 25 of labeling under these conditions could be assessed by labeling meant dithiol. No labeling occurred without prior reduction staining (Figure 2B) which was reversible with a few millimolar marker of transfected cells) gave distinct plasma membrane and intracellular staining. Only cells coexpressing BFP (as a in Supporting Information) to prevent membrane permeation structural information.13

Table 1. Relative Efficiency of the Fluorescent Staining of Tetracysteine-Tagged Gap Junctions and Subsequent Photoconversion of 3,3'-Diaminobenzidine by Biarsenical Dyes (Synthetic Details Given in Schemes 3 and 4 in Supporting Information)4

<table>
<thead>
<tr>
<th>name</th>
<th>W</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>fluorescent staining efficacy</th>
<th>DAB photoconversion efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlAsH</td>
<td>H</td>
<td>H</td>
<td>O</td>
<td>C--C6H4COOH</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>BrAsH</td>
<td>Br</td>
<td>H</td>
<td>O</td>
<td>C--C6H4COOH</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Br2AsH</td>
<td>Br</td>
<td>Br</td>
<td>O</td>
<td>C--C6H4COOH</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>tFlAsH</td>
<td>H</td>
<td>H</td>
<td>S</td>
<td>C--C6H4COOH</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>ReAsH</td>
<td>H</td>
<td>H</td>
<td>O</td>
<td>N</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Br,ReAsH</td>
<td>Br</td>
<td>Br</td>
<td>O</td>
<td>N</td>
<td>++</td>
<td>+++(+)</td>
</tr>
<tr>
<td>ThAsH</td>
<td>H</td>
<td>H</td>
<td>S</td>
<td>N</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

4 Hela cells transfected with a connexin43-tetracysteine construct13 were stained with 1–5 μM biarsenical and 50–100 μM EDT for 2 h, washed, and viewed by fluorescence microscopy and photoconverted as previously described.

FlAsH Labeling of Cell-Surface Tetracysteine-Containing Proteins. FlAsH binding to a tetracysteine motif requires that the thiols of the four cysteines are reduced, the prevalent form of cytoplasmic thiols. Other cellular compartments, such as the endoplasmic reticulum or the cell surface have more oxidizing environments, probably precluding FlAsH labeling unless the cystines are acutely reduced. To test this, we transiently expressed Hela cells a tetracysteine peptide (Figure 2) fused to the C terminus of VAMP2 (vesicle associated membrane protein 2 or synaptobrevin).14 The FlAsH tag would be either in the lumen of secretory vesicles or on the extracellular side of the plasma membrane. After brief reduction of any accessible disulfide bonds with the membrane-impermeant reductants, 2-mercaptopropanesulfonate (MES) and tris(carboxyethyl)phosphine (TCEP), the cells were stained with a sulfonated derivative of FlAsH (sFlAsH−EDT2; for synthetic details see Scheme 5 in Supporting Information) to prevent membrane permeation and intracellular staining. Only cells coexpressing BFP (as a marker of transfected cells) gave distinct plasma membrane staining (Figure 2B) which was reversible with a few millimolar 2,3-dimercaptopropanesulfonate (DMPS), a membrane-impermeant dithiol. No labeling occurred without prior reduction indicating the cysteines were oxidized as expected. The extent of labeling under these conditions could be assessed by labeling a VAMP2–F-cysteine construct with ReAsH which quenched approximately 25–40% of the GFP fluorescence by FRET. This percentage quench of the GFP was similar to unlabeled cells that were rapidly acidified to pH 5.2 to quench extracellular GFP. These results indicate that most, if not all, the extracellular tetracysteines were initially oxidized but full recovery by reduction was possible.

SDS-PAGE of FlAsH–Protein Complexes. The apparent high affinity of biarsenical/tetracysteine complexes suggested that they should be stable to typical denaturing gel electrophoresis conditions. Survival of fluorescence during SDS-PAGE would be useful to permit quick verification of the identity and integrity of tetracysteine-tagged proteins. For comparison, naturally fluorescent proteins do not retain fluorescence after denaturation; therefore, fusions to GFP can be detected in gels only by Western blotting with antiGFP antibodies. We therefore expressed Cys6,7,10,11-calmodulin in Escherichia coli and incubated crude bacterial lysates for 30–60 min at room temperature with FlAsH in a modified sample buffer for SDS-PAGE in which the usual thiol reductant (DTT dithiothreitol, or BME 2-mercaptoethanol) was replaced by TCEP. After electrophoresis, a single major fluorescent species running at the expected molecular weight for the calmodulin–FlAsH complex was visible under UV illumination (Figure 3, lanes 3–5). Other fluorescent bands, corresponding to <5% of the total fluorescence, include partially disulfide-linked multimers of Cys6,7,10,11-calmodulin which must still bind at least one FlAsH.

Subsequent staining with Coomassie Blue revealed the expected ladder of native protein bands present throughout the
Affinity Purification of Proteins. The high specificity of biarsenical dyes for the tetracysteine motif and the low apparent abundance of endogenous proteins containing such sites suggested that immobilized biarsenicals could be used for the affinity purification of tetracysteine-tagged proteins. Existing tags for affinity purification range from large proteins, such as glutathione-S-transferase or maltose-binding protein, to small peptides such as epitope or polyhistidine tags. Epitope tags require expensive antibody columns and harsh elution conditions, whereas polyhistidine tags often leave contamination with thiols, the minimal size of the binding site to FlAsH, the speed of the purification requiring one simple wash, and the high purity of the final product. The DMPS can be easily removed from the purified protein by dialysis. EDT may be used if a volatile dithiol is required, although its limited solubility and tendency to precipitate upon aerial oxidation are drawbacks.

Fluorescence Anisotropy of FlAsH–Protein Complexes. Conventional fluorescent labeling agents such as fluorescein isothiocyanate (FITC) or Texas Red are attached through rotationally mobile single bonds from the 9-(carboxyphenyl) substituent to the flexible side-chain of amino acid residues such as cysteine or lysine. Rapid rotation of these bonds within the excited-state lifetime diminishes fluorescence polarization or anisotropy. By contrast, the biarsenical dyes bind tetracysteine motifs via four covalent bonds to the arsenic substituents directly attached to fluorophore; therefore, the attachment of the fluorophore to the peptide backbone should be very rigid. Such rigidity should be ideal for fluorescence anisotropy measurements of the local rotational mobility of peptides or protein domains, including many popular assays in which binding of a fluorescently labeled ligand to a macromolecule is assessed by the resulting rotational immobilization of the ligand and adsorbed and could then be eluted with a high concentration of the competing ligand, DMPS. SDS-PAGE of these fractions (Figure 4) indicated a high purity in reasonable yield even without a wash of the adsorbed protein with a low concentration of eluting ligand. By comparison, the same protein construct was purified by a conventional polyhistidine:Ni2+-NTA method in good yield with comparable purity even with the inclusion of several washes before final elution with high concentrations of imidazole.

Figure 3. FlAsH–tetracysteine complexes are stable to reducing, denaturing polyacrylamide gel electrophoresis. Soluble lysate of bacterially expressed polyhistidine tagged Cys6,10,11-tagged calmodulin was treated with 10, 50, or 100 mM FlAsH–EDT2 dissolved in sample buffer containing either 20 mM TCEP (lanes 3, 4, 5) or 1M BME (lane 7) for 15–30 min at room temperature, loaded and run on a 12% SDS-PAGE gel. (A) Proteins were visualized by staining with Coomassie Blue. (B) The fluorescence of the unstained gel was collected with excitation at 480 nm (60 nm bandwidth) and emission at 545 nm (bandwidth 35 nm). The arrow marks the region in which the FlAsH complex was more clearly detected on Coomassie-stained gels with higher loadings of lysate.

Figure 4. Affinity purification by a tetracysteine tag yields protein of purity similar to that by conventional his-tag. Soluble lysate (load) of the bacterially expressed polyhistidine tagged Cys6,7,10,11-calmodulin was batch-bound to the two supports, separated by pouring into columns (flow through), washed (wash), and eluted with either 0.25 M imidazole or 10 mM DMPS (elution), respectively. The collected fractions were analyzed by a reducing 15% SDS-PAGE gel and stained with Coomassie Blue.

increased fluorescence anisotropy. We therefore examined the fluorescence anisotropy of FlAsH-labeled Cys6,7,10,11-calmodulin and compared it with calmodulin labeled with a conventional lysine-reactive fluorescein tag. Figure 5 shows a Perrin plot of the fluorescence anisotropies measured in aqueous sucrose at various viscosities and temperatures. The reciprocal of the anisotropy (r) is plotted against absolute temperature (T) divided by the viscosity (η) in centipoise. The viscosity is obtained from the table of the CRC Handbook, in which the buffer is assumed to contribute negligibly. The values for 40 °C were extrapolated from the data given for 30 °C in the table.

The intercept of the Perrin plot of FlAsH–Cys6,7,10,11-calmodulin indicates a limiting anisotropy r0 of 0.35. More significantly, the linearity of the points irrespective of temperature indicates a lack of segmental motions between the label and adjacent amino acid residues, which are known to cause the deviations normally observed in Perrin plots for proteins.4 From the slope (0.0081) of the regression line for FlAsH–Cys6,7,10,11-calmodulin and the excited-state lifetime of 4.37 ns measured in separate experiments by modulation fluorometry, a molecular diameter of 34 Å can be calculated, assuming a spheroidal shape.5 This estimate is in reasonable agreement with the known dimensions of calmodulin even though the protein shape is actually a dumbbell. Thus, FlAsH is indeed rigidly attached to the protein. By comparison, the fluorescence anisotropy of a fluorescein attached by a lysine residue of calmodulin showed a far greater dependence upon the temperature and viscosity of the solution with marked deviations from linearity in the Perrin plot. In nonscious solution, the calculated rotational correlation time is <0.5 ns, similar to that previously reported18 for the FITC conjugate of calmodulin, indicating free rotation of the dye independent of the protein. This contrasts with a rotational correlation time of 7.1 ± 0.3 ns for the FlAsH-labeled protein.

**Which Pairs of Thiols Bind to Each Arsenic?** In principle there are three ways that the two arsenics of FlAsH can plug into the four thiols. One As could bind the i, i + 1 cysteines while the other As binds the i + 4, i + 5 cysteines, or the pairings could be i, i + 4 and i + 1, i + 5, or i, i + 5 and i + 1, i + 4. A hint as to the most likely isomer resulted from experiments with a fluorescein with just one arsenic substituent, 4′-(1,3,2-dithiasolan-2-yl)-5-carboxyfluorescein, prepared by monomercuration of 5-carboxyfluorescein followed by transmetalation. This monoarsenical was reacted with model peptides AcWEEAARECCARA-amide and AcWEACARECAARA-amide containing either an i, i + 1 or an i, i + 4 arrangement of two cysteines. Both generated a single fluorescent product (shown by HPLC and ES-MS) but the complex with the i, i + 4 peptide formed more slowly and was considerably less fluorescent. On adding excess i, i + 1 peptide to this complex, a rapid increase in fluorescence resulted, indicating preferred formation of i, i + 1 complex. Thus, a single arsenic attached to fluorescein preferred to bind to a pair of adjacent cysteines rather than to separated cysteines, suggesting that the most likely pairing for FlAsH is i, i + 1 and i + 4, i + 5. Addition of FlAsH–EDT2 to AcWEEAARECCARA-amide gave either a two-peptide:1 FlAsH or a 1:1 complex depending on which component was in excess. The 1:1 complex was only weakly fluorescent, suggesting that one dithiarsolanyl substituent is sufficient to quench the fluorescein fluorescence.

**Kinetics and Dissociation Constants of FlAsH–Tetra-cysteine Complexes.** In our initial paper1 the dissociation constant of the FlAsH–tetra-cysteine peptide complex was estimated to be subnanomolar, because the fluorescence emission of 1 nM of the complex in the presence of millimolar monothiol (to mimic intracellular glutathione) remained unaltered for several days, and subsequent changes seemed consistent with decomposition rather than dissociation. We have now measured the rate constants for the dissociation and formation of the complex, whose ratio gives the equilibrium dissociation constant. In the absence of EDT, the reaction at neutral pH is:

\[
\text{FlAsH bis-arsenoxide + tetra-cysteine peptide} \rightleftharpoons \text{FlAsH–tetra-cysteine peptide + 2 H}_2\text{O} \quad (1)
\]

The bis-arsenoxide (named FlAsHO, to indicate the oxygen ligands of arsenic atoms) was generated from FlAsH–EDT2 by stoichiometric reaction with 2 equiv of Hg2+. Formation of the extremely stable Hg2–EDT2 complex (with an association constant of 10^{24} M^{-1})19 leads to irreversible hydrolysis of the As–S bonds (see Scheme 1 for an analogous reaction with ReAsH–EDT2). The reaction can be conveniently monitored by visible spectroscopy as stepwise addition of mercury (II) acetate at neutral pH gave an immediate reaction resulting in a hypsochromic shift of the absorption maximum of 508 nm to a new peak at 485 nm with sharp isobestic points. Analysis by reverse-phase HPLC indicated clean conversion to a more polar product whose NMR spectrum and molecular weight were consistent with the bis-arsenoxide. This spectroscopic change can be reversed by the addition of excess EDT, which regenerated the starting material (followed by visible spectroscopy and thin-layer chromatography). Addition of excess Hg2+ to the bis-arsenoxide gives a slower, subsequent decrease in the absorbance at 485 nm, consistent with decomposition. Solutions of FlAsHO in DMSO were stable in the absence of free Hg2+.  


---

**Figure 5.** Perrin plot of FlAsH–Cys6,7,10,11-calmodulin and fluorescein-labeled calmodulin. Fluorescence polarization of the two labeled proteins (1 μM) were measured in buffers of 50 mM K-MOPS, 2 mM MES, 1 mM EDTA, pH 7.4, adjusted to various viscosities with sucrose, at 10, 25, and 40 °C. The FlAsH complex shows a negligible decrease in polarization upon an increase in temperature and a decrease in viscosity indicating a rigidly bound complex, in contrast to the fluorescein-labeled protein.
The reaction of FlAsHO and tetracysteine peptides to form the complex was readily monitored by the resulting increase in fluorescence. The arsenoxide group strongly quenches the fluorescein fluorophore, albeit to a lesser extent than the 1,3,2-dithioarsolan-2-yl substituent. Formation of the tetracysteine peptide complex leads to a 50-fold fluorescence enhancement with a single-exponential rate in the presence of excess peptide (Figure 6A). Addition of millimolar monothiol such as MES gave a further small increase in fluorescence (less than 10% of the total fluorescence change) perhaps resulting from catalytic formation of a more stable (and more fluorescent) conformation. Pseudo-first-order kinetics from the initial rates in the absence of MES are found with large excesses of the 17-mer peptide, AcWEAAAREACCRECCARA-NH2 giving an on-rate of about 20,000 M$^{-1}$ s$^{-1}$ for the forward reaction 1. In the presence of MES (1–20 mM), a modest enhancement of the initial rate was observed (Figure 6A), giving a rate of 55,000 M$^{-1}$ s$^{-1}$ at 5 mM monothiol. MES, itself directly reacts with FlAsHO to generate a small increase in fluorescence at 530 nm (less than 10% of the total fluorescence change) but with a rate approximately 4–5 orders of magnitude slower than the peptide. The kinetic data was fitted to a single exponential for approximately the first 75% of the fluorescence increase to decrease the contribution of the reaction of FlAsHO with MES.

The reaction of FlAsHO and tetracysteine peptides to form the complex was readily monitored by the resulting increase in fluorescence. The arsenoxide group strongly quenches the fluorescein fluorophore, albeit to a lesser extent than the 1,3,2-dithioarsolan-2-yl substituent. Formation of the tetracysteine peptide complex leads to a 50-fold fluorescence enhancement with a single-exponential rate in the presence of excess peptide (Figure 6A). Addition of millimolar monothiol such as MES gave a further small increase in fluorescence (less than 10% of the total fluorescence change) perhaps resulting from catalytic formation of a more stable (and more fluorescent) conformation. Pseudo-first-order kinetics from the initial rates in the absence of MES are found with large excesses of the 17-mer peptide, AcWEAAAREACCRECCARA-NH2 giving an on-rate of about 20,000 M$^{-1}$ s$^{-1}$ for the forward reaction 1. In the presence of MES (1–20 mM), a modest enhancement of the initial rate was observed (Figure 6A), giving a rate of 55,000 M$^{-1}$ s$^{-1}$ at 5 mM monothiol. MES, itself directly reacts with FlAsHO to generate a small increase in fluorescence at 530 nm (less than 10% of the total fluorescence change) but with a rate approximately 4–5 orders of magnitude slower than the peptide. The kinetic data was fitted to a single exponential for approximately the first 75% of the fluorescence increase to decrease the contribution of the reaction of FlAsHO with MES.

Table 2. Apparent On-Rates, Exchange-Rates and Dissociation Constants of FlAsH Complexed to AcWEAAAREACCRECCARA-NH2 or AcWDCCPGCCK-NH2 at Different Monothiol Concentrations

<table>
<thead>
<tr>
<th>peptide</th>
<th>[MES] mM</th>
<th>$k_{on}^a$ M$^{-1}$ s$^{-1}$</th>
<th>$k_{exch}^a$ s$^{-1}$</th>
<th>$K_d^{app}$ pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcWEAAAREACCRECCARA-NH2</td>
<td>20</td>
<td>65000</td>
<td>$4.5 \times 10^{-6}$</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>55000</td>
<td>$1.1 \times 10^{-6}$</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>53000</td>
<td>$3.9 \times 10^{-7}$</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>23000</td>
<td>$[1.0 \times 10^{-7}]$</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>310000</td>
<td>$1.1 \times 10^{-6}$</td>
<td>3.6</td>
</tr>
<tr>
<td>AcWDCCPGCCK-NH2</td>
<td>5</td>
<td>72000</td>
<td>$2.6 \times 10^{-7}$</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>74000</td>
<td>$\sim 1 \times 10^{-7}$</td>
<td>$\sim 2$</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

$^a$ Values in brackets determined by extrapolation; nd, not determined.
Table 3. Fluorescent Properties and Stability of FIAsH-labeled Tetracysteine-Containing Peptides in 20 mM MES at pH 7a

<table>
<thead>
<tr>
<th>peptide sequence</th>
<th>CCXC</th>
<th>no. of</th>
<th>quantum</th>
<th>k_on</th>
<th>k_off</th>
<th>Kapp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcWDCCGCKC-NH₂</td>
<td>-</td>
<td>1</td>
<td>0.14</td>
<td>10000</td>
<td>3.8</td>
<td>67</td>
</tr>
<tr>
<td>AcWDCCACCK-NH₂</td>
<td>-</td>
<td>1</td>
<td>0.7</td>
<td>57000</td>
<td>2.6</td>
<td>60</td>
</tr>
<tr>
<td>AcWDCGGCCK-NH₂</td>
<td>G</td>
<td>3</td>
<td>0.66, 0.5, 0.5</td>
<td>35000</td>
<td>3.6 × 10⁻⁶</td>
<td>100</td>
</tr>
<tr>
<td>AcWDCCPCCK-NH₂</td>
<td>P</td>
<td>2</td>
<td>0.61, 0.58</td>
<td>100000</td>
<td>1.5 × 10⁻⁵</td>
<td>150</td>
</tr>
<tr>
<td>AcWEEAAAREACCRECCARA-NH₂</td>
<td>RE</td>
<td>2</td>
<td>0.5</td>
<td>65000</td>
<td>4.5 × 10⁻⁶</td>
<td>70</td>
</tr>
<tr>
<td>AcWDCCCAECK-NH₂</td>
<td>AE</td>
<td>2</td>
<td>0.58</td>
<td>25000</td>
<td>1.8 × 10⁻⁶</td>
<td>72</td>
</tr>
<tr>
<td>AcWDCCSECKC-NH₂</td>
<td>SE</td>
<td>2</td>
<td>0.58</td>
<td>50000</td>
<td>2.1 × 10⁻⁶</td>
<td>42</td>
</tr>
<tr>
<td>AcWDCCDEECK-NH₂</td>
<td>DE</td>
<td>2</td>
<td>0.5</td>
<td>32000</td>
<td>1.3 × 10⁻⁶</td>
<td>41</td>
</tr>
<tr>
<td>AcWDCCGPGCKC-NH₂</td>
<td>FG</td>
<td>2</td>
<td>0.71, 0.62</td>
<td>310000</td>
<td>1.2 × 10⁻⁵</td>
<td>4</td>
</tr>
<tr>
<td>AcWDCCGPGCCK-NH₂</td>
<td>GP</td>
<td>1</td>
<td>0.44</td>
<td>50000</td>
<td>3.6 × 10⁻⁶</td>
<td>72</td>
</tr>
<tr>
<td>AcWDCCDEACCK-NH₂</td>
<td>DEA</td>
<td>3</td>
<td>0.1, 0.13, 0.46</td>
<td>65000</td>
<td>6.0 × 10⁻³</td>
<td>92000</td>
</tr>
</tbody>
</table>

*The products from the reaction of FlAsH−EDT2 and peptides at pH 7 were separated by HPLC. Each peak was collected and gave the expected molecular weight of the complex.

Breaking the As−S bonds or sequentially trapping each cysteine as it dissociated stepwise. We chose as the trapping agent a 10-fold excess of ReAsH bis-arsenoxide (ReAsH2O; see Scheme 1) rather than ReAsH−EDT2 so that initial dissociation of EDT does not limit trapping and to avoid the competing reactions involving EDT. The reaction was followed by a decrease in the fluorescence at 530 nm (from the FlAsH−peptide complex) with a concomitant increase in fluorescence at 640 nm (from the ReAsH−peptide complex). The reaction kinetics fitted a single exponential as expected for a first-order reaction, and the rate was moderately sensitive to the concentration of monothiol (in the tested range of 1−20 mM), requiring 20 mM MES to complete the reaction in several days (Figure 6C). Under conditions mimicking intracellular monothiol concentrations (e.g., 5 mM reduced glutathione), the exchange rate was 1 × 10⁻⁶ s⁻¹. Dividing this by the on-rate of FlAsHO and the peptide at this concentration of MES yields an apparent dissociation constant of about 10 pM under these conditions (Figure 6D, Table 2).

The dissociation constant of arsenite or 4-sulfonamidophenylnarsenoxide for vicinal thiols (cysteines or dithiols) has been reported to be 0.2−1.4 μM. For comparison with this method, which is too insensitive to measure the much tighter binding of FlAsH complexes, we measured the on- and off-rate for a fluorescein containing one arsenoxide complexed with a peptide containing two cysteines spaced at the i, i + 1 positions. The on-rate was similar to that found for FlAsH and the tetracysteine peptide, but the off-rate was increased by about 4 orders of magnitude, giving an apparent dissociation constant of 72 nM (at 5 mM MES), only a little lower than the above-reported values. Thus, the cooperation between the two arsenic-dithiol binding steps raises the affinity about 7200-fold. This increase is quite considerable and is essential for the practical utility of the biarsenical/tetracysteine system. However, it leaves considerable room for improvement, because once one arsenic and pair of cysteines have bonded, the second pair of cysteines is presented to the other arsenic at an effective concentration of only about 7200 × 72 nM = 0.5 mM. If the peptide were well preorganized to make both arsenic-dithiol interactions strain-free, one might expect effective molarities closer to 1 M.

Optimizing the Tetracysteine Motif. The sequence of the original tetracysteine peptide AcWEAAAREACCRECCARA-

speed of FlAsH staining of tetracysteines has already found practical application for monitoring protein synthesis kinetics. 23

Reversing the order of the proline and glycine hindered FlAsH binding and resulted in formation of a less fluorescent complex, suggesting that a specific conformation allowable by PG, but not GP, is preferred. This observation may correlate with the strong preference for proline in the $i+1$ and glycine in the $i+2$ positions of Type-I and -II $\beta$-turns. 24 Although Gly-Pro can form a Type-II $\beta$-turn, this geometry is apparently not favored for binding of FlAsH to the tetracysteine motif. When the two pairs of cysteines were separated by just one amino acid (CCXXC where $X = A$, G, or P), the stability of the complex was only slightly impaired relative to most of the CCXXCC motifs except $XX = PG$. The CCPCC peptide had a significantly faster on-rate than the alanine or glycine containing peptides, but also a 5- to 10-fold increase in off-rate. The proline may form a turn in the tetracysteine motif, thereby preordering the sulfhydryl groups for FlAsH binding and resulting in the faster on-rate. The increased off-rate with proline may be due to the inability of the FlAsH—CCXCC complex to adapt a stable conformation that is allowed only for the conformationally more flexible alanine and glycine residues. Eliminating the spacer amino acids altogether to give CCC decreased the stability of the complex 10-fold and the fluorescence quantum yield about 4-fold. Increasing the number of spacer residues to three in CCDEACC was even more drastically detrimental due to 3 orders of magnitude increase in the off-rate with no increase in the on-rate. The stability of the FlAsH—CCDEACC complex was comparable to that of a single arsenoxide with two cysteines, even though the mass spectrum of this complex was consistent with both arsenoxides reacting with the cysteines.

Comparison of FlAsH Binding to Different Tetracysteine Motifs in Living Cells. On the basis of these kinetic studies with model peptides in vitro, the CCPGCC peptide appended to a recombinant protein should form more stable complexes with FlAsH—EDT$_2$ in living cells than could our original CCRECC peptide motif. To test this prediction, we expressed fusions of cyan fluorescent protein (CFP) with either EAAAREACCRECC or EAAAREACCPGCCA in HeLa cells. Binding of FlAsH to the tetracysteine motifs was monitored by FRET from CFP to FlAsH, which quenches CFP emission. Both constructs reacted with FlAsH at similar rates but the complex formed with CCPGCC had greater resistance to high concentrations of competing dithiol. For example, FlAsH was about 50% displaced from the CCPGCC construct at 1.6 mM EDT or 0.7 mM BAL, compared to 0.35 mM EDT for the original CCRECC construct (Figure 7A). The CCPGCC motif resists about 4.6-fold higher concentrations of EDT than its CCRECC counterpart, which translates to a 21-fold ratio of apparent affinities because two EDT molecules should be required to displace a tetracysteine peptide. Thus the Pro-Gly spacer confers increased affinity in live cells as well as in vitro. Direct excitation of FlAsH at 495 nm, a wavelength that avoids excitation of CFP, permitted comparison of fluorescence from transfected cells expressing the constructs and nontransfected neighboring cells. Nonspecific staining (background) could then be compared to specific staining upon labeling with FlAsH and subsequent washing out under various regimens designed to give the largest specific signal with the smallest background.

The majority (~90–95%) of FlAsH background staining in nontransfected cells can be competitively suppressed by addition of sufficient concentrations of dithiol (1.3 mM EDT or 1.2 mM BAL). The residual thiol-independent background staining (~5–10%) persists even at concentrations of dithiol as high as several millimolar. Addition of the hydrophobic dye Disperse Blue (20 $\mu M$) suppresses the majority of this thiol-independent back-

---

inside cells because the chelators would release the toxic transition metals and lose any selectivity for hexahistidines. Still’s group has developed interesting dye-labeled macrocyclic polyamides that bind peptides with micromolar dissociation constants in chloroform and increase fluorescence severalfold. Other polyamides at 5–8 μM showed qualitatively detectable binding to peptides on beads in water at pH 4, but no binding at physiological pH has yet been reported, let alone cellular applications. The peptides contain D-amino acids and thus are not genetically encodable. Rozinov and Nolan used phase display to evolve 12-mer peptides (“fluorettes”) of natural amino acids to bind commercially available fluoresceins and rhodamines. The resulting change in dye fluorescence was at most a few percent, and the lowest dissociation constant was 1.6 μM. Rao et al. attached three D-Ala-D-Ala moieties to a trimeric scaffold and showed that this ligand would bind a trimer of vancomycin with a $K_d \approx 4 \times 10^{-17}$ M at neutral pH. Although none of these components are fluorescent, genetically encodable, or readily attachable to proteins, this system is analogous to the biarsenical-tetracysteine system in exploiting multivalency to attain extremely high affinity yet reversibility by ligands of lesser valency.

Thus, the biarsenical-tetracysteine system offers the following advantageous properties: Short (1–3 step), facile synthetic routes have been developed to a wide variety of tricyclic biarsenicals, which can be chosen either to be membrane-permeant or -impermeant. The complementary peptide motif is genetically encodable and small, as few as six amino acids, for which the current preferred sequence is Cys-Cys-Pro-Gly-Cys-Cys. The affinities are unprecedented for such small partners, <10 pM dissociation constants, with reasonably fast association rates (seconds). Time scales for dissociation are weeks in the absence of 1,2-dithiols, yet can be accelerated to minutes at millimolar dithiol concentrations. Fluorescence of the biarsenical dyes is greatly enhanced by binding to the target tetracysteine, and the anisotropy of the resulting fluorescence reflects the overall rotational freedom of the peptide domain rather than the flexibility of a linker. Labeling remains visible after denaturing gel electrophoresis and can be used for affinity purification or electron-microscopic visualization of tetracysteine-tagged proteins.

Compared to a natively fluorescent protein (Table 4) such as GFP or DsRed, the tetracysteine motif needs a biarsenical small molecule partner, which adds one step and a potential for toxicity, but allows a much wider range of spectroscopic properties. Also the association between the biarsenical and the unstructured tetracysteine motif develops fluorescence within seconds, considerably faster than the tens of minutes required for cyclization and autoxidation to form the fluorescent protein chromophores. Therefore, if the biarsenical is already present, its fluorescence can monitor the biosynthesis of a tetracysteine-tagged protein with faster and more faithful kinetics than that of an autofluorescent protein. Despite the above advantages, there is still considerable need and scope for improvements and alternatives. The most important improvement for fluorescence labeling inside live cells would be a further increase in the ratio

of the intensity of intended fluorescence relative to background staining, especially the dithiol-independent component of the latter. This signal-to-background ratio has already improved considerably since our initial report and those using similar staining conditions, due to optimization of the peptide sequence, staining protocols, and use of nonfluorescent dyes to compete for unwanted binding. Nevertheless, the detection limits for biarsenical dyes in live cells are currently significantly higher than the estimate of 1 μM required to detect GFP diffusely expressed in the cytosol. Further reduction in the number and fluorescence brightness of biarsenical molecules bound to nonspecific sites would still be desirable. Meanwhile, it should be possible to increase the specific brightness of tagged proteins simply by concatenating multiple copies of the tetracysteine motif, so that each molecule of protein attracts multiple copies of the biarsenical dye. Beyond such refinements in the current biarsenical-tetracysteine system, it would still be worthwhile to devise a second, chemically orthogonal attachment system to permit simultaneous attachment of two different tags or a heterodimerizing cross-link to pairs of distinct proteins. Another attachment system might be created either by changing the steric heterodimerizing cross-link to pairs of distinct proteins. Another approach to permit simultaneous attachment of two different tags or a motif, so that each molecule of protein attracts multiple copies of the tetracysteine peptide motif, or by finding a system not reliant on cysteines and trivalent arsenic. Efforts to devise a second, chemically orthogonal attachment system to biarsenical-tetracysteine system, it would still be worthwhile to consider generalizability to other systems not reliant on cysteines and trivalent arsenic. Efforts to devise a second, chemically orthogonal attachment system to biarsenical-tetracysteine system, it would still be worthwhile to consider generalizability to other constructs.

**Experimental Section**

**Materials.** Chemicals were from Aldrich and general solvents (HPLC-grade) were from Fisher, and were used directly as received unless otherwise noted. NMP and DMSO were dried by storage over 4 Å molecular sieve. Solvents, activated amino acids, and coupling reagents for peptide synthesis were from Perceptive Biosystems. Thin-layer chromatography and column chromatography used Merck precoated silica gel 60 F-254 plates and silica gel 60 (230–400 mesh), respectively.

**Instrumentation.** Proton nuclear magnetic resonance spectra were collected on a Varian Gemini 200 MHz spectrometer in the solvent(s) indicated. UV absorbance and fluorescence spectra were recorded on a Cary 3E (Varian) spectrophotometer and Fluorolog 2 (Spex) fluorimeter, respectively. Fluorescence polarization was recorded on a Hewlett-Packard Electrospray 5989B mass spectrometer in the Howard Hughes Medical Institute facility at the University of California, San Diego. Peptides were synthesized by standard Fmoc solid-phase techniques using a Multiple Peptide Synthesis system attached to a Pioneer peptide synthesizer (Perceptive Biosciences). Peptides were acetylated on the N termini with acetic anhydride in pyridine and DMF, and cleaved from the support with TFA, triturated with 95% EtOH, chilled, and filtered to give the product as an orange solid. Yield, 1.5 mg (34%). The solid appears stable for at least 6 months when stored at 0°.

**Table 4. Comparison between Autofluorescent Proteins and Tetracysteine–Biarsenical System**

<table>
<thead>
<tr>
<th></th>
<th>Autofluorescent proteins derived from Aequorea and Discosoma</th>
<th>Tetracysteine–biarsenical system</th>
</tr>
</thead>
<tbody>
<tr>
<td>length of polypeptide</td>
<td>238–900</td>
<td>6–10</td>
</tr>
<tr>
<td>tendency to aggregate</td>
<td>weak dimerization to strong tetramerization</td>
<td>none known, provided cysteines</td>
</tr>
<tr>
<td>time to become fluorescent</td>
<td>minutes to days</td>
<td>are reduced or bound to biarsenical</td>
</tr>
<tr>
<td>chemical requirements</td>
<td>O₂ must be present</td>
<td>cysteines must be reduced</td>
</tr>
<tr>
<td>toxicity concerns</td>
<td>H₂O₂ generated</td>
<td>1,2-dithiol antidote must be present</td>
</tr>
<tr>
<td>emission wavelengths</td>
<td>blue to red</td>
<td>blue to red</td>
</tr>
<tr>
<td>extinction coefficients, (mM)⁻¹cm⁻¹</td>
<td>20–60</td>
<td>30–80</td>
</tr>
<tr>
<td>fluorescence quantum efficiencies</td>
<td>0.2–0.8</td>
<td>0.1–0.6</td>
</tr>
<tr>
<td>photosbleach resistance</td>
<td>low to very high</td>
<td>low to good</td>
</tr>
<tr>
<td>fluorescence detection limits</td>
<td>&lt; 1 μM</td>
<td>several μM</td>
</tr>
<tr>
<td>for diffuse cytosolic tag</td>
<td>limited because protein itself is large</td>
<td>very promising because tag is small</td>
</tr>
<tr>
<td>usefulness for fluorescence polarization assays</td>
<td>regardless of host</td>
<td>and rigidly attached to its host</td>
</tr>
<tr>
<td>fluorescence remaining after denaturing gel electrophoresis</td>
<td>none to slight</td>
<td>good, if boiling with excess thiols avoided</td>
</tr>
<tr>
<td>simultaneous multicolor labeling of different proteins</td>
<td>yes</td>
<td>not yet possible</td>
</tr>
<tr>
<td>sequential (pulse-chase) multicolor labeling of a single protein</td>
<td>possible by photobleaching or without precise temporal control</td>
<td>yes</td>
</tr>
<tr>
<td>photoconvertible into electron-microscopic image</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>generalizability to other spectroscopic properties</td>
<td>probably not</td>
<td>probably yes</td>
</tr>
<tr>
<td>usability for affinity chromatography</td>
<td>no</td>
<td>yes</td>
</tr>
</tbody>
</table>
4.5-Bis(1,3,2-dithiosarolan-2-yl)-resorufin; ReASH—EDT2. Resorufin bis(mercuric trifluoroacetate) (73 mg, 87 μmol) was suspended in dry NMP (1.5 mL) under argon. Arsenic trichloride (Caution: toxic, handle only in a fume hood!) (170 mL, 4.0 mmol), palladium acetate (few mg), and DIEA (140 μL, 1.6 mmol) were added, and the reaction mixture was stirred at 60–70 °C for 2 h. After cooling, the reaction mixture was poured into acetone (~0.25 M phosphate buffer pH 7 (25 mL, 1:1 v/v), treated with excess 1.2-ethanedithiol (0.5 mL), extracted with CHCl3 (3 × 30 mL), dried over Na2SO4, evaporated, and purified by chromatography on silica (20 g packed in toluene, eluted with 10% EtOAc–toluene). Care should be taken not to evaporate purified fractions excessively as this leads to partial loss of EDT. After trituration with water, dissolved in DMSO, filtered, and stored frozen. ES-MS (50% MeOH, 0.2% HOAc) +ve mode: (M+H)0.1 m/z 391.6. Calcd for C55H65As2N12O18S5 1517.16. Fluorescence quantum yield of peptide complex in pH 7 buffer, 0.18.

4.5-Bis(1,2,3-dithiosarolan-2-yl)-2',7'-dibromo-fluorescein, Br2AsH—EDT2. Dimercurated intermediate ‘H NMR (d2-DMSO) 1.85 (s, 3H, OAc), 1.86 (s, 3H, OAc), 7.26 (s, 2H, H-1,8'), 7.32 (s, 1H, H-7), 7.53 (m, 2H, H-5,6), 7.68 (s, 1H, H-4). Bismercurial ‘H NMR (d2-DMSO) 3.5 (EDT peaks part obscured by H2O), 7.71 (br s, 2H, OAc), 8.05 (s, 2H, H-1,8), 8.15 (dd, 1H, H-4), 8.12 (dd, 1H, H-14), 8.04 (dd, 1H, H-14). ES-MS (50% MeOH, 1% NHAc) +ve mode: (M+H)0.1 m/z 819.4. Calcd for C24H18As2Br2O5S 822.7. Complex with AcWEAAAREACCRECCARA-NH2 ES-MS (50% MeOH, 1% HOAc) +ve mode: (M+H)+ 1497.0. Calcd for C56H65As2N12O18S5 1517.16. Fluorescence quantum yield of peptide complex in pH 7 buffer, 0.35.

4.5-Bis(1,2,3-dithiosarolan-2-yl)-2',7'-dibromo-fluorescein, Br2AsH—EDT2. Dimercurated intermediate ‘H NMR (d2-DMSO) 1.85 (s, 3H, OAc), 1.86 (s, 3H, OAc), 7.26 (s, 2H, H-1,8'), 7.32 (s, 1H, H-7), 7.53 (m, 2H, H-5,6), 7.68 (s, 1H, H-4). Bismercurial ‘H NMR (d2-DMSO) 3.5 (EDT peaks part obscured by H2O), 7.71 (br s, 2H, OAc), 8.05 (s, 2H, H-1,8), 8.15 (dd, 1H, H-4), 8.12 (dd, 1H, H-14), 8.04 (dd, 1H, H-14). ES-MS (50% MeOH, 1% NHAc) +ve mode: (M+H)0.1 m/z 819.4. Calcd for C24H18As2Br2O5S 822.7. Complex with AcWEAAAREACCRECCARA-NH2 ES-MS (50% MeOH, 1% HOAc) +ve mode: (M+H)+ 1497.0. Calcd for C56H65As2N12O18S5 1517.16. Fluorescence quantum yield of peptide complex in pH 7 buffer, 0.18.

4.5-Bis(1,2,3-dithiosarolan-2-yl)-2',7'-dibromo-fluorescein, Br2AsH—EDT2. Bismercurial NMR (CDCl3) 3.6 (m, 8H, S–CH3), 6.52 (d, 1H, H-7), 6.61 (d, 1H, H-8'), 6.90 (s, 1H, H-1'), 7.21 (dd, 1H, H-7), 7.69 (m, 2H, H-5,6), 8.03 (dd, 1H, H-4), 9.90 (s, 1H, 3°-OH), 10.55 (s, 1H, 6°-OH). ES-MS (50% MeOH) +ve mode: (M+H)+ 1427.5. Calcd for C46H37As2N10O12S6 1427.27. Fluorescence quantum yield of peptide complex in pH 7 buffer, <0.01.

4.5-Bis(1,2,3-dithiosarolan-2-yl)-fluorescein, sFlAsH—EDT2. Dimercurated intermediate ‘H NMR (D2O + Na2CO3): 1.85 (s, 3H, OAc), 1.86 (s, 3H, OAc), 6.62 (d, 2H, H-2,7'), 7.09 (d, 2H, H-1,8'), 7.3 (m, 1H, H-7), 7.63 (m, 2H, H-5,6), 7.92 (m, 1H, H-4). ES-MS (50% MeOH, 1% HOAc) +ve mode: (M+H)+ 1427.5. Calcd for C46H37As2N10O12S6 1427.27. Fluorescence quantum yield of peptide complex in pH 7 buffer, <0.01.

4.5-Bis(1,2,3-dithiosarolan-2-yl)-fluorescein, sFlAsH—EDT2. Dimercurated intermediate ‘H NMR (D2O + Na2CO3): 1.85 (s, 3H, OAc), 1.86 (s, 3H, OAc), 6.62 (d, 2H, H-2,7'), 7.09 (d, 2H, H-1,8'), 7.3 (m, 1H, H-7), 7.63 (m, 2H, H-5,6), 7.92 (m, 1H, H-4). ES-MS (50% MeOH, 1% HOAc) +ve mode: (M+H)+ 1427.5. Calcd for C46H37As2N10O12S6 1427.27. Fluorescence quantum yield of peptide complex in pH 7 buffer, <0.01.

4.5-Bis(1,2,3-dithiosarolan-2-yl)-fluorescein, sFlAsH—EDT2. Dimercurated intermediate ‘H NMR (D2O + Na2CO3): 1.85 (s, 3H, OAc), 1.86 (s, 3H, OAc), 6.62 (d, 2H, H-2,7'), 7.09 (d, 2H, H-1,8'), 7.3 (m, 1H, H-7), 7.63 (m, 2H, H-5,6), 7.92 (m, 1H, H-4). ES-MS (50% MeOH, 1% HOAc) +ve mode: (M+H)+ 1427.5. Calcd for C46H37As2N10O12S6 1427.27. Fluorescence quantum yield of peptide complex in pH 7 buffer, <0.01.

4.5-Bis(1,2,3-dithiosarolan-2-yl)-fluorescein, sFlAsH—EDT2. Dimercurated intermediate ‘H NMR (D2O + Na2CO3): 1.85 (s, 3H, OAc), 1.86 (s, 3H, OAc), 6.62 (d, 2H, H-2,7'), 7.09 (d, 2H, H-1,8'), 7.3 (m, 1H, H-7), 7.63 (m, 2H, H-5,6), 7.92 (m, 1H, H-4). ES-MS (50% MeOH, 1% HOAc) +ve mode: (M+H)+ 1427.5. Calcd for C46H37As2N10O12S6 1427.27. Fluorescence quantum yield of peptide complex in pH 7 buffer, <0.01.
Preparation of Immobilized FlAsH–EDT$_2$. Crude 5-carboxyfluorescein-4',5'-bis(mercuric acetate) (89 mg, 0.10 mmol) was suspended in dry NMP (1 mL) under Ar and treated with arsenic trichloride (170 mL, 2.0 mmol), DIEA (140 µL, 0.80 mmol), and palladium (II) acetate (a few mg). After overnight reaction, the reaction mix was added to aqueous phosphate buffer, pH 7; acetone (1:1 v/v 50 mL, 0.2 M KPO$_4$) and ethanedithiol (0.5 mL) under Ar and diisopropylcarbodiimide (1 mmol) and stirred for 1 h and separated, and the aqueous layer was extracted (2 × 30 mL) with CHCl$_3$. The combined organic layers were dried (Na$_2$CO$_3$): 1.84 (s, 6H, CH$_3$), 6.62 (d, 2H, J = 9 Hz), 7.11 (d, 2H, J = 9 Hz), 7.67 (s, 1H, H-4), 7.78 (d, 1H, J = 8 Hz, H-7), 8.00 (d, 1H, J = 8 Hz, H-6). 5'-Bi(1,2,3-dithioarosalan-2-y1)-fluorescein-5-carboxylic Acid, Succinimidyl Ester: 5-Carboxyfluorescein–EDT$_2$, Succinimidyl Ester. 5-CarboxyFlAsH–EDT$_2$ (3.5 mg, 5.0 µmol) was dissolved in dry THF (0.5 mL) under Ar and diisopropylcarbodiimide (1 µL, 6 µmol) and N-hydroxysuccinimide (0.7 mg, 6 µmol) were added. After 3 h, the mixture was evaporated and the product was purified by chromatography on silica (7 g SiO$_2$, eluted with 30% ethyl acetate–0.5% HOAc–toluene to elute the product. After trituration with 95% EtOH, 14 mg (20% yield) of a whitish-pink solid was obtained. $^1$H NMR (CDCl$_3$–CD$_2$OD): 3.5 (m, partially obscured by solvent), 6.42 (d, 2H, J = 9 Hz), 6.50 (d, 2H, J = 9 Hz), 7.78 (s, 1H, H-4), 7.97 (d, 1H, J = 8 Hz, H-7), 8.21 (d, 1H, J = 8 Hz, H-6). ES-MS (50% MeOH, 0.04% NH$_3$) -ve mode; (M – 1)$_{-}$ 708.4. Calcd for C$_{35}$H$_{27}$O$_{16}$AsS$_{5}$ 708.5.

Further elution with 25–50% ethyl acetate–0.5% HOAc–toluene yielded the monosubstituted product as a yellow solid (15 mg, 28% yield). ES-MS (50% MeOH, 0.04% NH$_3$) -ve mode; (M – 1)$_{-}$ 541.5. Calcd for C$_{23}$H$_{14}$O$_{7}$AsS$_{2}$ 541.5. Conversion to the arsenoxide with EDT$_2$ was as described for ReAsHO.

4',5'-Bi(1,2,3-dithioarosalan-2-y1)-fluorescein-5-carboxylic Acid, Succinimidyl Ester: 5-CarboxyFlAsH–EDT$_2$, Succinimidyl Ester. 5-CarboxyFlAsH–EDT$_2$ (3.5 mg, 5.0 µmol) was dissolved in dry THF (0.5 mL) under Ar and diisopropylcarbodiimide (1 µL, 6 µmol) and N-hydroxysuccinimide (0.7 mg, 6 µmol) were added. After 3 h, the mixture was evaporated and the product was purified by chromatography on silica (7 g SiO$_2$, eluted with 30% ethyl acetate–0.5% HOAc–toluene to give a white solid (3.7 g, 92%). $^1$H NMR (CDCl$_3$–CD$_2$OD): 2.91 (s, 4H, OCH$_2$), 3.60 (m, 8H, SCH$_2$), 6.54 (d, 2H, J = 9 Hz), 6.60 (d, 2H, J = 9 Hz), 7.95 (s, 1H, H-4), 8.15 (d, 1H, J = 8 Hz, H-7), 8.38 (d, 1H, J = 8 Hz, H-6), 9.97 (s, 2H, OH).

Coupling to Amino Agarose. 5-CarboxyFlAsH–EDT$_2$ succinimidyl ester (0.2 mL of 2.4 mM solution in DMSO, 0.50 µmol) was added to 1 mL of amino-agarose (Affi-Gel 102; Biorad, or Sepharose-EAH; Pharmacia), which had been washed thoroughly with 50 mM K$_2$HPO$_4$. 0.1 mM EDTA, pH 7.5; 5 mM BME and 0.1 mM EDTA can be added to maintain the integrity of the FlAsH–EDT$_2$, moiety without affecting coupling significantly but is not necessary. The mixture was mixed by slow rotation at 4 °C overnight, poured into a short column, and washed extensively with the same buffer. Measurement of the absorbance of the washings at 508 nm indicated >80% coupling efficiency. Capping of free amines was achieved by treatment with a 4-fold excess of N-acetoxyseuccinimide (prepared from acetic acid and N-hydroxysuccinimide with dicyclohexylcarbodiimide in CH$_2$Cl$_2$) over total amines (approx 15 µmol/ml) in the same phosphate buffer with slow rotation overnight. After washing with buffer, a sample of the agarose tested negative for free amines by reaction with ninhydrin. The immobilized FlAsH–EDT$_2$ was washed and stored in 50% EtOH at 4 °C.

Staining of Extracellular Tetracysteine Motif by Impermanent FlAsH$_2$. A gene encoding a fusion of the peptide AEAAARECCREC-CARA to the C-terminus of VAMP 2 (vesicle associated membrane protein 2;14 was created by the polymerase chain reaction (PCR). The resulting gene was inserted into pcDNA3 vector (Invitrogen) and cotransfected with an EBFP plasmid into HeLa cells with lipofectin. After 24–48 h, the cells were treated with 5 mM MES and 0.5 mM TCEP in HBSS for 20–30 min; 2–5 µM sFlAsH–EDT$_2$ was added for a further 30 min, and the cells were rinsed with fresh HBSS. Imaging experiments were performed as described above with excitation at 480 nm (30 nm bandwidth), emission at 535 nm (25 nm bandwidth), and dichroic mirror at 505 nm for sFlAsH$^-$ and excitation of 380 nm (10 nm bandwidth), emission at 440 (bandwidth 40 nm), and dichroic at 420 nm for EBFP.

Affinity Purification of Proteins Using Immobilized FlAsH–EDT$_2$. Crude bis-tagged Cys$_{8,12,13}$ calmodulin$^-$ containing an N-terminal polylys tag was prepared by cloning Xenopus Cys$_{8,12,13}$-calmodulin into pRSETB vector, expression in E. coli, lysis by French press in 250 mM KCl, 50 mM KPO$_4$, 1 mM EDTA, 10 mM MES, 1 mM TCEP, pH 7.5, containing the protease inhibitors, leupeptin, apotinin, TLCK, TPCK, and PMSF, followed by high-speed centrifugation. The supernatant was stored frozen at –80 °C.

Supernatant (1 mL) was mixed by slow rotation at 4 °C for 3–12 h with 0.5% argease–FlAsH–EDT$_2$, which had been washed in 250 mM KCl, 50 mM KPO$_4$, 1 mM EDTA, pH 7.5. The mixture was transferred to a short column, the unbound material was collected, and the resin was washed with buffer (5–20 mL) and then eluted with buffer containing 10 mM DMPS (3 × 0.2 mL). Fractions were analyzed by SDS-PAGE (15% separating gel).

Purification by the poly-histidine tag used standard immobilized metal affinity chromatography methods (Qiagen) except that 1 mM NiCl$_2$ was added to the bacterial supernatant before binding to prevent the EDTA from removing Ni$^{2+}$ from the support. The protein was bound to 100 µL of resin at 4 °C for 3–4 h, washed with buffer (0.25 M NaCl, 10 mM Tris, pH 8, containing 10 mM and 20 mM imidazole (each 10 mL), and eluted with buffer containing 0.25 M imidazole (3 × 0.2 mL). SDS-PAGE of Protein–FlAsH Complexes. FlAsH–EDT$_2$, (10–100 µM) was added to Laemmli sample buffer with SDS, glycerol, Tris-Cl pH 6.8, bromophenol blue, but containing 50 mM TCEP (diluted from a 100 mM stock neutralized with 3.5 equiv of Tris base) as the reductant instead of BME or DTT. The protein and sample buffer were kept at room temperature for 15–30 min and loaded onto the gel that was run as usual. The samples can be briefly boiled (3 min only) but did cause a slight decrease in the intensity of the fluorescent band. Alternatively, if the protein requires heat to denature, the sample combined with sample buffer lacking FlAsH–EDT$_2$, can be boiled and cooled, and FlAsH–EDT$_2$ can be added. High (>1mM) concentrations of DTT and BME should be avoided in the sample buffer. The protein–FlAsH complexes were visualized on the unstained or unfixed gel by illumination with either an ultraviolet light-box or by using a CCD camera and Xe-light source with appropriate filters for FlAsH fluorescence (excitation at 480 nm, with emission at 535 nm).

Fluorescence Polarization of FlAsH and Fluorescein-Labeled Calmodulin. Measurements of fluorescence anisotropy were made on an ISS Inc. K2 fluorometer with Glan-Thompson polarizing prisms, 1 mm slits, 18 A lamp current, and excitation monochromator set at 500 nm, and measuring emission at 533 nm, for FlAsH–Cy5.7,12,13, calmodulin. For fluorescein-labeled calmodulin, the excitation setting...
was 490 nm, and emission was at 520 nm. The samples were prepared in 50 mM MOPS adjusted to pH 7.4 with potassium hydroxide, also containing 2 mM mercaptoethanesulfonate (MES) and 1 mM ethylendiaminetetraacetic acid (EDTA). To adjust viscosity, the buffer above was used to prepare a solution of 60% sucrose by weight and then mixed with unsweetened buffer in 0.3-mL divisions to obtain a series of sucrose concentrations of 0, 10, 20, 30, 40, and 50%. In each cuvette 0.3 mL of the reduced and labeled stock protein solution was diluted 6-fold to obtain a concentration of approximately 1 μM.

The labeling of the Cys6,7,10,11-calmodulin sample was accomplished as follows: 144 μM of the recombinant Cys6,7,10,11-calmodulin1 stock (200 μM) was mixed with 1056 μl of 50 mM K-MOPS pH 7.4 buffer (no MES or EDTA) containing 1 mM (TCEP) and allowed to stand at room temperature for 2.5 h to reduce any disulfide bonds. Then 4 μl of 1 mM FlAsH–EDT (in DMSO) was added so that 24 μM of Cys6,7,10,11-calmodulin was labeled with 3.3 μM FlAsH–EDT. The sample was kept at room temperature for 2 h and then at 4 °C for 2 days. Prior to diluting the samples, the 1.2 mL of sample was gel-filtered with 0.4 g G25 Sephadex (Sigma) in which the void volume was determined to be 0.5 mL. The buffer used for elution was the MOPS–MES–EDTA used to dilute the samples. Several of the 0.5-mL fractions were fluorescent and were combined and diluted to 2 mL. The specificity of labeling was verified by electrospray ionization mass spectrometry. It was found that the TCEP reduction step was necessary to observe distinct, deconvolved mass peaks for the labeled and unlabeled Cys6,7,10,11-calmodulin components in the sample. The gel-filtered complex was stable for at least several days even though a nonoptimal FlAsH site (CCQICC) was used. For the control sample of fluorescein-labeled native calmodulin, 5(6)-carboxyfluorescein succinimidyl ester (Molecular Probes) was reacted with recombinant bovine CaM at pH 7, gel-filtered, and dialyzed to remove any free fluorescein. It was then diluted into a similar series of six stepped sucrose percentage cuvette samples as described above. The effect of FlAsH or fluorescein labeling upon the biological activity or structure of calmodulin has not yet been determined.

Complex Stability and Kinetics. On-rate: 2–5 nM FlAsH was added to a stirred, degassed solution of peptide (0.015–1 μM) and MES (0, 1, 5, or 20 mM) in 100 mM KCl, 10 mM K-MOPS, pH 7.3, at 20 ± 2 °C in a Spex fluorimeter. The fluorescence intensity at 530 nm was monitored with time (30–300 s), with excitation at 508 nm, and the initial part was fitted to a single exponential using GraphPad Prism software. The determined rate plotted against at least three different peptide concentrations gave a straight line with gradient equal to the rate constant.

Exchange-rate: FlAsH–peptide complex (0.1 μM isolated by HPLC) in degassed 100 mM KCl, 10 mM K-MOPS, pH 7.3, was treated with 1 μM ReAsH and MES (1, 5, or 20 mM) and sealed in an ampule. Fluorescent emission spectra (with excitation at 508 nm) were acquired periodically over 3–4 weeks. Long-term variations (±10%) in the fluorimeter stability were corrected for by referencing each time point to a rhodamine B block. Plots of emission intensity at 530 nm with time were fitted to single exponential decays.

FlAsH–EDT, Staining in Live Cells. The CFP–CCRECC (CFP–AEAAAREACCPGCCARA) construct was prepared by PCR of the previously reported CFP–CCRECC (CFP–AEAAAREACCRECCARA)1 with an antisense primer that contained the RE to PG mismatch. The gene was cloned into the mammalian expression vector pcDNA3 (Invitrogen) and either it or CFP–CCRECC were transected into HeLa cells with Fugene (Roche). Imaging experiments were performed 24 h after transfection with a cooled charge-coupled device camera (Photometrics) controlled by Metaflour software (Universal Imaging). Fluorescence was monitored in three channels; CFP, excite at 440 nm (20 nm bandwidth) with emission at 480 nm (30 nm bandwidth); FRET, excite CFP with emission at 635 nm (55 nm bandwidth); FlAsH, excite at 495 nm (10 nm bandwidth) with emission at 535 nm (25 nm bandwidth). The 455 nm dichroic mirror reflected sufficient 495 nm light. Cells were washed with Hank’s buffered saline solution (HBSS) containing glucose and were stained by addition of a premixed DMSO stock solution to give a final concentration of 2.5 μM FlAsH–EDT and 10 μM EDT. Staining was generally allowed to proceed for 60–90 min before the cells were thoroughly washed with HBSS and allowed to equilibrate. Concentrated DMSO stock solutions of EDT and Disperse Blue were generally added in volumes of less than 5 μL. CFP concentration was calibrated using a wedge of height 0.14 mm and length 7.4 mm that contained a 3 μM solution of purified CFP in HBSS. Under conditions identical to those used for live cell imaging, the objective lens was moved through the thickness of the wedge at a constant rate, and the start and end of the fluorescent signal were taken as the start and end of the physical wedge. The fluorescence at a wedge thickness of 5 μm was interpolated and used as the scaling factor for calibrating CFP concentration in HeLa cells.

Photoconversion and Fluorescent Staining of FlAsH Derivative-Labeled Connexin 43. A gene encoding a fusion of the peptide AEAAARECCRECCARA to the C terminus of connexin 4317 was created by the polymerase chain reaction (PCR). The resulting gene was inserted into pcDNA3 vector (Invitrogen) and cotransfected with a EBFP plasmid into HeLa cells with lipofectin. Fluorescent imaging and photoconversion staining were performed as described (Gaietta et al., submitted) with excitation of bound FlAsH, BrAsH, Br2AsH, and tFlAsH at 480 nm emission and emission at 535 nm, and with excitation of ReAsH, Br2ReAsH, and ThAsH at 580 nm with emission at 630 nm.

Acknowledgment. We thank Jianghong Rao and Oded Tour for helpful discussions and Guido Gaietta and Tom Deerinck for photoconversion procedures. This work was supported by National Institutes of Health Grant NS27177 (to R.Y.T.), the Howard Hughes Medical Institute (to R.Y.T.), Office of Naval Research, Order No. N00014-98-F-0402 to the Molecular Design Institute through the U.S. Department of Energy under Contract No. DE-AC03-76SF00098 (S.R.A., J.L., and R.Y.T.) and National Institutes of Health Grant P01 DK54441 (to Susan S. Taylor). The award of a NIH Training Grant to G.K.W. (GM198404-02) is gratefully acknowledged. Some of the work included here was conducted at the National Center for Microscopy and Imaging Research, which is supported by National Institutes of Health Grant RR04050 (to Mark H. Ellisman).

Supporting Information Available: Synthetic details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA017687N