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The Biarsenical-Tetracysteine Motif as a Fluorescent Tag for Detection in Capillary Electrophoresis

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Abstract

Biarsenical dyes complexed to tetracysteine motifs have proven to be highly useful fluorescent dyes in labeling specific cellular proteins for microscopic imaging. Their many advantages include membrane permeability, relatively small size, stoichiometric labeling, high affinity, and an assortment of excitation/emission wavelengths. The goal of the current study was to determine whether the biarsenical labeling scheme could be extended to fluorescent detection of analytes in capillary electrophoresis. Recombinant protein or synthesized peptides containing the optimized tetracysteine motif "-C-C-P-G-C-C-" were labeled with biarsenical dyes and then analyzed by MEKC. The biarsenical-tetracysteine complex was stable and remained fluorescent under standard micellar electrokinetic capillary chromatography (MEKC) conditions for peptide and protein separations. The detection limit following electrophoresis in a capillary was less than 3×10^{-20} moles with a simple laser-induced fluorescence system. A mixture of multiple biarsenical-labeled peptides and a protein were easily resolved. Demonstrating that the label did not interfere with bioactivity, a peptide-based enzyme substrate conjugated to the tetracysteine motif and labeled with a biarsenical dye retained its ability to be phosphorylated by the parent kinase. The feasibility of using this label for chemical cytometry experiments was shown by intracellular labeling and subsequent analysis of a recombinant protein possessing the tetracysteine motif expressed in living cells. The extension of the biarsenical-tetracysteine tag to fluorescent labeling of peptides and proteins in chemical separations is a valuable addition to biochemical and cell-based investigations.

INTRODUCTION

Capillary electrophoresis (CE) is becoming increasingly important for biochemical analysis of peptides and proteins.[1, 2] The facility with which nanoliter to picoliter sample injections can be performed and the exquisite sensitivity made possible by laser-induced fluorescence (LIF) detection have advanced CE's application to the analysis of peptides, proteins, and other analytes in single cells, a technique commonly referred to as chemical cytometry.[3-5] In analyzing fluorescent species from cells, small organic dyes and fluorescent proteins are commonly used as tags for analyte labeling.[6,7] The use of organic dyes, such as derivatives of fluorescein and rhodamine, is limited by the need either to label analytes prior to their introduction into the cell or to derivatize them after cell sampling. Pre-labeling provides a highly pure analyte that can be used as an intracellular probe, but the cell membrane tends to be impermeant to the labeled molecules making introduction into the cell a challenge.[8,9] Labeling of native peptides or proteins after cell sampling is an alternative approach. Amine-reactive fluorescent tags are commonly used to covalently attach fluorophores to both the ε-amine of lysine and the amino terminus of proteins, but these dyes also react with amino acids and other biogenic amines.[6] This approach has been used to great success for seminal

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investigations of cellular proteins; nevertheless, limitations exist due to nonspecific and non-stoichiometric binding of the dyes, and problems with the large excess of unbound dye that frequently remains after derivatization.[10-12]

Recombinant proteins composed of a target molecule and a fluorescent protein (*e.g.* green fluorescent protein [GFP]) have proven effective as reporters of intracellular biochemical activity.[13] Cell loading can be bypassed because the cell itself produces the fluorescently-tagged analyte, but this approach is not without its drawbacks. The fluorescent proteins are large molecules, for example the molecular weight of GFP is 27kD, so that its conjugation may perturb the bioactivity and cellular location of the protein to which it is fused.[14,15] In addition, after expression these proteins can require many hours to mature in order to become fluorescent.

Biaresenical dyes were developed by Tsien and colleagues for the specific labeling of target peptides or proteins with small fluorescent dyes *in vivo*.[16] The method relies on introduction of a genetically encodable motif of four cyteines (-C-C-X-X-C-C- where -X-X-consists of any two non-cysteine amino acids) that binds with high affinity to a fluorescent dye incorporating two arsenic moieties (Fig. S1).[16] The cell-permeable fluorescent dye FlAsH [4',5'-bis(1,3,2,-dithioarsolan-2-yl)fluorescein] contains two As(III) substituents that pair with the four cysteine thiol groups located in the motif. The dye increases in fluorescence upon binding the motif. An optimized tetracysteine (TC) sequence (-C-C-P-G-C-C-) provides high affinity binding with a dissociation constant of 10 pM.[17] The specificity of the genetically encoded tetracysteine binding site and the high affinity binding enable quantitative labeling of the target.[16,17] A number of TC sequences have been described all of which are significantly smaller than a fluorescent protein, thus being less likely to perturb the bioactivity of the tagged molecule.[14,17] Also in contrast to fluorescent proteins, the TC motif in the presence of a biarsenical dye is detectable immediately after synthesis as no folding or post-translational modifications are required. Additionally, the excitation and emission maxima of the FlAsH-TC complex are 508 nm and 528 nm, respectively, which is compatible with commonly used detection schemes for CE-LIF. Other cell permeable biarsenical dyes are available with different spectral properties. ReAsH (ex/em 593nm/608nm) and CHoXAsH (380nm/430nm) have been shown to effectively bind the TC motif in the intracellular environment, thus increasing the flexibility of the TC-labeling scheme.[18] Specific binding and membrane permeability of these biarsenical dyes are key advantages of in-cell labeling over other conventional methods. Since they are membrane permeant, the biarsenical dyes are easily loaded into intact cells. When simultaneously loaded into cells with micromolar concentrations of 1,2-dithiols, non-specific binding of As(III) to endogenous thiols is decreased which also minimizes cell toxicity.[17] After cell labeling, unbound dye can be removed in the presence of sub-millimolar concentrations of dithiols, such as 1,2-ethanedithiol (EDT), since the high affinity biarsenical-TC complexes is not disrupted by low concentrations of dithiols.

Despite the advantages of the biarsenical-TC system for intracellular labeling, detection of tagged proteins expressed at low levels remains limited due to background fluorescence from unbound dye and spontaneous binding to non-target biomolecules within the cell even with the use of 1,2-dithiols during labeling.[19] Random mutagenesis of the residues surrounding the optimized TC binding motif has produced two sequences of 12 amino acids (FLNCCPGCCMEP and HRWCCPGCCKTF) that display 20-fold higher fluorescence quantum yields and improved binding in the presence of dithiols. These characteristics provide improved contrast for imaging of the biarsenical-TC complex in the cellular environment.[19] Nevertheless, dithiol competition with biarsenical-TC binding and persistent nonspecific binding of the dye remain limiting issues in the use of this system for microscopy. These problems would be negated if the biarsenical-TC tag was used in labeling peptides or proteins to be analyzed in chemical separations. By employing a separation format of high sensitivity, detection of very low amounts of biarsenical-TC labeled analyte from cells should be possible

due to physical separation of the species of interest from either free or nonspecifically bound biarsenical.

While GFP-fusion proteins have been used as fluorescent analytes for chemical cytometry, the use of the biarsenical-TC-complex for analyte labeling in chemical separations has distinct advantages. Fluorescent proteins do not remain fluorescent upon being denatured. CE separations of GFP-tagged proteins under mildly denaturing conditions have been reported; however, the need to maintain the protein tag in its fluorescent state severely limits the choice of separation conditions. GFP also significantly increases the molar mass of the target protein further limiting its applications in chemical separations. Conversely, the biarsenical-TC complex is stable under the denaturing conditions typically used for gel electrophoresis of proteins and has a molecular weight less than 2kDa when bound to the biarsenical dye.[17] This stability suggests that its use as a fluorescent tag in most common high-resolution electrophoretic techniques, such as MEKC or capillary sieving electrophoresis (CSE), could prove a valuable tool for high sensitivity analyses of peptides and proteins.[1]

The goal of this publication was to determine whether the biarsenical dyes could be used as a fluorescent probe for *in vitro* and cellular peptide and protein studies by CE. A genetically engineered protein containing the TC motif as well as synthesized peptides were used as analytes in this study. The biarsenical label was shown to remain bound to analytes and remain fluorescent under MEKC conditions. A peptide labeled with FlAsH and separated under these conditions could be detected at better than 10^{-20} mol by LIF detection in a capillary and over a greater than 100-fold dynamic range. A peptide known to be a kinase substrate and labeled with the FlAsH reagent could be phosphorylated by the parent kinase indicating that the biarsenical-TC complex did not block the bioactivity of the peptide. Furthermore, in-cell-labeled recombinant protein possessing a TC motif and expressed in living cells could be directly analyzed by MEKC with greater sensitivity than with conventional slab gel techniques. The potential for analysis of multiple species of biarsenical labeled analytes was demonstrated by the simultaneous separation by MEKC of a mixture of TC-containing peptides and a protein. These studies indicate that the biarsenical-TC labeling technique is a valid and advantageous method for cellular analysis of peptides and proteins by CE.

EXPERIMENTAL SECTION

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) unless specified otherwise. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was from Pierce Biotechnology Inc. (Rockford, IL). The biarsenical FlAsH reagent (4',5'-bis[1,2,3-dithioarsolan-2-yl]-fluorescein) used in this study was synthesized by the Van Vranken Lab, Dept. of Chemistry, University of California at Irvine. Fluorescent protein standards (FPS) and Lumio™ Red In-Cell Labeling Kit were purchased from Invitrogen, Inc. (Carlsbad, CA).

Proteins and Peptides with the Tetracysteine Motif

. The protein and peptides containing the TC motif used in this investigation were expressed in bacteria or synthesized (Table 1). The recombinant protein was expressed in *E. coli*, and was utilized for both *in vitro* and cellular studies. Peptides were synthesized either by the Van Vranken Lab (UC Irvine) or by Anaspec, Inc. (San Jose, CA), and were used for *in vitro* labeling studies.

Construction of Erk1-TC

The recombinant protein Erk1-TC composed of a TC motif conjugated to the C-terminus of extracellular signal-regulated protein kinase 1 (Erk1) and an N-terminal 6-histidine tag (His_6) for Ni-column-based affinity purification was engineered for expression in *E. coli* as follows. The recombinant plasmid pHis_6-Erk1-TC was constructed from the Erk1 plasmid p$ETHis_6$/MEK1R4F+ERK1 (a kind gift from Dr. Melanie Cobb).[20] Codons encoding the His_6 tag and the TC motif LNCCPGCCMEP were introduced into the 5'and 3' ends, respectively, of the original plasmid by standard polymerase chain reaction (PCR). The PCR product was inserted into p$ETHis_6$/MEK1R4F+ERK1 at NdeI and BamHI (New England Bio Labs; Beverly, MA) restriction sites, which resulted in the loss of the MEK1R4F and the unmodified Erk1 genes from the plasmid. For proper translation, the existing stop codon of the Erk1 gene was moved to the 3' end of the TC sequence.

Protein Expression and Purification

The recombinant protein was expressed in the *E. coli* strain BL21(DE3) pLysS (Novagen; La Jolla, CA). BL21 competent cells transformed with the pHis_6-Erk1-TC plasmid were grown in Terrific Broth at 37°C. Protein expression was induced with isopropyl β-D-thiogalactoside (IPTG) (Anatrace; Maumee, OH) at an A_{600} of 1, and grown for 4 hours to allow expression. Non-phosphorylated Erk1-TC was purified *via* its His_6 tag using a nickel-nitrilotriacetic acid column, eluting with a 100 − 300 mM imidazole gradient. Collected fractions of Erk1-TC were dialyzed against 50 mM Tris, pH 8.5, 1 mM phenylmethylsulfonyl fluoride, and 5 mM beta-mercaptoethanol (βME), concentrated by ultrafiltration (12 μM), and stored at −70°C.

Peptide and Protein Labeling with FlAsH-EDT_2

The labeling procedure closely followed the method described previously by the Tsien group.[17,21] The FlAsH (*Fl*uorescein *A*r*s*enical *H*airpin binding) reagent was dissolved and stored in dimethyl sulfoxide (DMSO) with excess EDT, at a final concentration of 1μM FlAsH, 10μM EDT. Tetracysteine-containing peptides were dissolved in phosphate buffered saline (PBS, pH 7.2) in the presence of TCEP (1 mole of TC-peptide : 25 moles of TCEP). The presence of TCEP was used to prevent oxidation of the thiol group in the cysteine residues and improve labeling efficiency.[22,23] Labeling reactions were carried out in phosphate buffered saline (PBS) at pH 7.2. TC-peptides used in excess (10-to-1000 fold) were reacted with the FlAsH-EDT_2 reagent and incubated for 15 to 30 min at room temperature to assure complete labeling. For protein labeling the incubation period was extended to 90 min. The binding efficiency of the FlAsH reagent with the peptide or protein was improved by adding 1 mM βME to the PBS reaction media.[21] After incubation, the labeled samples were analyzed by CE or SDS-PAGE.

In-Cell FlAsH Labeling

Intracellular labeling of the recombinant protein was performed in the *E. coli* expression system. As described above, BL21 cells were transformed with pHis_6-Erk1-TC. After 4 hours of IPTG treatment, the cells were centrifuged and washed in a physiologic buffer (ECB, *e*xtra*c*ellular *b*uffer; 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM MgCl , 2 mM CaCl, pH 7.4). Then bacterial cells were incubated for 30 min at room temperature in the presence of 1 μM FlAsH-EDT_2. After incubation, excess reagent was removed by wash of the *E. coli* in ECB (×3). In-cell FlAsH labeling was confirmed by fluorescence microscopy. The cells were then lysed in buffer containing 2% SDS, 1 mM TCEP, 0.6% glycerol, 0.1 M Tris, pH 6.8. As a control, non-transformed BL21 cells were incubated with the FlAsH reagent in parallel using the same labeling protocol.

Capillary Electrophoresis

FlAsH- and ReAsH-labeled samples were analyzed by CE with LIF detection on a system that was built in-house. The following modifications have been made from an earlier version of this reported CE-LIF system.[24] The open space excitation path was replaced by single-mode fiber-optic cable that coupled a focusing lens of 3.5-cm focal length to the end of the fiber (Oz optics, Ottawa, ON, Canada). Separations were performed in a 50/360-μm (inner diameter/ outer diameter) fused silica capillary (Polymicro Technologies, Phoenix, AZ) at a field strength of −300 V/cm unless specified otherwise. The labeled samples were injected gravimetrically and the volume loaded was calculated using Poiseulle's equation.[25] All separations were performed by MEKC. The run buffer consisted of 1% SDS, 1 mM DTT, 0.1 M borate at pH 8.5, unless specified otherwise. DTT was added to the CE run buffer to maintain a reducing environment during the separation. While TCEP is a more effective reducing agent than DTT, preliminary studies showed that 1 mM or higher concentrations of TCEP adversely affected the CE separation; therefore, the concentration of TCEP used in buffers prior to CE analysis was maintained at ≤ 0.5 mM.

Detection Limits of the Biarsenical-Tetracysteine Complexes

Derivatization of the TC-FLN peptide was performed as described above. Peptide aliquots containing molar excess of TC-FLN (10 μM) were reacted with the FlAsH reagent over the concentration range of 5 pM to 500 pM. To maximize consumption of the FlAsH reagent, the 20 μL reaction mixture was incubated overnight at 4°C as previously described.[19] Samples were then stored at −70°C to inhibit further reaction prior to analysis by CE-LIF. At least 3 consecutive electropherograms were generated for each sample. The detection limit was that yielding a peak height 3-fold the height of the baseline noise.

Fluorescence Resonance Energy Transfer (FRET)

A peptide (TC-ABL-FAM, Table 1) was composed of an N-terminal TC motif conjugated *via* a poly(ethylene glycol) linker (PEG_6) to a substrate peptide for the tyrosine kinase ABL.[26] The substrate peptide was labeled on its C-terminus with fluorescein (FAM). TC-ABL-FAM (0.5 μM) was reacted with 1.0 μM ReAsH (Invitrogen Co., Carlsbad, CA) using the identical protocol as that for FlAsH-TC labeling described above. In FRET, the FAM tag acted as the FRET donor and the ReAsH-TC complex the acceptor.[27] Emission spectra of both the FAM-only peptide and the ReAsH labeled peptide were obtained using a fluorescence spectrophotometer (SpectraMax M5, Molecular Devices). Detection of FRET was also performed in a capillary. Using the 488 nm line of an Argon-ion laser (JDS Uniphase, Fremont, CA), fluorescence emission of the FAM label was measured at 535-nm using a bandpass filter (535 ± 50 nm, Chroma Technology Corp., Rockingham, VT). Similarly for FRET, samples were excited at 488 nM and emission was measured at 628 nm using a bandpass filter (628 ± 40 nm, Semrock, Rochester, NY).

ABL Kinase Assay

An identical peptide to that described in the previous paragraph, but lacking the C-terminal FAM label (TC-ABL, Table 1), was synthesized as a test substrate for the ABL kinase. Enzyme activity (peptide phosphorylation) of ABL kinase was studied for pre- and post-FlAsH-labeled TC-ABL peptide. Labeling reactions were preformed as described above, but incubation time was extended to 90 min to assure complete labeling. The reaction mixture of the enzyme assay contained 50 nanomoles of TC-ABL and 1μg ABL kinase (specific activity of 2800 U/mg: 1 U equals the incorporation of 1 nmol phosphate per min [Upstate, Charlottesville, VA]) in a reaction buffer composed of 75 mM $MgCl_2$, 0.5 mM ATP, 5 mM EGTA (ethylenediaminetetraacetic acid), 1 mM Na_3VO_4, 1 mM DTT, in 20 mM MOPS buffer, pH 7.2. Samples were incubated for 120 min at room temperature. Control experiments for both

FlAsH-labeled and unlabeled TC-ABL substrates were performed in which ATP was omitted from the reaction mixtures. Unlabeled TC-ABL was labeled with FlAsH after the kinase assay was performed and then measured by CE analysis. After completion of both labeling and enzyme assays, samples were stored in −70°C prior to CE analysis.

SDS-PAGE

Bacterial expression of the Erk1-TC protein was confirmed by SDS-PAGE. SDS-PAGE separation of FlAsH and ReAsH labeled Erk1-TC was performed using the BioRad Mini-Protean II system (Bio-Rad Inc., Hercules, CA). Separations were performed on 10% or 15% acrylamide/bis-acrylamide SDS-PAGE gels at a constant 200 V for 50 to 70 min using a run buffer of 0.1% SDS, 196 mM glycine and 50 mM Tris-HCL. The FlAsH/ReAsH/Erk1-TC complexes were visualized using a Typhoon gel imager, Model 9400 (Amersham Bioscience, Piscataway, NJ). For detection of total protein, gels were stained with Coomassie Blue.

RESULTS AND DISCUSSION

CE Analysis of the TC-FlAsH Complex

CE-LIF with MEKC-based separation conditions were employed to test the potential of using FlAsH-TC labeling for CE analysis of peptides and proteins. The optimized 12-amino-acid peptide TC-FLN was tested initially (see Table 1). The result of the CE analysis of the FlAsH labeling of TC-FLN is shown in Figure 1a. A molar excess of the TC-FLN peptide was incubated with the FlAsH reagent in a final concentration of 10 μM TC-FLN to 1 μM FlAsH. The peptide was used in excess because other investigators have shown that a 10-to-1000-fold excess of peptide in the labeling reaction results in pseudo-first-order kinetics and gives a higher on-rate for the forward reaction.[17] A sample of the reaction mixture was injected onto the CE column and electrophoresed. The resulting electropherogram revealed two partially separated peaks at 163±3 s (migration time based on the major peak, n=16). A sample of the FlAsH reagent (1 μM) was also injected onto the CE column and electrophoresed. The resulting electropherogram revealed a single peak having a migration time of 212±4 s (n=9). No fluorescent peaks were present in the CE analysis of a sample of the unlabeled TC-FLN peptide (10 μm). Notably, the signal-to-noise ratio of the peak seen in the FlAsH/TC-FLN sample was 32±1 (n=3) greater than that of FlAsH (1 nM) alone. This finding is similar to the enhanced fluorescence of the FlAsH-TC complex observed by other investigators.[17] The doublet peak for the FlAsH/TC-FLN complex was also consistent with prior data demonstrating isomeric formation of the complex. The two As(III) atoms of the FlAsH reagent can be bound in more than one orientation with the four thiols of the tetracysteine motif.[16,17] A theoretical plate number of 123,000±10,000 plates/m (n=5) was calculated for the FlAsH/TC-FLN complex based on the initial peak of the paired isomers. These data showed that MEKC-based CE separations could readily separate FlAsH-TC complexes from unlabeled FlAsH.

Confirmation of Biarsenical-Tetracysteine Binding by FRET

A FRET analysis of a dual labeled model peptide was used to confirm that the biarsenical remained bound under MEKC conditions. FRET using the biarsenical-TC complex as one member of the FRET pair has been previously demonstrated.[14, 19, 28, 29] The peptide TC-ABL-FAM (Table 1) provided a C-terminal fluorescein molecule that served as the FRET donor (see Supplemental Data Fig. S2a). In this experiment, ReAsH was used as the FRET acceptor since the emission spectrum of FAM and the absorbance spectrum of the ReAsH-TC overlap. By virtue of the highly flexible PEG6 domain linking the TC and ABL peptide domains, the two fluorophores within the ReAsH-labeled TC-ABL-FAM peptide should readily be able to move to within the 1−10 nm distance needed for effective energy transfer.[27] Measurement of the emission spectra of the FAM-only labeled peptide, the ReAsH/FAM-labeled peptide, and the ReAsH only peptide confirmed the presence of FRET in the ReAsH/

FAM-labeled peptide (Supplemental Figure S2b). LIF detection in a capillary was then performed for samples of FAM-only labeled and ReAsH-labeled peptides injected into the capillary and electrophoresed. TC-ABL-FAM that had not been incubated with the ReAsH labeling agent showed a high fluorescence signal at 535 nm, but minimal fluorescence at 628 nm (Fig. 1b). The electropherogram of ReAsH alone showed negligible fluorescence at both emission wavelengths. In two independent trials in which the peptide was incubated with ReAsH, the relative fluorescence of the peptide was reduced 70% and 74% while the fluorescence peak at 628 nm increased 330- and 360-fold respectively. An identical amount of TC-ABL-FAM was used in the analysis of both unlabeled and ReAsH-labeled peptides. These data are consistent with energy transfer from FAM to the TC-ReAsH complex demonstrating that the biarsenical dye remains bound to the TC motif during MEKC.

CE-LIF Detection Limits of Biarsenical-Tetracysteine Labeling

A potential advantage of the combination of CE-LIF with biarsenical labeling is that it may be possible to analyze peptides and proteins present in very low amounts within a cell by virtue of the exquisite detection limits engendered by this technique. For this reason, the limit of detection in a capillary of the biarsenical-TC complex was tested. In these experiments, a limiting amount of FlAsH (5 pM to 500 pM) was reacted with an excess amount of TC-FLN (10 μM) and incubated overnight. Samples of the reaction mixture were then analyzed by CE. Assuming complete reaction of the FlAsH reagent under the conditions used, the limit of detection of the FlAsH-TC-FLN complex was found to be 18,400±500 molecules (n=3), corresponding to 3×10^{-20} moles (Fig. 1c). Detection was linear over a range of at least 3×10^{-20} to 10^{-18} mol (the highest tested).

Effect of Biarsenical Binding on the Phosphorylation of a Peptide Substrate

In this study, the phosphorylation of a peptide was evaluated to assess whether FlAsH binding eliminated bioactivity of the peptide. A synthetic peptide based on a known peptide substrate (EAIYAAPFAKKK) for the ABL tyrosine kinase was utilized. The peptide TC-ABL was identical to the TC-ABL-FAM peptide used in the FRET studies, except that it lacked the C-terminal fluorescein (Table 1). The influence of FlAsH binding on phosphorylation of the peptide was determined by comparing the percentages of pre-labeled and unlabeled TC-ABL phosphorylated in an *in vitro* kinase assay. The amounts of phosphorylated and nonphosphorylated peptide were determined by CE-LIF and quantified in comparison to standards. FlAsH-labeled or unlabeled TC-ABL was incubated in the presence of the ABL kinase. Phosphorylation was confirmed by conducting the phosphorylation reaction in the presence and absence of ATP. In the absence of ATP, a single peak was observed on the electrophoresis trace that co-migrated with the peptide standard whether pre-labeled or unlabeled peptide was used in the reaction mixture (Fig. 2a). In the presence of ATP, electropherograms of the reaction mixture for both the pre-labeled and unlabeled samples displayed a peak with a prolonged migration time compared with non-phosphorylated standard (Fig. 2b). This peak was most likely the phosphorylated form of the peptide. In both samples, conversion to the second peak was almost complete after 120 min of incubation in the presence of ATP. Mixing experiments of the ATP and ATP-free reaction mixtures were used to confirm that a new peak had indeed been generated in the ATP-containing reaction mixture (Fig. 2c). Calculation of peak areas of the phosphorylated and non-phosphorylated peaks in the pre-labeled and unlabeled samples showed that 83% of the pre-labeled and 96% of the unlabeled peptide were phosphorylated by 120 min. Although there was a small difference in the amount of phosphorylated peptide between the pre-labeled and unlabeled samples, the data suggest that the addition of the TC motif and its bound biarsenical tag do not prevent a small peptide substrate from being phosphorylated by its kinase.

CE Analysis of FlAsH Labeled Recombinant Protein

Erk1 was used as a model protein for these studies. Erk1 belongs to the mitogen-activated protein (MAP) kinase family and is composed of 379 amino acids with a molecular weight of 43 kDa.[30] Using standard recombinant DNA techniques, the TC motif was introduced into the C-terminus of a previously cloned Erk1 recombinant protein containing a His_6 tag.[20] The final protein (Erk1-TC) possessed a molecular weight of 45.4 kDa. The Erk1-TC protein was purified and then incubated with either FlAsH or ReAsH in parallel experiments. Each sample was separated by SDS gel electrophoresis, and fluorescence scans of the unstained gels were performed. In each sample, a fluorescent band of the expected molecular weight was present when the gel was interrogated at the appropriate excitation/emission spectra (Fig. 3a). Coomassie blue staining also demonstrated a band of the correct molecular weight (n=4).

Despite denaturing the samples by boiling and exposure to 10% SDS and 20% βME for SDS page separation, the FlAsH and ReAsH fluorescence persisted as has been previously described.[17] These results along with the data using peptides strongly suggested that the biarsenical label could be used as a protein label under MEKC conditions. MEKC could then be used as a more sensitive means for protein analysis than SDS page. A sample of the FlAsH-labeled Erk1-TC was injected into a capillary and electrophoresed in a run buffer containing 5% SDS. The resulting electropherogram revealed one major and two minor peaks (Fig. 3b). Comparison with a trace obtained from the electrophoresis of FlAsH reagent alone revealed the minor peaks at 395 s and 575 s were due to the unbound FlAsH and the major peak at 675 s was due to the FlAsH-labeled Erk1-TC. Similar results were obtained when replicated on different days (n>6). The fluorescence intensity of the major peak present in the FlAsH incubated Erk1-TC sample was 10±5-fold (n=6) that of the peak seen in the sample of the biarsenical reagent alone consistent with enhanced fluorescence of the biarsenical when bound. Of note, the unbound FlAsH and labeled protein were well separated (by 275 s) demonstrating that the electrophoretic separation circumvented problems with unbound dye. The number of theoretical plates for the FlAsH labeled Erk1-TC protein was 204,000±7000 plates/m (n=3) which is similar to that published for protein separation by MEKC.[6, 31] Although a somewhat low value compared with the number of theoretical plates typically reported for MEKC-based separations, this value was calculated based on the width of the doublet peak (or broadened single peak) which as stated previously is due to isomers of FlAsH-TC.[32] It may be possible to further improve plate number by optimization of the separation buffer. Nevertheless, these data clearly demonstrate that the biarsenical tag is a valid labeling technique for protein analysis by CE.

Fluorescent Labeling of Recombinant Proteins in Living Cells

Since the biarsenical reagents are membrane permeable, Erk1-TC expressed in BL21 cells (*E. coli*) was analyzed after incubating the intact cells in FlAsh. BL21 cells that were either wild-type or transfected with the plasmid carrying the Erk1-TC cDNA were incubated for 30 min with the FlAsH reagent and then washed. After FlAsH treatment, the cells were imaged by epifluorescence microscopy. Cells transfected with the Erk1-TC plasmid were brightly fluorescent while wild-type BL21 cells were weakly fluorescent (data not shown). The cells were then lysed and samples of the cell lysates were analyzed by SDS-PAGE and by CE (Fig. 4). SDS-PAGE revealed a large band with the appropriate molecular weight for Erk1-TC (Fig. 4a). This band also co-migrated with a standard of Erk1-TC. SDS page analysis of the cell lysate from wild-type cells incubated with FlAsH lacked this protein band. CE-based analysis (Fig. 4b) was consistent with the result seen by SDS-PAGE, though the enhanced sensitivity of CE-LIF required 100-fold dilution of the lysate samples prior to analysis (n=3). The electropherogram of the wild-type cells incubated with the FlAsH reagent displayed three peaks consistent with those seen in a sample of the FlAsH reagent dissolved in the lysis buffer. In contrast, the electropherogram obtained from the cell lysate sample of BL21 expressing the

Erk1-TC that had been pre-incubated with FlAsH revealed a major peak co-migrating with the Erk1-TC standard. These data from cell lysates demonstrated the feasibility of employing a CE-based technique to analyze biarsenical-labeled cellular protein. To demonstrate that labeled cells could be directly analyzed by CE, intact wild-type and Erk1-TC-expressing BL21 cells were pre-incubated with the FlAsH reagent as above. The cells were pelleted by centrifugation then resuspended and a sample of the cell suspension hydrodynamically injected into the capillary. Under these conditions, lysis of the cells occurred within the capillary as a result of mixing of the cells with the SDS-containing alkaline run buffer. Previous reports have demonstrated that bacterial cells can be lysed within 30 − 40 s of exposure to an SDS-alkaline media.[33] The electropherograms obtained from these experiments showed a large, but broad, peak from the Erk1-TC-expressing cells that co-migrated with the Erk1-TC standard, but no such peak was present in the wild-type cells (Fig. 4c) (n=4) . The broadening of the FlAsH labeled Erk1-TC peak under these circumstances was likely due to the relatively slow lysis of multiple cells over time as the sample plug mixed with the run buffer upon injection into the capillary. This peak broadening may be improved by reducing the volume of the injected sample and modifying the composition of the run buffer in order to achieve faster on-column cell lysis.

Separation of Multiple Biarsenical-TC Complexes

An advantage to the use of chemical separation techniques in the analysis of biarsenical-labeled peptides and proteins is the ability to analyze multiple species simultaneously. To demonstrate this potential, the three peptides TC-ABL, TC-FLN, and TC-HRW along with the protein Erk1-TC were labeled with FlAsh. The peptide and protein complexes were then co-mixed. A sample of this mixture was injected onto a capillary and separated by MEKC. The electropherogram revealed four major analytes and unlabeled FlAsH (Fig. 5) (n=3). Doublet peaks consistent with isomers of the biarsenical-TC complexes were also present. The identities of the peaks were determined in separate runs of the analytes in isolation and in various mixtures (data not shown). These data demonstrate the simultaneous identification of multiple biarsenical-TC-labeled species using a single fluorophore.

CONCLUSION

The biarsenical-TC labeling method has been an important contribution to microscopic studies of cell biology. In the present work, we demonstrate that the biarsenical-TC complex is a useful fluorescent tag for CE as well. Studies with model peptides using LIF detection in a capillary demonstrated that the biarsenical tag remained fluorescent and bound to the TC motif during separations by MEKC. In most separations, the labeled analyte was seen as a doublet peak consistent with isomer formation as previously described.[16,17] Buffer conditions and sample source (purified reagent or cell lysate) were variables that impacted the ability to resolve the isomeric forms of the TC-FlAsH labeled analyte. The label also displayed zeptomolar detection limits under standard MEKC conditions. Experiments to assess the impact of this motif on phosphorylation of a peptide-based kinase substrate indicated that the small tag did not prevent the peptide from being phosphorylated by the kinase. Thus, this labeling method should prove useful for the growing number of CE-based biochemical assays utilizing peptides. New methods for fluorescent labeling of proteins continue to be developed, but to date remain limited by the requirement for protein-based tags or are restricted to the labeling of extracellular proteins.[34] The biarsenical-TC motif remains unique in its use for site-directed labeling of a small intracellular peptide motif by cell permeable small molecules. Thus, the successful demonstration of this fluorescence labeling scheme for analysis of proteins by CE is significant for chemical cytometry. It was shown that a genetically engineered protein containing the TC motif could be labeled in the cell, and the expressed protein could then be analyzed by CE in cell lysates or in cells sampled directly into the capillary. This approach provided a number of

advantages for the analysis of cellular proteins. Labeling of the target protein was stoichiometric and took place within the living cell; consequently, there was no need for more complex pre- or on-column labeling protocols. The unbound biarsenical dye could be separated from the labeled protein to avoid the problem of background fluorescence. The improved sensitivity of CE-LIF also enhanced the detection limits of a target protein compared with that of conventional gel electrophoresis techniques. Since the fluorescence of the biarsenical increased as soon as it bound to the TC motif, analysis could be performed soon after labeling without the need for a prolonged time period for maturation of fluorescence properties. The demonstration of the simultaneous identification of multiple species all labeled with the same biarsenical was an important advantage. In this regard is the potential of using the biarsenical system for quantitative dual color pulse chase experiments similar to those described by Tsien's group for microscopic analysis.[35] To investigate the dynamics of protein expression, a protein under control of a specific promoter and containing the tetracysteine motif could be employed for dual color labeling experiments. After activation of the promoter, treatment of the cells with one color of biarsenical dye, and then switching to a second color for a second period of time would identify those proteins made during the two time periods. Two-color CE analysis could then be used to accurately quantify the amount of protein made during each period of time. The implementation of chemical separations with or without dual-color labeling extends the potential for analyzing numerous targets within the cell by virtue of the resolving power of CE-based separations. The biarsenical-TC labeling scheme for analytical chemical separations can be expected to be of considerable value as a tool for biochemical and cell-based studies.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

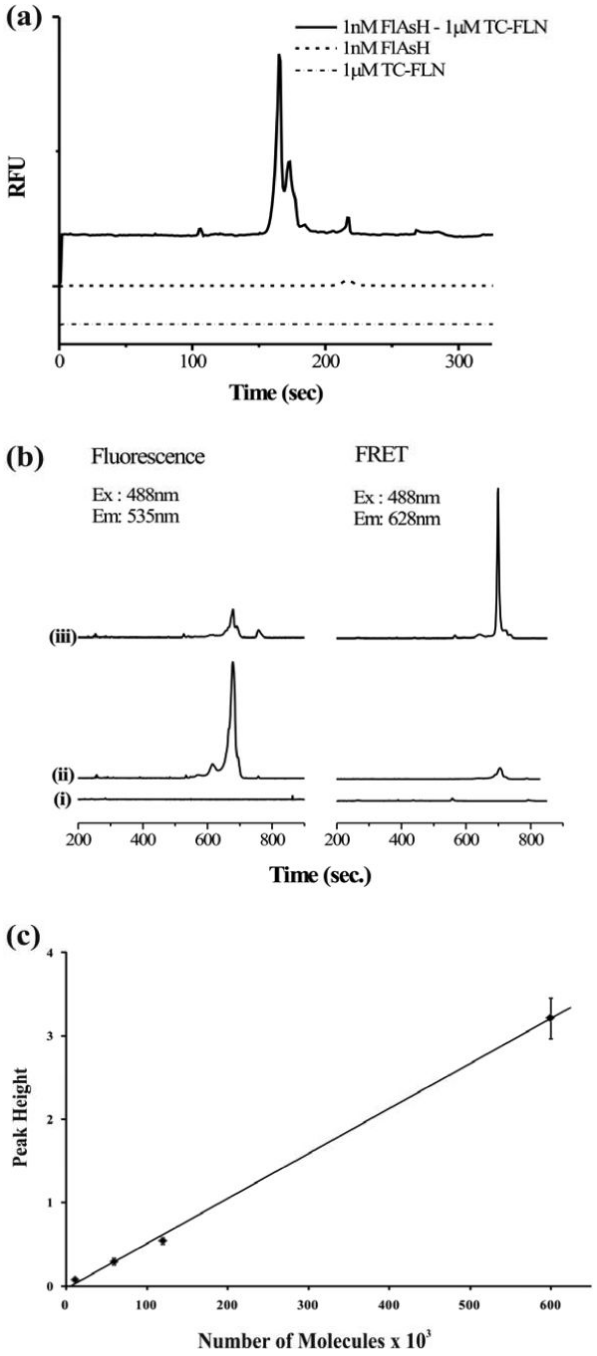

Figure 1.

CE-based analysis of biarsenical binding to the TC complex. (a) Shown are electropherograms of FlAsH, TC-FLN and the FlAsH/TC-FLN complex. The doublet peak seen in the FlAsH/TC-FLN complex is likely the result of isomer formation (see text). (b) Electropherograms of *i*) ReAsH, *ii*) TC-ABL-FAM, and *iii*) TC-ABL-FAM after incubation with ReAsH. Peptides were excited at 488 nm and emission was measured at 535 nm for fluorescence (left) and 628 nm for FRET (right). (c) Detection limits and linearity of the FlAsH/TC-FLN complex in a capillary are shown. The separations in (a - c) were conducted in a bare silica capillary; 50 μm i.d., effective length 19 cm with an overall length of 34 cm. The voltage was −10.2 kV

with a run buffer composed of: 1% SDS, 1 mM DTT and 0.1 M borate buffer, pH 8.5. Samples were injected hydrodynamically with a calculated injection volume of 2 nL.

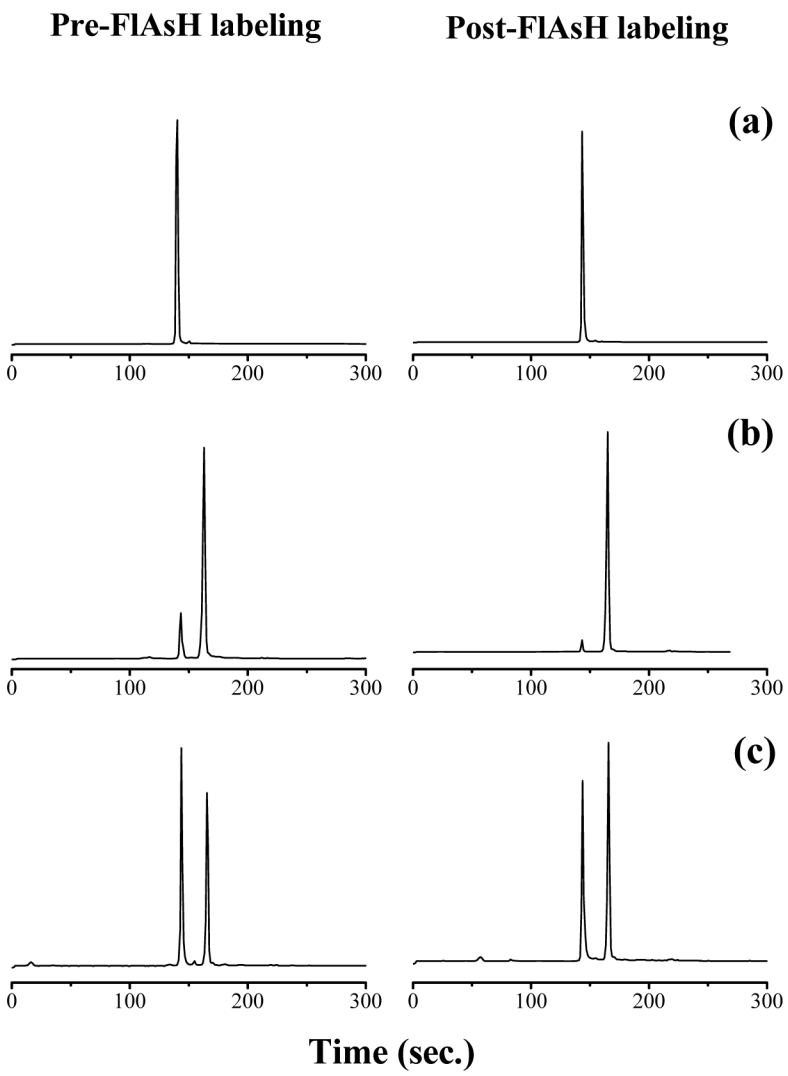

Figure 2.

Phosphorylation of FlAsH-labeled and unlabeled TC-ABL substrate peptide by ABL kinase. The electropherograms in the left column show the CE-LIF analysis of the reaction mixture when TC-ABL pre-labeled with FlAsH was the substrate. The right column shows the results of the assay when unlabeled TC-ABL was the substrate (see text). (a) TC-ABL; 2 h kinase reaction mixture without ATP. (b) TC-ABL; 2 h kinase reaction mixture with ATP. (c) Mixture of samples from “a” and “b”. CE conditions: run buffer; 0.1M tris-tricine, pH 8.2.

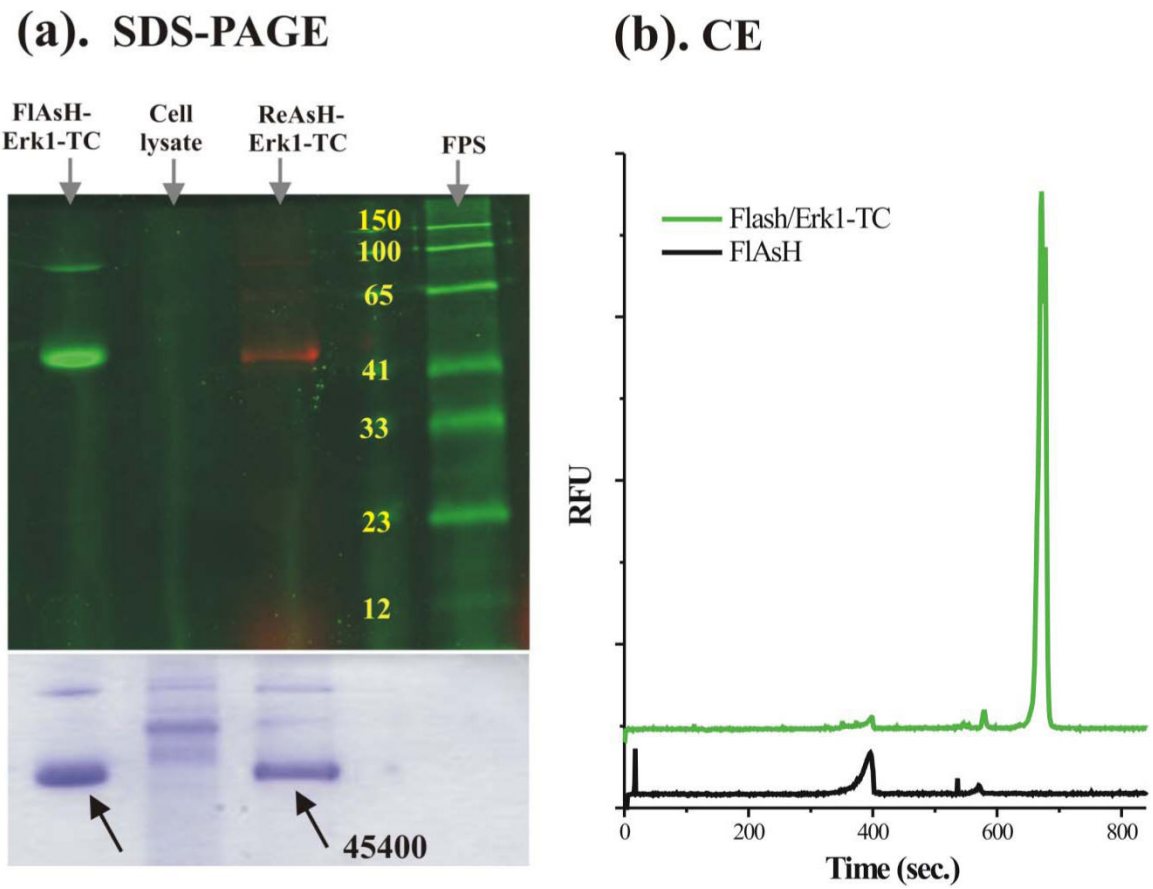

Figure 3.

Electrophoretic separations of FlAsH or ReAsH labeled recombinant protein Erk1-TC. (a) Shown are fluorescence scans (upper) and Coomassie blue stain (lower) of an SDS-PAGE gel separation of Erk1-TC labeled with FlAsH (lane 1) or ReAsH (lane 3). Lane 2 is the cell lysate from the non-transformed cells incubated with the biarsenical reagent. The fluorescence image is an overlap of two scans- *i*) em/ex 488 nm/535 nm (green), and *ii*) ex/em 633 nm/640 nm (red). The fluorescent protein standards (lane 5) were too low in concentration to be seen in the Coomassie blue stained gel as they were optimized for the fluorescence scan. In the Coomassie stain, arrows indicate the bands for the recombinant protein. (b) CE-based separation of FlAsH-labeled Erk1-TC. Run buffer: 5% SDS, 1 mM DDT, 0.1 M borate buffer, pH 8.5; −250 V/cm; LIF detection: ex/em 488 nm/535 nm.

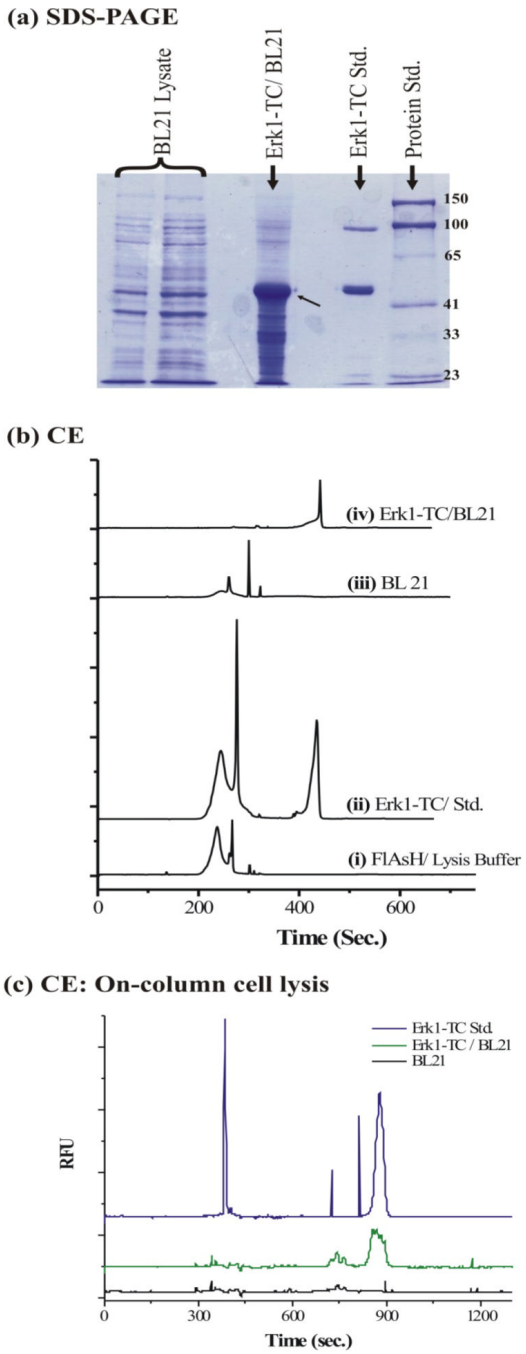

Figure 4.

Intracellular labeling with FlAsH of Erk1-TC expressed in BL21 cells. (a) Coomassie blue stained SDS-PAGE gel. Arrow indicates band of expected M.W. for Erk1-TC. Lanes contain (from left to right) wild type BL21, Erk1-TC expressing BL21, Erk1-TC standard, and protein standard. (b) Shown are the electropherograms from the CE analyses of standards and cell lysates (n=3): i) FlAsH in lysis buffer; ii) FlAsH labeled Erk1-TC standard; *iii*) cell lysate of wild-type BL21 pre-incubated with FlAsH; and *iv*) 100-fold dilution of cell lysate obtained from BL21 expressing Erk1-TC and pre-incubated with FlAsH. The separation conditions are the same as in Fig. 3, except that E = −300 V/cm. (c) On-column cell lysis and separation of the in-cell labeled Erk1-TC (n=4). Based on the known injection volume and cell density of

the sample, approximately 50 ng of the labeled cells were hydrodynamically loaded into the capillary. CE separation conditions were identical to those in Fig. 3.

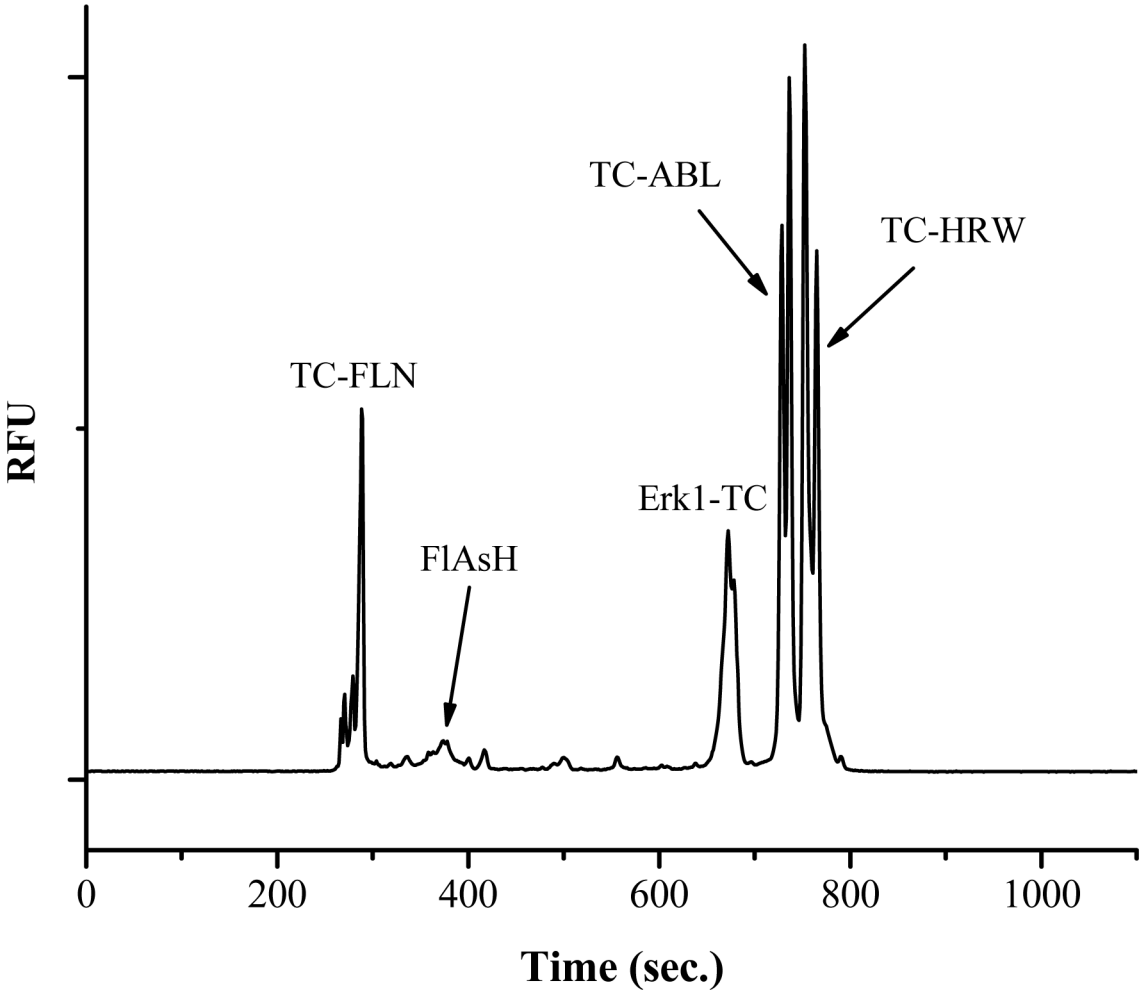

Figure 5.

Capillary electrophoresis of FlAsH labeled protein and peptides (n=3). Purified analytes were labeled with the biarsenical (10 μM TC-peptide or TC-protein incubated with 10 nM FlAsH) and then mixed. Doublet peaks are isomers of the biarsenical-TC complex (see text). Run buffer: 5% SDS, 1 mM DDT, 0.1 M borate buffer, pH 8.5; −250 V/cm; LIF detection: ex/em 488 nm/535 nm.

Table 1

Peptides and Proteins with a Tetracysteine Motif

Name	Sequence
TC-FLN	FLNCCPGCCMEP
TC-HRW	HRWCCPGCCKTF
TC-ABL	GCCPGCCG-*PEG6*-EAIYAAPFAKKK
TC-ABL-FAM	GCCPGCCG-*PEG6*-EAIYAAPFAKKK-*FAM*
Erk1-TC	Erk1-LNCCPGCCMEP