

Published in final edited form as:

*Bioconjug Chem.* 2011 July 20; 22(7): 1330–1336. doi:10.1021/bc100560c.

## Rapid Identification of Fluorochrome Modification Sites in Proteins by LC ESI-Q-TOF Mass Spectrometry

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### Abstract

Conjugation of either a fluorescent dye or a drug molecule to the  $\epsilon$ -amino groups of lysine residues of proteins has many applications in biology and medicine. However, this type of conjugation produces a heterogeneous population of protein conjugates. Because conjugation of fluorochrome or drug molecule to a protein may have deleterious effects on protein function, the identification of conjugation sites is necessary. Unfortunately, the identification process can be time-consuming and laborious; therefore, there is a need to develop a rapid and reliable way to determine the conjugation sites of the fluorescent label or drug molecule. In this study, the sites of conjugation of fluorescein-5'-isothiocyanate and rhodamine-B-isothiocyanate to free amino groups on the insert-domain (I-domain) protein derived from the  $\alpha$ -subunit of lymphocyte function-associated antigen-1 (LFA-1) were determined by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS) along with peptide mapping using trypsin digestion. A reporter fragment of the fluorochrome moiety that is generated in the collision cell of the Q-TOF without explicit MS/MS precursor selection was used to identify the conjugation site. Selected ion plots of the reporter ion readily mark modified peptides in chromatograms of the complex digest. Interrogation of these spectra reveals a neutral loss/precursor pair that identifies the modified peptide. The results show that one to seven fluorescein molecules or one to four rhodamine molecules were attached to the lysine residue(s) of the I-domain protein. No modifications were found in the metal ion-dependent adhesion site (MIDAS), which is an important binding region of the I-domain.

### Introduction

Proteins (e.g., antibodies) have been modified with fluorochromes with high quantum yields such as fluorescein-5'-isothiocyanate (FITC) or rhodamine-B-isothiocyanate (RITC) to maximize the detection sensitivity in binding assay or cell imaging (1). The isothiocyanate group on the fluorescent dye is reacted with the primary amines and thiol groups of proteins to yield thiourea or dithiourethane adducts (2). Frequently, the reaction product is a

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#### Supporting Information Available

The sequence of LFA-1 I-domain protein, chemical structures of RITC and RTC-I-domain, deconvoluted mass spectrum of RTC-I-domain, ion chromatograms of tryptic digest of RTC-I-domain, and an example of a processed spectra. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

heterogeneous mixture of conjugates with different number of fluorochromes attached to the protein. It may be worthwhile to determine the modification sites because the conjugation of fluorochrome on the protein-binding site may alter protein affinity to its receptors. Unfortunately, this task can be challenging and laborious because of sample heterogeneity and low abundance of the modified residues. In this study, a rapid and reliable method was evaluated to determine the site(s) of conjugation of FITC and RITC groups on the insert-domain (I-domain) protein derived from the  $\alpha$ -subunit of lymphocyte function-associated antigen-1 (LFA-1) receptor.

T-cell adhesion to vascular endothelial cell is mediated by LFA-1 and intercellular adhesion molecule-1 (ICAM-1) interactions via the I-domain of LFA-1 to the domain-1 (D1) of ICAM-1. I-domain interacts via the metal ion-dependent adhesion site (MIDAS) to a glutamate residue of D1 of ICAM-1 (3–5). In autoimmune diseases such as multiple sclerosis (6–8), rheumatoid arthritis (9), type 1 diabetes (10), and lupus (11), the cell surface expression of ICAM-1 is upregulated on different types of cells. Thus, there is a possibility of conjugating drug molecules to the I-domain protein. The resulting drug-I-domain conjugate can be used to target cells with upregulated ICAM-1 expression to lower the side effects of the drugs. To evaluate the utility of the I-domain for targeting the upregulated ICAM-1, the I-domain was conjugated with FITC and RITC at lysine residues and the N-terminus (Figure 1 and supporting information Figure S2) to produce the FTC- and RTC-I-domains, respectively. FTC- and RTC-I-domains have been shown to bind ICAM-1 and are also transported into the intracellular space of leukocytes. Because the I-domain has 20 lysine residues and modification of certain lysine residues could influence the binding and cellular uptake of I-domain conjugates, it is important to determine the sites of modification on the I-domain. The conjugation reaction was optimized to introduce an average of three-to-four fluorescein molecules per I-domain molecule or two-to-three rhodamine molecules per I-domain molecule.

A combination of tryptic digestion and mass spectrometry was used to identify the conjugation sites of the fluorophores because it is a sensitive and flexible method for the characterization of modified peptides. The thiocarbamoyl modification of proteins has been detected by mass spectrometry since the early use of soft ionization such as thermospray, or chemical ionization, to detect phenyl thiohydantoin (PTH) derivatives of amino acids, the result of phenyl isothiocyanate (PTC) coupling to amines (12). While the focus of this early work was differentiating between the amino acids in the classic Edman degradation, the spectra also presented a “reporter ion” of the modifying group. The related reporter ion from isothiocyanate (ITC) dyes is readily generated in an electrospray ionization source with cone fragmentation or in the collision cell of quadrupole time-of-flight (ESI-Q-TOF) instrument. A strategy of using fragmentation in the collision cell of a Q-TOF without explicit MS/MS has been exploited in the fatty acid analysis of complex lipids (13) and provides the fundamental data used in so called MSE, or high and low energy off sets of the collision cell on alternation scan cycles by WATERS for proteomics (14). This data acquisition strategy for generating fragmentation information has expanded to metabolite identification (15) and quantitative proteomics (16). A description of a data reduction algorithm for chromatographic time alignment to determine precursor/fragments relationship in MSE data (17) can be instructive in appreciating a MSE data set. In this study, we initially employed high and low energy cycles on the collision cell of a Q-TOF, the usual MSE acquisition method, and observed that at a low to intermediate energy a reporter ion for the dye and corresponding neutral loss dominated fragmentation in spectra. The classic uses of reporter ions in structure determination include amino acid indicating from peptides (18), neutral loss of hexose groups from glycopeptides (19–21) and phosphate from phosphorylation (22–25). All these aid rapid identification of modification sites. Previously, Schnaible and Przybylski demonstrated the possibility of the identification of fluorescent dye-modified sites by

increasing the declustering potential (so called “cone” fragmentation) in the electrospray ion (ESI) source to generate a reporter ion from the dye on a peptide (26). This specific fragmentation was utilized for assigning the fluorescent dye modification sites in peptides obtained from enzymatic digestion of fluorescein-modified hen egg white lysozyme (6). This work is an adaptation of emerging method of using a Q-TOF for improved information content during LC/MS with a MSE like approach. We used ESI-Q-TOF MS to readily generate reporter ions in the collision cell of the Q-TOF and, along with LC/MS, expand the earlier observations of Schnaible and Przybylski to assess the fluorochrome distribution and determine the fluorescent dye conjugation sites in the modified I-domain after trypsin digestion.

## Experimental Procedures

### Materials

FITC isomer I, RITC mixed isomers, solvents, and reagents of highest available purity were purchased from Sigma-Aldrich (St. Louis, MO). Sequence-grade modified trypsin was purchased from Promega (Madison, WI).

### Peptide synthesis and purification

The model peptides cyclo(1,12)Pen-ITDGEATDSGC (cLABL) and cyclo(1,12)Pen-PRGGSVLVTGC (cIBR) were synthesized as linear peptides by standard Fmoc solid-phase peptide chemistry using the automated peptide synthesis system (Pioneer™ PerSpective Biosystems, Framingham, MA) as described elsewhere (27). The sequences of cLABL (ITDGEATDSG) and cIBR (PRGGSVLVTG) peptides were derived from the LFA-1 I-domain and domain-1 (D1) of ICAM-1 proteins, respectively. LFA-1 I-domain serves as a ligand for ICAM-1 receptor. The model peptides also showed that the dye modified peptide were hydrophobic. The product from synthesis was purified by HPLC followed by cyclization and purification as previously described (27). The molecular weight of the peptide was confirmed by electrospray ionization mass spectrometry ( $M + H^1 = 1197.3$  for cLABL and 1174.5 for cIBR).

### Fluorochrome modification of the peptides

Conjugation of FITC with cLABL was carried out using the procedure previously published by our laboratory (28). Briefly, pure peptide (0.04 mmol) was dissolved in 5 mL Nanopure water followed by addition of FITC (0.08 mmol). The pH of the solution was adjusted to 10 with a 1.0 N NaOH solution and it was stirred for 1 h. Then, the reaction mixture was neutralized by the addition of a 10% v/v acetic acid solution followed by lyophilization. The lyophilized crude peptide was then purified by semi-preparative  $C_{18}$  reversed-phase HPLC. The pure fractions of the peptide were pooled and lyophilized. The lyophilized FITC-peptide was analyzed by analytical  $C_{18}$  reversed-phase HPLC for its purity, and its identity was confirmed by ESI-MS ( $M + H^1$ ). Conjugation of rhodamine with cIBR was done in a manner identical to that described above with the exception that FITC was replaced by RITC.

### LFA-1 I-domain protein expression and fluorochrome modification

The LFA-1 I-domain protein (residues 128–307, with an addition of N-terminal methionine) was expressed, refolded, and purified as previously described (29). The sequence of the I-domain protein is provided in the supporting information (Figure S1). Briefly, the expression vector pET-11d containing the gene of LFA-1 I-domain<sub>128–307</sub> was used in *E. coli* BL21 (Stratagene, La Jolla, CA) competent cells, where the protein was found in

inclusion bodies, which were subsequently solubilized in guanidine-HCl and refolded by dilution (29). The identity of the pure protein was confirmed by mass spectrometry.

The procedure for conjugation of fluorescent dyes (FITC/RITC) with the I-domain protein was adopted from the previously published method with some modifications (1). To a total of 6.0 mg of the I-domain in PBS containing 10 mM MgSO<sub>4</sub>, a one-fourth volume of 1.0 M NaHCO<sub>3</sub>-Na<sub>2</sub>CO<sub>3</sub> buffer at pH 9.0 was added. FITC (5 mg/mL in DMSO) was added to the protein solution to a 25-fold molar excess over the I-domain protein with a final concentration of 1.5 mg/mL. The reaction was carried out for 2 h at 25 °C in the dark with constant stirring. The reaction was quenched with addition of 0.1 N HCl. Separation of the free dye from dye-coupled protein was done using a Superdex 200 size-exclusion column. The fractions belonging to the conjugated protein were collected and concentrated using an ultrafiltration device. The concentrated protein was filtered using a 0.2-micron filter and stored at 4 °C. Modification of I-domain with rhodamine was done in a manner identical to that described above with the exception that a 25-fold molar excess FITC was replaced by a 20-fold molar excess of RITC. The number of FITC or RITC groups conjugated to the I-domain protein was determined by electrospray mass spectrometry.

### **In-gel tryptic digestion and LC ESI-Q-TOF mass spectrometry analysis**

The protein (100 µg) was separated by SDS-PAGE and stained with Coomassie blue. The stained gel spot of interest was excised and cut into smaller pieces for better solvent penetration into the gel. Then, the gel pieces were transferred to a clean microcentrifuge tube, washed twice with a solution containing a 1:1 mixture of acetonitrile and 50 mM ammonium bicarbonate buffer at pH 8.0 for 45 min at 37 °C with gentle agitation. After discarding the washing solution, the gel pieces were shrunk with 250 µL of neat acetonitrile. After 10–15 min the residual solvent was discarded, and the gel pieces were dried completely. The gel pieces were re-swelled with 40 µL of digestion buffer A (0.2 M ammonium bicarbonate buffer at pH 8 and 5 mM CaCl<sub>2</sub>) containing 2 µg trypsin. Then, 50 µL of the digestion buffer B (0.2 M ammonium bicarbonate buffer at pH 8 and 10% acetonitrile) was added to keep the gel pieces immersed throughout the digestion. The digestion was carried out overnight at 37 °C, and the reaction was stopped by the addition of 0.1% formic acid. The gel pieces were sonicated and the supernatant containing the digested peptides was collected as primary sample fraction and analyzed by LC ESI-QTOF MS.

All HPLC separations were performed with a Water Acquity solvent delivery system using a binary gradient of solvent A composed of 98.92:1:0.08 H<sub>2</sub>O/acetonitrile/formic acid (vol/vol/vol) and solvent B containing 98.92:1:0.08 acetonitrile/H<sub>2</sub>O/formic acid (vol/vol/vol). The primary sample fraction (20 µL) containing the digested peptides was separated on a C<sub>4</sub> (5 cm × 1 mm i.d.) reversed-phase HPLC column (VM5C4W, packed by Micro-Tech Scientific, Vista, CA) with a linear gradient from 10% to 70% B in 60 min with a flow rate of 100 µL/min followed by a wash and re-equilibration step. Furthermore, the HPLC system was coupled online to the electrospray source of a Q-TOF-2 mass spectrometer (Micromass UK Ltd., Manchester, UK). Mass spectra were acquired with instrument cone voltage 35 eV, collision energy 10 eV with Ar in the collision cell. The instrument was set up such that ESI-MS spectra were acquired in positive reflector mode with a scan time of 6 s and in the mass range of 200–3000. The instrument was calibrated using NaI.

### **Analysis of the peptide fragments**

The reporter ion chromatograms (*m/z* of 390.0 ± 0.1 Da for FITC and 500.2 ± 0.1 Da for RITC) were processed to obtain the molecular weights of the modified peptides using MaxEnt3 in the MassLynx V4.1 software (Micromass UK Ltd.) to reduce the spectral complexity to single charge representation. A theoretical list of all the possible peptides

(including missed cleavages) as products of tryptic digestion and their corresponding masses was generated using BioLynx. Under each peak of the reporter ion we interrogated for the missed cleavage peptide that was in the theoretical list. To this we added the molecular weight of FITC (389.0) or RITC (500.2) to identify the modified peptide.

## Results

### Analysis of I-domain conjugates by ESI-MS

The I-domain protein was modified with fluorescent dye (FITC or RITC) in an aqueous buffer solution using a molar ratio of fluorescent dye:I-domain of 25:1 or 20:1 respectively, for 2 hrs at 25 °C at pH 9.0 as shown in Figure 1 (and supporting information Figure S2). Under such conditions, the isothiocyanate group of the fluorescent dye preferentially reacts with primary amino and thiol groups on proteins forming stable thiourea and dithiourethane adducts. Because the I-domain does not contain any cysteine residues, it is expected that only thiourea adducts are formed. After removal of the unreacted fluorescent dye by SEC, the number of conjugated FITC molecules was ascertained using ESI-Q-TOF MS. The deconvoluted spectrum shows seven prominent peaks (Figure 2). Each adjacent peak is separated by a mass difference of 388.0 Da to 390.0 Da with a mean of 389.0 Da. This consistent mass difference between the adjacent peaks in the spectra is due to the covalent link of the FITC group (molecular weight 389.0 Da) on the I-domain protein. The mass of the first peak in the series is 21,072.0 Da (1F, one fluorescein), which is associated with I-domain conjugated with one FITC molecule. The parent I-domain protein has a molecular weight of 20,682.0 Da (0F, no fluorescein). In this case, the associated error is less than 0.01%, which is typical for such mass measurements. The rest of the peaks can be assigned to the I-domain conjugated with two, three, four, five, six, and seven covalently linked FITC (2–7F) molecules, respectively. The spectrum also shows the disappearance of the peak for the parent I-domain (0F), indicating a complete conversion. A similar analysis done using RTC-I-domain shows 0–4 covalently linked RITC molecules (supporting information Figure S3).

### Analysis of model peptide conjugates by ESI-MS

Before analyzing the tryptic-digest products of I-domain conjugates, the fragmentation profile of model peptide-conjugated with fluorescent dye was first investigated using the Q-TOF. cLABL peptide was reacted with FITC at the free amino group to give FTC-cLABL and cIBR peptide was reacted with RITC to produce RTC-cIBR. After HPLC purification, pure FTC-cLABL or RTC-cIBR was used for ESI-MS analysis. Under the conditions employed, the presence of the reporter ion (i.e., free dye molecule) was detected. In this case, several fragments were detected, including the free dye (i.e., FTC,  $m/z = 390.0$  or RTC,  $m/z = 500.3$ ), the parent peptide fragment, and the modified peptide. The fluorescent dye demonstrated facile fragmentation at low energies readily generating reporter ions. Selected ion profiles of the dye reporter ions enabled the peptides to which the dyes are attached to be found chromatographically and the mass of the peptides was revealed in a fragment resulting from neutral loss of the dye. These results suggest the possibility of identifying the conjugation sites in the I-domain protein by tracking the modified and unmodified peptide fragments.

### Determination of conjugation sites in the I-domain

For determination of FITC conjugation sites, a classical peptide-mapping technique involving trypsin digestion and ESI-Q-TOF mass spectrometry followed by computer-facilitated data analysis of peptides was performed. It is well known that trypsin cleaves the C-terminal Lys and Arg residues; however, the modified Lys residues in the I-domain cannot be clipped by trypsin. Thus, the total ion chromatograms (TIC) from the tryptic

digest of FTC-I-domain and parent I-domain proteins were compared (Figure 3A–B). The TIC shows differences in the profiles, peak intensities, and retention times. For example, the increase in the retention time of the dye-modified peptides is due to the increase in hydrophobicity of the peptide fragment conjugate to the dye. Because of the large differences between the chromatograms of the tryptic digests of FTC-I-domain and I-domain, the identification of the conjugation sites using UV and/or fluorescence traces was laborious and time-consuming. Therefore, MS data were used to identify the modified peptides. During the analysis, the assumptions were that the modifications occurred at lysine residues and the expected mass of the uncleaved tryptic peptides increased by 390.0 Da for FTC-modified peptides and 500.2 Da for RTC-modified peptides. Interestingly, MS data of the dye-modified proteins showed a unique peak that could be assigned to the mass of the free fluorescent dye molecule (reporter ion with a mass of 390.0 Da or 500.3 Da), similar to that found in the model peptide analysis. Thus, the observed free dye molecule was derived from a specific peptide fragment upon cleaving the dye moiety (i.e., FTC or RTC) during collision-induced dissociation (CID).

Extracted ion chromatograms (EICs) of  $m/z$  390.0 Da (FITC) or of 500.2 (RITC) were generated from the full scan data with an error tolerance of 0.1 Da. A similar extraction was also done from unmodified protein profile (data not shown). The EIC of the FTC-modified protein shows several peaks that appeared between 7.2 and 30.0 min (Figure 3C), indicating that the amino group of the lysine side chain and/or the N-terminus of methionine could be modified in these peptides. Each of the peaks from the reporter ion chromatogram was processed using MaxEnt3 charge state deconvolution algorithm. The processed spectra were then searched for three ions: reporter ion (FTC), peptide fragment ion, and its corresponding FTC-modified peptide.

The ESI-MS spectrum of a peak eluting at 22.0 min from the tryptic-digest of FTC-I-domain is shown in Figure 4A. The deconvoluted spectrum shows three prominent ions (Figure 4B), including  $m/z$  390.0 Da (Figure 4A), which corresponded to the FTC moiety,  $m/z$  2056.7 Da, which correlated with the peptide fragment ion (T18-19;  $^{151}\text{FAS}\dots\text{FE}^{168}\text{K}$ ; Cal: 2057.1 Da), and  $m/z$  2445.7 Da, which corresponded to the modified FTC-peptide T18-19 ( $^{151}\text{FAS}\dots^{154}\text{K}\dots\text{FE}^{168}\text{K}$  or  $^{151}\text{FAS}\dots^{161}\text{K}\dots\text{FE}^{168}\text{K}$ ). The FTC-peptide T18-19 has modification either at  $^{154}\text{K}$  or  $^{161}\text{K}$ . In addition, the ESI-MS spectrum of another peak eluting at 28.0 min shows a fourth ion with  $m/z$  2834.6 Da, which corresponds to an FTC-peptide with modification at both the  $^{154}\text{K}$  and  $^{161}\text{K}$  residues. Peaks corresponding to the mass of 2445.7 Da and 2834.6 Da were found in the modified-peptide mapping profile but not in that of the unmodified I-domain profile, suggesting both  $^{154}\text{K}$  and  $^{161}\text{K}$  were indeed modification sites. Further, the ability to find the peptide T18-19 (Figure 3B) along with its FITC-peptide at the same retention time in the modified-peptide profile supports that the peak at 2056.7 Da is a result of fragmentation from its corresponding FITC-peptide. However, there are populations of protein that were not modified at both  $^{154}\text{K}$  and  $^{161}\text{K}$  residues. This is due to the observation of peptides T18 ( $m/z$  1210.4) and T19 ( $m/z$  865.3) derived from complete trypsin cleavage. We were not able to find the mass corresponding to the unmodified peptide ( $^{151}\text{FAS}\dots\text{FE}^{168}\text{K}$ ), 2056.7 Da, in the LC/MS profile of the unmodified I-domain as a result of trypsin cleavage at  $^{161}\text{K}$ , however we were able to find the smaller portions of the peptide, T18 and T19 (Figure 3A). Similar conclusions were drawn from the analysis of a peak eluting at 24.0 min from the tryptic-digest of RTC-I-domain as shown in the supporting information Figure S4 and S5.

In summary, a total of 8 and 6 modification sites were identified in the FTC-I-domain and RTC-I-domain, respectively. Tables 1 and 2 show a complete list of modified peptides obtained from the FTC-I-domain and RTC-I-domain. In all the identified peptides, the conjugation sites were assigned to the internal lysine residue except for peptide T1, where

the conjugation site was assigned to the primary amino group or the N-terminus of the first amino acid residue, methionine. Interestingly, most of the conjugation sites found in the RTC-I-domain were similar to those found in the FTC-I-domain.

## Discussion

The conjugation method adopted for the preparation of the I-domain-fluorescent dye conjugates yields samples with a heterogeneous distribution of fluorochrome groups covalently linked to the lysine residues. However, the reaction conditions have been optimized to maintain batch-to-batch reproducibility. Each batch consistently showed the same number of fluorochrome modifications per molecule of protein as determined by whole protein MS analysis and UV. Also, as a quality control tool, CD spectroscopy results showed reproducibility of the spectra of modified proteins.

The FTC-I-domain preparation contained one to seven linked FTC molecules (Figure 2), while the RTC-I-domain preparation contained one to four linked RTC molecules (supporting information Figure S3). Out of 21 modifiable residues (20 lysine + N-terminus methionine), there are eight sites modified by FTC and six sites modified by RTC (Tables 1 and 2). In addition, all of the identified sites were partially modified. There are possibly low abundant modification sites that cannot be detected during LC-MS analysis. The MS data analysis using the reporter ion can identify almost all of the modified peptides in relatively short time. Also, such analysis can aid in identifying low-abundance modified peptides that fall within the detectable range of detecting either dye + peptide or the neutral loss peptide only ion. However, very low levels of modified peptides were flagged by the presence of the reporter ion but precursor peptides mass were inconclusive. Using single intermediate collision energy, compared to the low/high energies used in MSE, simplified the data reduction such that only one set of spectra required interrogation. This study shows a combination of tryptic digest and LC ESI-MS with elevated collision energy in the cell of a Q-TOF analysis is proven to be a powerful and reasonably rapid technique in identifying the modification sites.

Fluorescent dye labeling of the I-domain protein serves two main purposes. The first purpose is that the labeling of I-domain increases the sensitivity of detection of the protein during *in vitro* binding studies. It is hoped that conjugation with the fluorescein and rhodamine does not alter the structure and binding site of the I-domain. Secondly, characterization of the modification sites provides an idea regarding the reactivity and availability of certain lysine residues for conjugation with drug molecules within the I-domain. Neither of these goals may be accomplished if the residues at the MIDAS region and the structure of I-domain have been altered to make the molecule inactive.

Our results indicate that FTC-moieties are found at lysine residues away from the MIDAS region (Figure 5). In a separate study, the FTC-I-domain has been shown to bind to ICAM-1 on Raji cells, indicating the conjugation of the I-domain with fluorochrome does not alter its binding properties to ICAM-1. The binding properties of FTC-I-domain to ICAM-1 on the cell surface are concentration and temperature dependent. Binding of FTC-I-domain to its receptor suggests structural competence. Recently, the I-domain has also been conjugated to antigenic peptides for targeting to antigen-presenting cells (APC) for controlling T-cell differentiation in autoimmune diseases. The antigen-I-domain conjugate can suppress the progress of experimental autoimmune encephalomyelitis (EAE) in the mouse model, a model for multiple sclerosis. The results from the biological studies suggest that I-domain can be used to target drug molecules to ICAM-1-expressing cells.

In conclusion, the reporter ion quickly allowed identification of peaks modified with peptides in a complex peptide chromatogram. While this approach may be similar to the cone fragmentation study (26) the activation in the collision cell provided superior data quality. The observation of reporter ion and the peptide fragment observed as a neutral loss suggests ion trap instruments using a data dependant neutral loss strategy can also rapidly screen digests for these types of dyes. This method can be extended to determine the sites of conjugation of drug molecules or antigenic peptides on the I-domain or other proteins.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

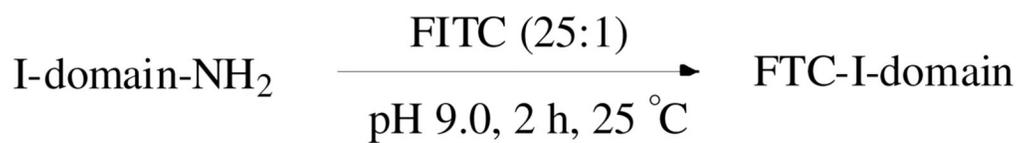
This work is supported by the National Institutes of Health (R01-AI-063002 and R56 AI-063002), National Multiple Sclerosis Society, and Institute for Advancing Medical Innovation, University of Kansas Cancer Center. We thank Amgen Inc. Predoctoral Fellowships to Prakash Manikwar. The Q-ToF2™ was purchased with support from KSTAR, Kansas administered NSF EPSCoR and the University of Kansas. The Acquity UPLC was purchased with partial support from K-INBRE (www.kumc.edu/kinbre/) and Protein Lynx Global Server software was obtained with support from KCALSI (www.kclifesciences.org). We also thank Nancy Harmony for proofreading the manuscript.

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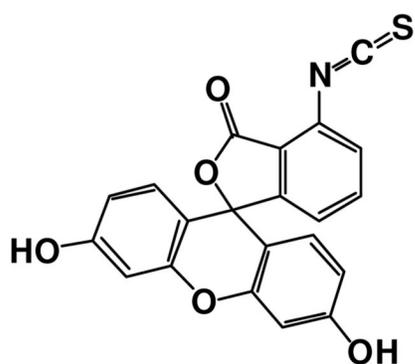
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A



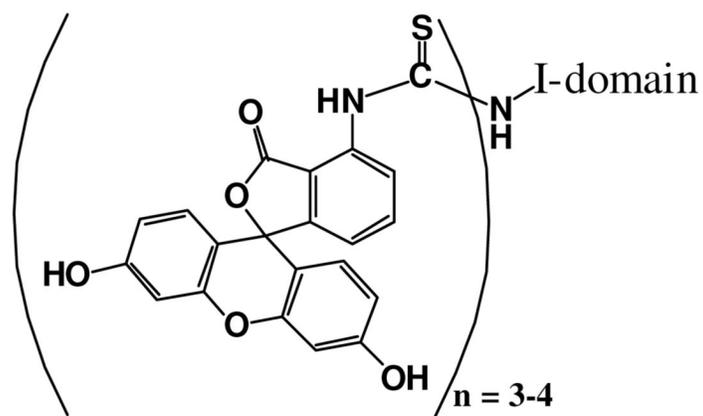
B



Fluorescein-5'-isothiocyanate  
(FITC)

Exact Mass: 389.0

Molecular Weight: 389.4

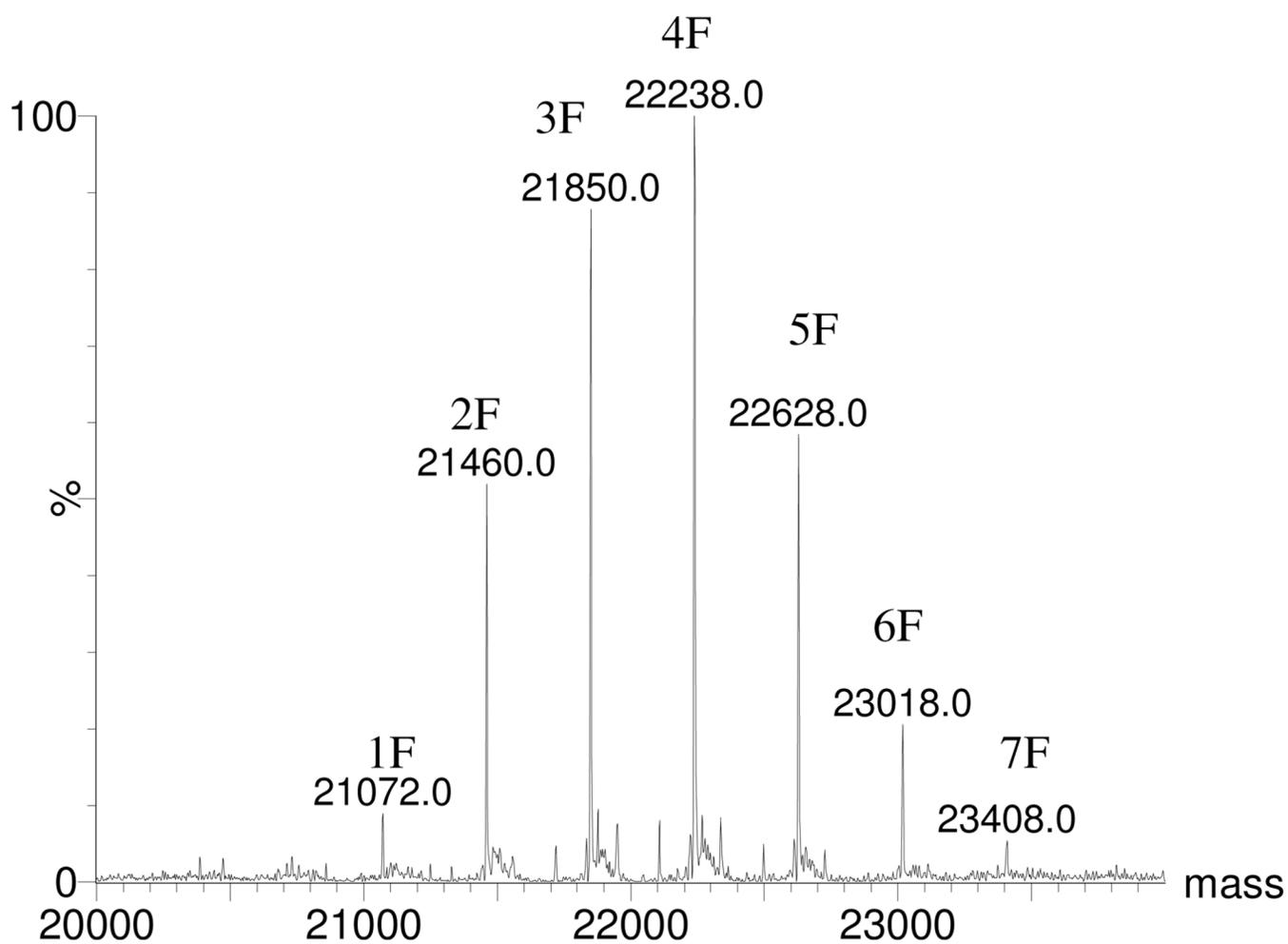


Fluorescein-5'-thiocarbamoyl (FTC)-  
modified I-domain

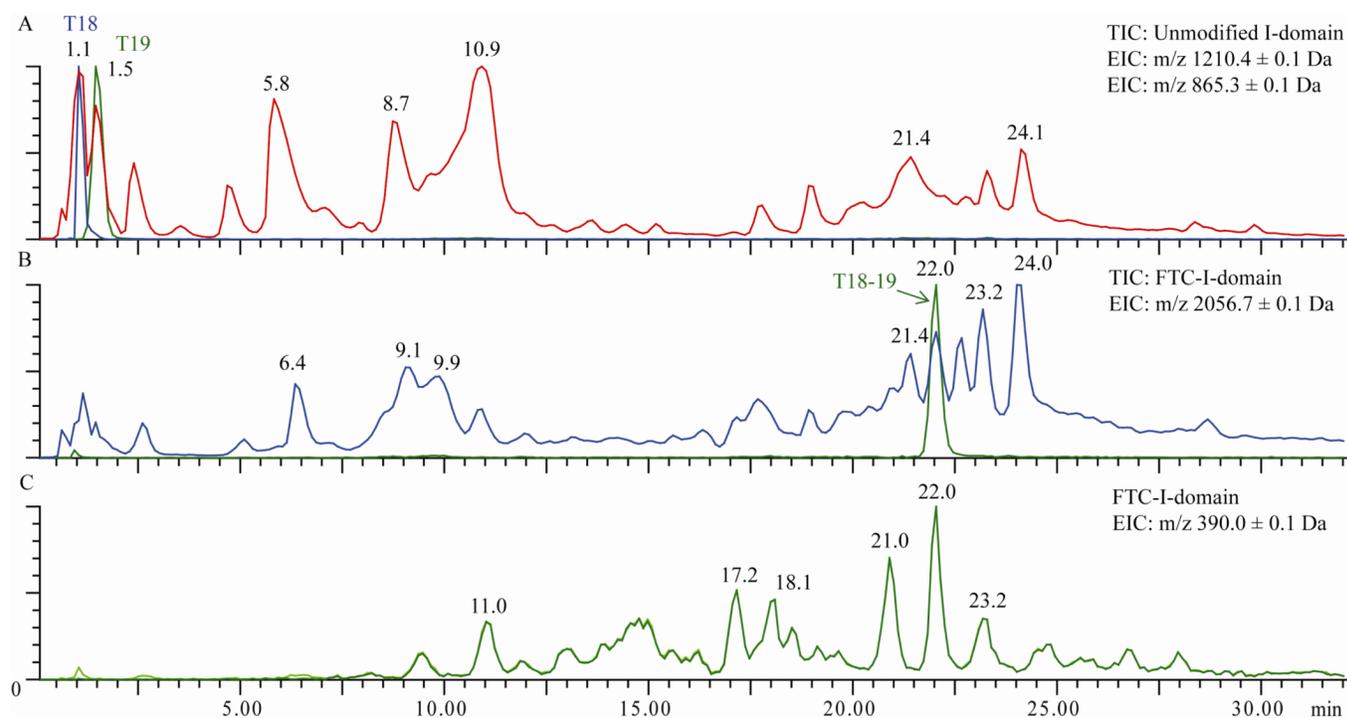
**Figure 1.**

(A) Schematic representation of a single-step modification of I-domain protein with FITC.

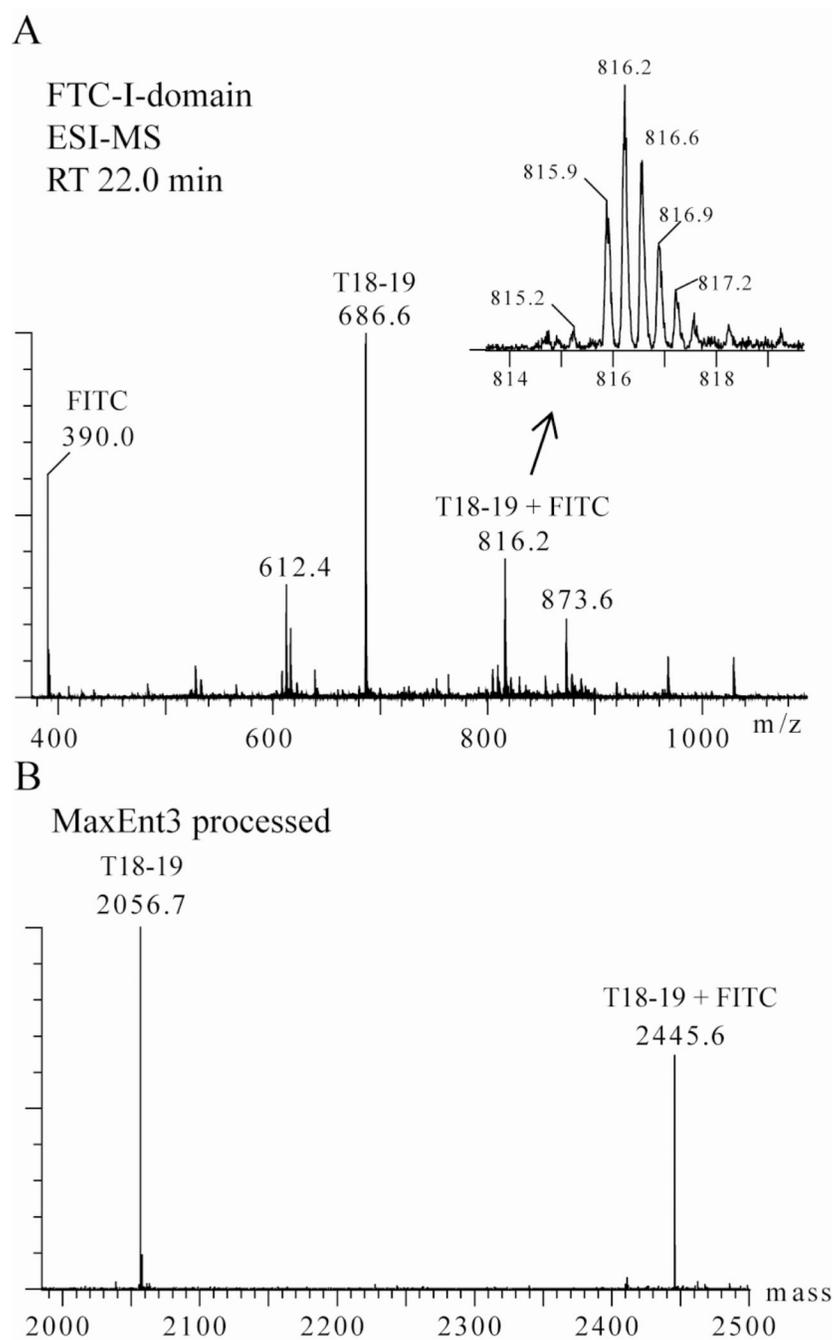
(B) Illustration of chemical structures of FITC and FTC-I-domain.



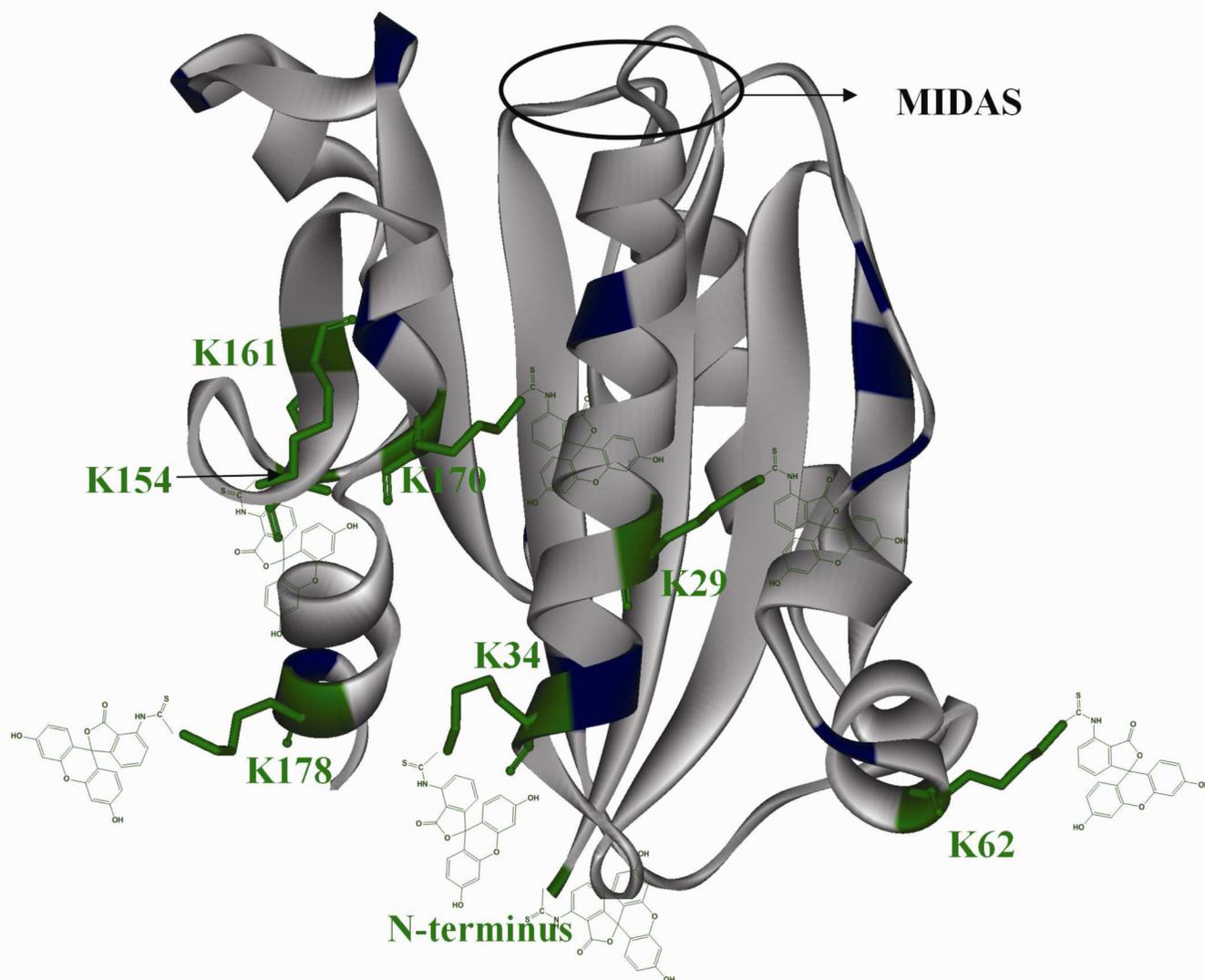
**Figure 2.** Deconvoluted mass spectrum of FTC-I-domain. The modification of one FITC molecule to either lysine or the N-terminus amino group increases the theoretical mass of the I-domain protein by 389.0 Da. F, is the number of FITC molecules conjugated.



**Figure 3.** The ion chromatograms of tryptic digest of unmodified I-domain and FTC-I-domain. **(A)** The total ion chromatogram (TIC) of unmodified I-domain superimposed with the extracted ion chromatograms (EIC) of the peptide T18 ( $m/z$  1210.4  $\pm$  0.1 Da) in blue and T19 ( $m/z$  865.3  $\pm$  0.1 Da) in green. **(B)** The TIC of FTC-I-domain superimposed with the EIC of the peptide fragment T18-19 ( $m/z$  2056.7  $\pm$  0.1 Da) in green. **(C)** The EIC of the reporter ion, indicating all components whose spectra contain an ion of  $m/z$  390.



**Figure 4.** The ESI-MS and MaxEnt3 processed spectra of the peak eluting at 22.0 min derived from the LC-MS analysis of the tryptic digests of FITC labeled I-domain showing the MW of the parent compound and the fragments representing the peptide and the fluorescent dye. **(A)** The ESI-MS of the peak eluting at 22.0 min derived from the LC-MS analysis of the tryptic digests of FTC-I-domain, shows the reporter ion (FITC) and the inset shows the spectrum of FITC modified T18-19 peptide. **(B)** The MaxEnt3 processed spectrum of peak eluting at 22.0 min derived from the ESI-MS spectra of the tryptic digests of FTC-I-domain showing the tryptic digest of the peptide fragment and the FITC labeled peptide.



**Figure 5.** The X-ray structure of I-domain (PDB code 1ZON). The residues in green are the FITC modification sites and the residues in blue are the unmodified lysine modification sites. The modified lysine residues and the N-terminus are labeled. The protein images were created using Accelrys DS Visualizer 1.7.

**Table 1**

FITC modification sites in the FTC-I-domain detected in peptides from trypsin digestion

| Modified peptide | Sequence   | RT (min) | Modified sites  |
|------------------|--|----------|-----------------|
| T1               | <sup>1</sup> MGNVDLVFLFDGMSLQDEFQ <sup>23</sup> K              | 28.0     | 1M (N-terminus) |
| T2-3             | <sup>24</sup> ILDFM <sup>K</sup> DVM <sup>33</sup> K           | 21.0     | 29K             |
| T4-5             | <sup>34</sup> KLSNTSYQFAAVQFSTSY <sup>52</sup> K               | 17.5     | 34K             |
| T6-7             | <sup>53</sup> TEFDFSDYV <sup>K</sup> <sup>63</sup> R           | 18.1     | 62K             |
| T18-19           | <sup>151</sup> FASKPASEFV <sup>K</sup> ILDTFE <sup>168</sup> K | 22.0     | 154K, 161K      |
| T20-21           | <sup>169</sup> L <sup>K</sup> DLFTELQ <sup>178</sup> K         | 19.7     | 170K            |
| T21-22           | <sup>171</sup> DLFTELQ <sup>K</sup> <sup>179</sup> K           | 17.2     | 178K            |

**Table 2**

RTC modification sites in the RTC-I-domain detected in peptides from trypsin digestion

| Modified peptide | Sequence   | RT (min) | Modified sites  |
|------------------|--|----------|-----------------|
| T1               | <sup>1</sup> MGNVDLVFLFDGMSLQDEFQ <sup>23</sup> K                          | 29.2     | 1M (N-terminus) |
| T2-3             | <sup>24</sup> ILDFM <sup>K</sup> DVM <sup>33</sup> K                       | 22.2     | 29K             |
| T4-5             | <sup>34</sup> KLSNTSYQFAAVQFSTSY <sup>52</sup> K                           | 18.9     | 34K             |
| T15-16           | <sup>131</sup> YIIGIG <sup>K</sup> HFQT <sup>142</sup> K                   | 23.1     | 137K            |
| T18-19           | <sup>151</sup> FASKPASEFV <sup>K</sup> ILD <sup>TFE</sup> <sup>168</sup> K | 24.0     | 154K, 161K      |