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Mass spectrometry-based carboxyl footprinting of proteins: Method evaluation

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ABSTRACT

Protein structure determines function in biology, and a variety of approaches have been employed to obtain structural information about proteins. Mass spectrometry-based protein footprinting is one fast-growing approach. One labeling-based footprinting approach is the use of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and glycine ethyl ester (GEE) to modify solvent-accessible carboxyl groups on glutamate (E) and aspartate (D). This paper describes method development of carboxyl-group modification in protein footprinting. The modification protocol was evaluated by using the protein calmodulin as a model. Because carboxyl-group modification is a slow reaction relative to protein folding and unfolding, there is an issue that modifications at certain sites may induce protein unfolding and lead to additional modification at sites that are not solvent-accessible in the wild-type protein. We investigated this possibility by using hydrogen deuterium amide exchange (H/DX). The study demonstrated that application of carboxyl group modification in probing conformational changes in calmodulin induced by Ca^{2+} binding provides useful information that is not compromised by modification-induced protein unfolding.

1. Introduction

Converting gene-sequence information into functional information about a protein is a major challenge of post-genomic biology [1]. Proteins have a variety of functions from serving as catalysts to acting as structural components; all these functions are closely related to protein structure. The first step to understand protein function is often a structural study of that protein. Two major approaches, NMR spectroscopy [2] and X-ray crystallography [3], can provide an atomic-level, 3D structural model of a protein. The applications of these high resolution approaches, however, can be limited by protein size and available quantity, conformational flexibility, existence in complex media, and aggregation propensity or presence in a protein complex [4]. To obtain complementary structural information about proteins, a variety of approaches from traditional structural biology (e.g., circular dichroism, fluorescence spectroscopy) to new advances (e.g., computational prediction, protein footprinting) are required [1].

Protein footprinting as a complementary approach to probing protein conformation has rapidly developed during the last two decades [5]. Different footprinting strategies include acetylation and other side-chain derivatizations, hydrogen deuterium amide exchange (H/DX), and hydroxyl-radical modification [6–9]. A labeling process induces a mass shift in the protein's molecular weight. The resulting shift makes mass spectrometry (MS) a suitable analytical tool for measuring the extent of protein footprinting and locating its site. Coupled with the labeling strategy, MS has greater sensitivity and speed than many other approaches in protein biochemistry. Several successful MS-based protein footprinting methods have been demonstrated for monitoring the dynamics of protein folding/unfolding [4,10,11], measuring protein–ligand binding affinity [12], locating the interface of protein–protein interactions, and probing protein structural information [13,14].

To analyze the outcome of protein footprinting by MS, the protein must be separated from a biological matrix. The matrix can be as simple as aqueous solution containing appropriate buffers and salts to as complex as a protein in a membrane as part of a complex assembly. The matrix is often essential for its activity and for maintaining its active conformation. Separation from matrix can affect the footprinting product if the labeling is reversible. Both MS-based H/DX and hydroxyl-radical footprinting are limited in some applications that require extensive post-labeling separation [15–17]. For example, MS-based H/DX requires a rapid separation to avoid the back exchange, and this requirement challenges the need to study a protein in a complicated biological system (e.g., embedded in a membrane). Extensive post-labeling separation and storage can induce a false readout in hydroxyl radical footprinting by adding additional oxidations [18,19]. Even with

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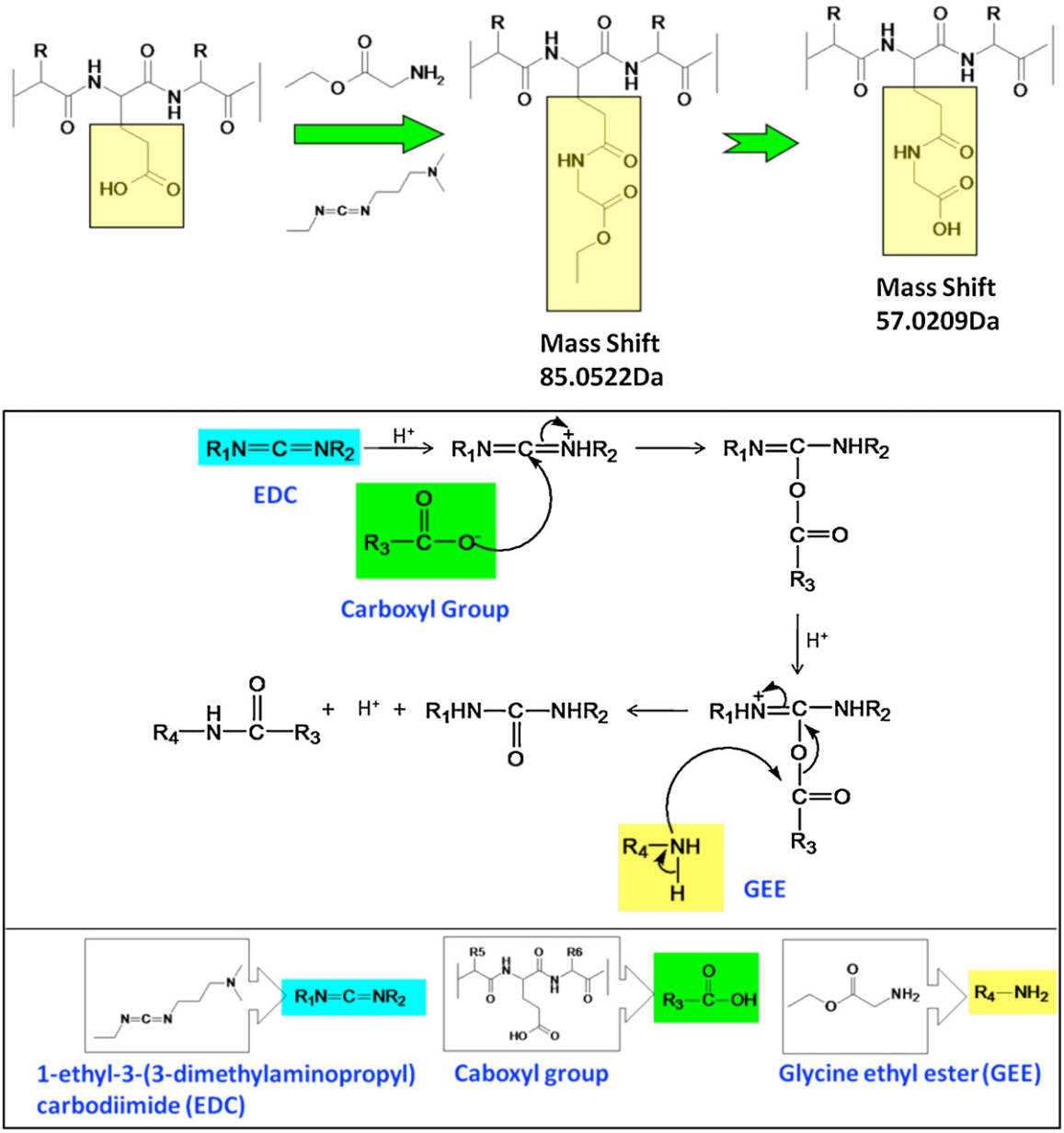

Fig. 1. Modification reaction by the GEE coupling reaction (top). A reaction mechanism of EDC-mediated GEE coupling is shown at the bottom.

the development of efficient separation techniques including UPLC [20,21], studies of complicated membrane proteins by MS-based H/DX and hydroxyl radical footprinting are difficult to conduct [15,17,22–24].

One applicable approach for specific, irreversible modification is the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide(EDC)-mediated coupling reaction between glycine ethyl ester (GEE) and the carboxyl group of a protein [7,25] (Fig. 1). The carboxyl groups of protein are activated by EDC (commonly used as a zero-length, cross-linking reagent), and then the nucleophilic modifying reagent, GEE, attacks the activated carboxyl group to yield the product. This reaction can lead to relatively rapid and quantitative modification of solvent-accessible carboxyl groups under mild conditions [25]. Any protein cross-linking by-products can be minimized in this process by providing excess GEE as the primary amide source for the nucleophilic attachment. The covalently labeled amide product and its hydrolyzed form are relatively stable in solution and can readily remain intact during extensive sample handling and separation.

A footprinting strategy based on modifying carboxyl groups appears appropriate because aspartate and glutamate side chains play important roles in electrostatic interactions, are often located on the surface of proteins, and are essential for enzymatic activities in cells. This GEE coupling reaction has been successfully used in probing the enzymatic activity of the mammalian polyamine transport system, pancreatic phospholipase, thymidylate synthase and cytochrome c oxidase [26–29].

Coupled with MS analysis, carboxyl-group modification can probe protein conformation. A previous study from us demonstrated the application of carboxyl-group modification in determining the membrane orientation of the FMO antenna protein in the green sulfur bacterial photosynthetic system from *Chlorobaculum tepidum* [30]. After footprinting, the modified FMO protein was separated and analyzed by MS. Information about FMO orientation

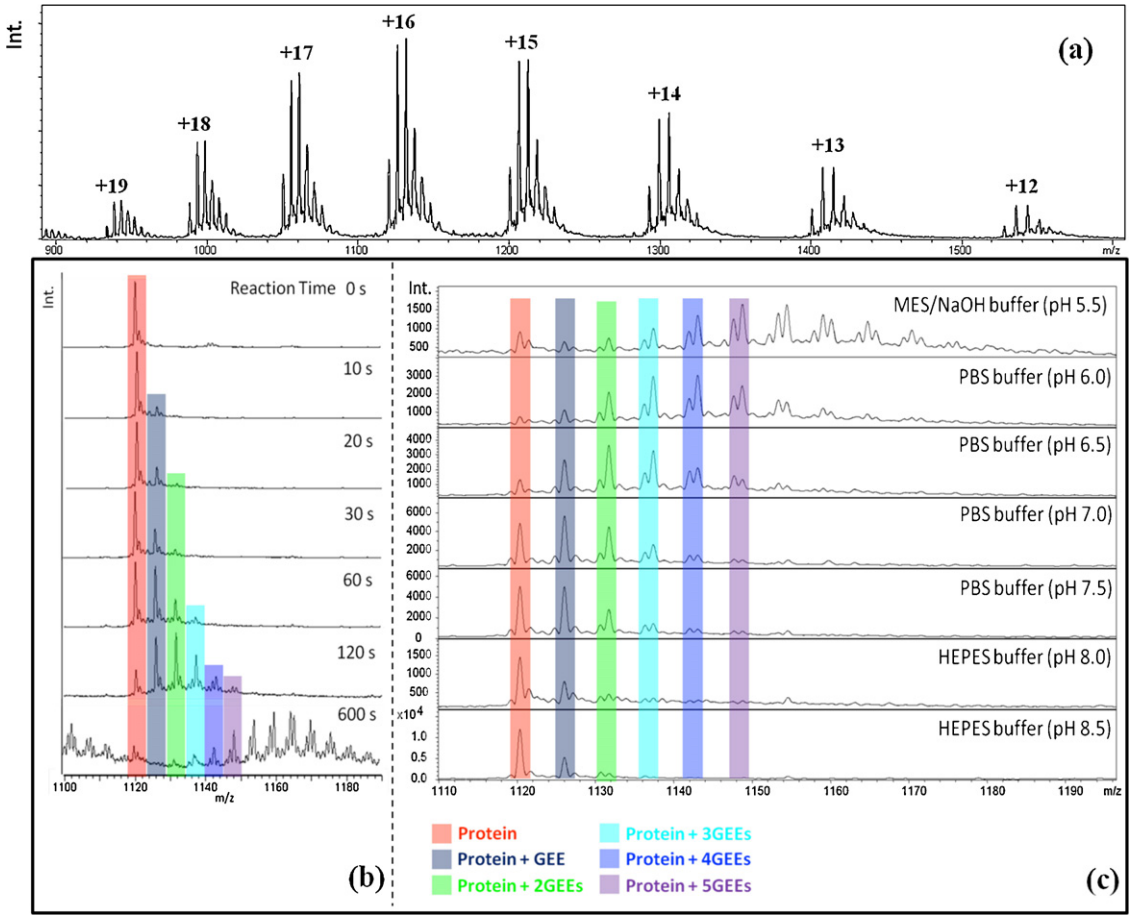

Fig. 2. Mass spectra of modified CaM samples. (a) Spectrum of modified CaM sample. (b) Mass spectra (+15 charge state) after modification of CaM samples at different reaction times ([CaM]:[GEE]:[EDC] = 1:20000:500) in PBS buffer. The unmodified protein state and each modified protein state are highlighted by different colors. (c) Mass spectra (+15 charge state) of modified CaM in different pH buffer systems were labeled in the same way by the carboxyl-group modification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in the membrane was elucidated by determining the modification extent at the peptide level by using MS. Recently, we also reported, in collaboration with Bose, the application of this MS-based protein carboxyl-group footprinting in the study of membrane associated tyrosine kinase dimerization [31]. Two advantages of carboxyl-group modification—it is specific to carboxyl groups of protein and results in a relatively stable modified product—make this approach attractive for the study of protein conformational.

Here, we describe a systematic method development and test of this protein-footprinting approach. The reaction rate of carboxyl-group modification was examined under different conditions. The protocol was specifically evaluated by using model proteins, calmodulin (CaM), β-lactoglobulin (BlG) and troponin C (TnC), and the approach we used should be appropriate for evaluating other new footprinting reagents. We used H/DX as a protein structure integrity check method during the carboxyl-group modification. We wish to confirm that carboxyl-group modification provides conformational information for aspartates and glutamates at the residue level in a manner that is reliable and complementary to the other protein footprinting methods.

2. Experimental

2.1. Chemicals and proteins

Calcium free calmodulin (CaM) from bovine and troponin C (TnC) from rabbit skeletal muscle were obtained from Ocean Biologics Co. (Edmonds, WA). Water, acetonitrile, calcium chloride, phosphate-buffered saline powder, MES (2-(N-morpholino)ethanesulfonic acid), HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), formic acid, glycine ethyl ester, EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, (ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid)(EGTA), ammonium acetate, urea solution (8 M), trifluoroacetic acid, β-lactoglobulin A from bovine milk (BlG), trypsin from porcine pancreas were all obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Protein sample preparations

The protein stock solution was prepared as 10 μM protein in 10 mM phosphate buffered saline (PBS buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4). The calcium-free CaM samples were prepared by incubating with EGTA as per a previously reported protocol [32].

2.3. Carboxyl group modification

Protein samples (10 μL) were mixed with 1 μL GEE (2 M in water) and 1 μL EDC (50 mM in water) to initiate the reaction at room temperature (22 °C). The reaction was quenched by adding 20 μL ammonium acetate (1 M). After modification, all samples were kept at 4 °C. In the pH-dependence experiments, protein samples in a variety of buffer systems, MES/NaOH (pH 5.5), PBS (pH 6,

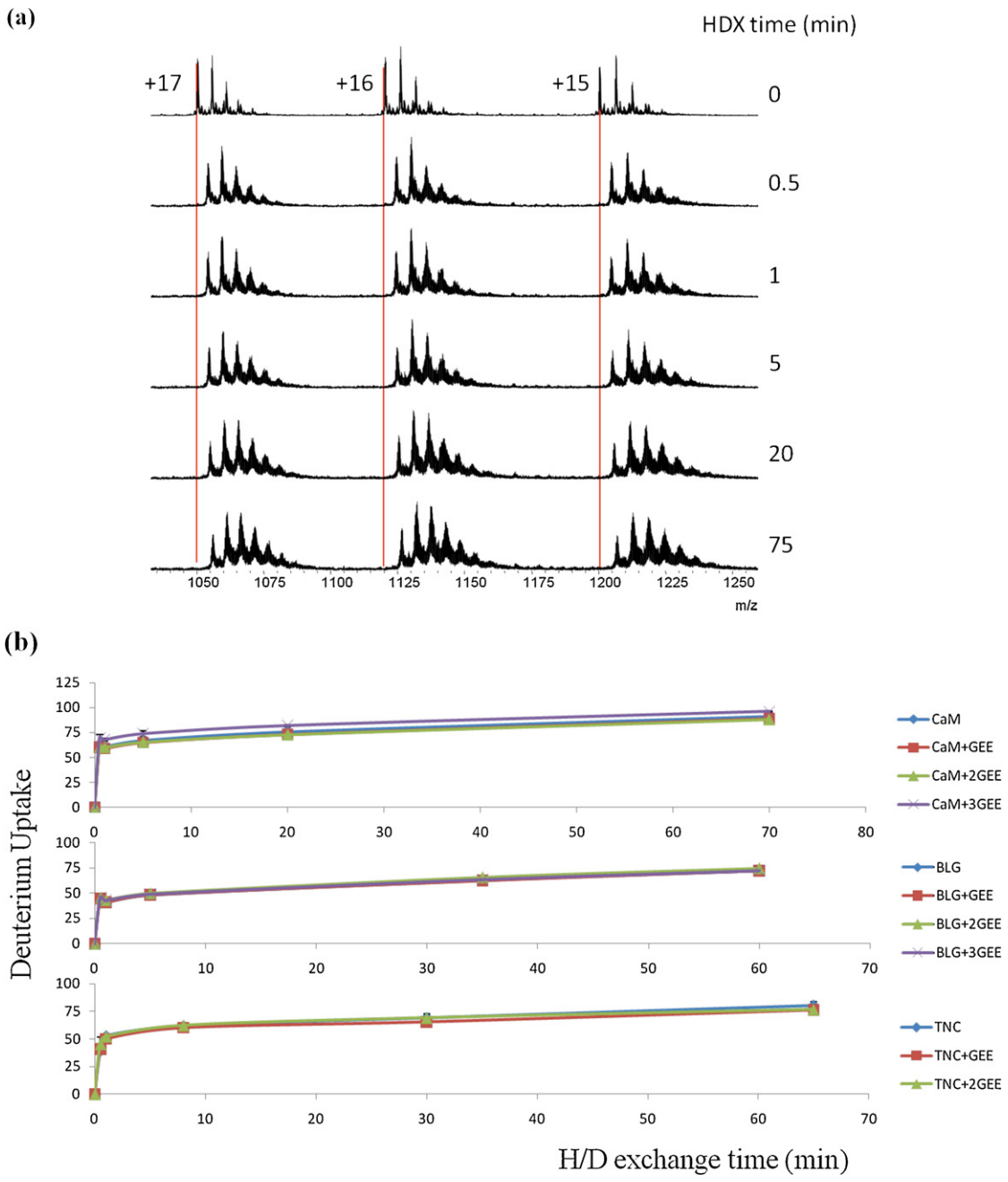

Fig. 3. H/DX kinetics of modified CaM, BlG and TnC. (a) Extended views of the modified-CaM spectra at different exchange times. (b) The deuterium uptakes of unmodified and modified protein state were monitored separately.

6.5, 7 and 7.5), HEPES (pH 8 and 8.5) were prepared and modified in an identical way.

2.4. Mass spectrometry of the modified protein

ESI mass spectra were acquired in the positive-ion mode on a Waters (MicroMass) Q-TOF Ultima (Manchester, U.K.) and a Bruker MaXis Q-TOF (Bremen, Germany). The instrument setup for Waters Q-TOF for protein analysis was similar to the previous protocol [32].

For the Bruker MaXis Q-TOF, the capillary voltage was 3.8–4 kV, nebulizer gas was 0.6 bar, dry gas was 6.0 L/min, and the source temperature (dry temperature) was 180–200 °C. The protein sample (10 pmole) was loaded on a C8 rapid-resolution cartridge (ZORBAX Eclipse XDB-C8, 2.1 × 15 mm, 3.5 μm, Agilent Technologies, Santa Clara, CA). The protein was eluted at 200 μL/min with a LC gradient (Agilent 1200 HPLC, 5–15% B in 0.3 min, 50% B in 5.5 min, 100% B in 6–7.5 min then back to 5% B in 9.5 min, solvent A: water, 0.3% formic acid; solvent B: 80% acetonitrile, 20% water, 0.3% formic acid).

2.5. Circular dichroism

CaM samples, unmodified native and modified (reaction time 90 s), were desalted to remove excess ammonium acetate. CD spectra were measured at room temperature over the wavelength range of 195–300 nm at 1 nm intervals by using a JASCO J815 CD spectrometer (JASCO Analytical Instruments, Tokyo, Japan).

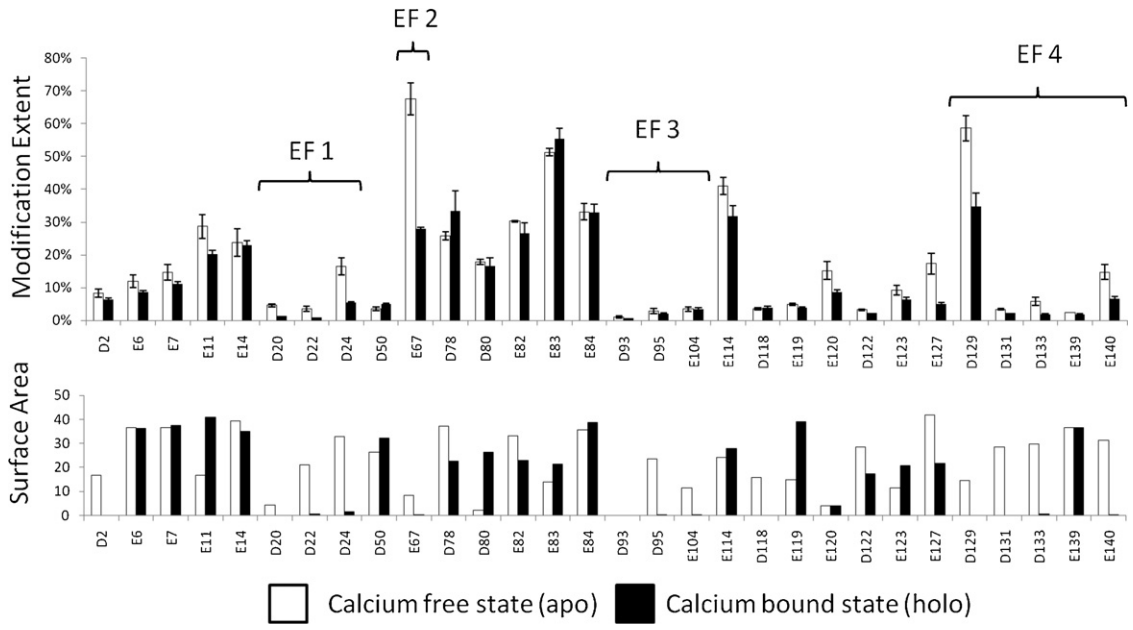

Fig. 4. The modification extent and calculated SASA of aspartate and glutamate residues detected by LC–MS/MS experiment for calcium-free and calcium-bound CaM samples. The modification extents of each detected aspartate and glutamate residue are in the top panel, and calculated SASA values from calcium-free CaM (PDB ID: 1CFC) and calcium-bound CaM (PDB ID: 1CLL) are in the bottom for reference. Those SASA values not depicted as bars are calculated to be zero.

2.6. H/DX protocol

Protein samples (2 μL, 10 μM) were mixed with PBS D_2O buffer (18 μL). The hydrogen/deuterium (H/D) exchange was conducted on ice. Exchange was quenched by adding 30 μL 3 M urea solutions with 0.1% trifluoroacetic acid at 0 °C. H/D exchanged samples were directly desalted by LC at 0 °C and analyzed by MS to minimize any back exchange.

2.7. Typsin digestion, LC–MS/MS and data analysis

The digestion of the protein CaM samples was conducted according to a previously reported protocol with minor changes [32]. The labeled protein sample was mixed with trypsin (trypsin:protein = 1:10) without heating or denaturation. The protein digest was analyzed by nanoLC–MS/MS with an LTQ Orbitrap (Thermo-Scientific, San Jose, CA) coupled with Eksigent

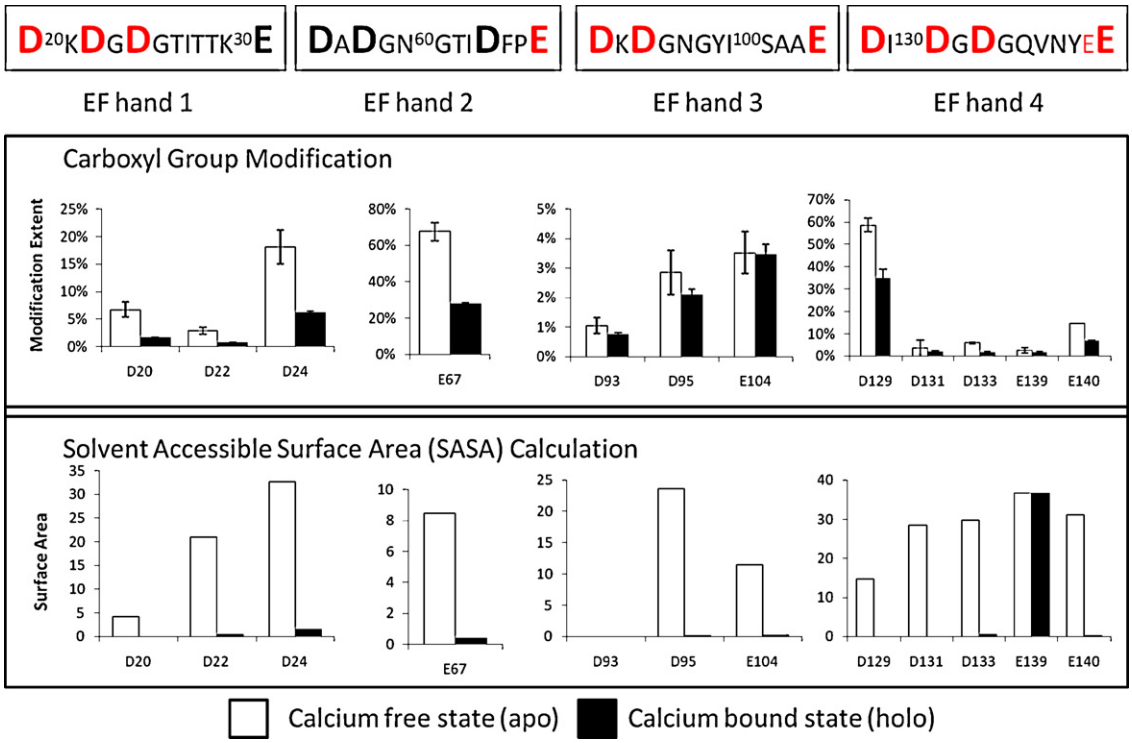

Fig. 5. The modification extents and calculated SASA of aspartate and glutamate residues in the CaM EF hand regions. For those SASAs for which there is no bar, the value is not detected.

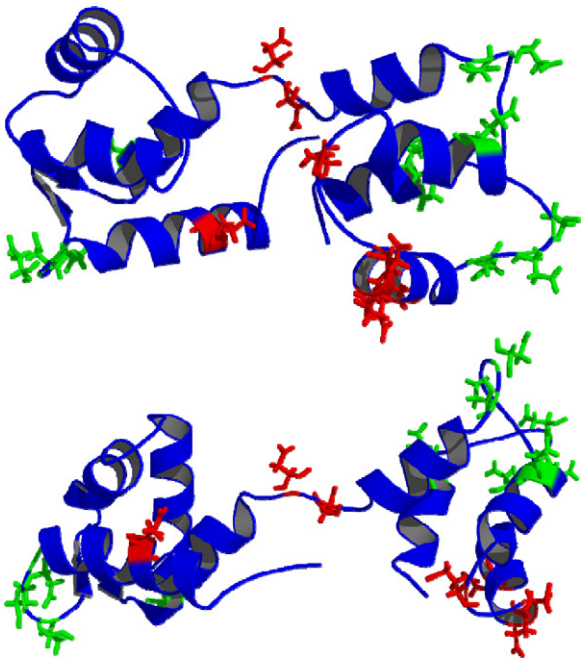

Fig. 6. Two conformations of calcium-free CaM as determined by NMR spectroscopy. The aspartate and glutamate residues from EF hand regions are labeled in the stick model in green. The aspartate and glutamate residues with inconsistent outcomes between modification extents and calculated SASA values (E11, D78, D80, E114, E118, E119, and E123) are labeled in stick model in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

NanoLC-Ultra 1D (Eksigent Technologies, Inc. Livermore, CA), as previous reported [33]. LC–MS/MS data were processed by Rosetta Elucidator system (v3.3.0.0.220, Rosetta Biosoftware, Seattle, WA) for retention time alignment of shared LC–MS features. The method for calculation of modification extent by using modified peptide peak intensity was same as that used in FPOP footprinting. Changes were made to search for carboxyl-group modifications instead of hydroxyl radical-induced modifications.

2.8. Solvent accessible surface area (SASA) calculation

The side-chain solvent accessibility of calcium-free and bound CaM were calculated by using the structures determined by NMR spectroscopy or X-ray crystallography. PDB files of calcium-free CaM (PDB ID: 1CFC) and calcium-bound CaM (PDB ID: 1CLL) were submitted to GETAREA 1.0 (http://curie.utmb.edu/getarea.html) for calculation of individual side chain SASA [34].

3. Results and discussion

3.1. Workflow of post labeling MS analysis

We analyzed the outcomes of protein footprinting MS at both the protein and the amino-acid residue levels. At the protein level, we used desalted protein samples and directly analyzed them by ESI MS. Peaks corresponding to unmodified and modified proteins allowed a comparison of the modification extents for different protein states. In the method development, we optimized the modification conditions based on MS analysis at the protein level even though the results cannot provide information about local conformational changes of the protein. To probe changes in local conformations, we digested the protein and analyzed the resulting peptides by LC–MS/MS, using a typical label-free, quantitative-analysis approach from proteomics. We used both calcium-free and calcium-bound CaM samples to obtain information at the specific amino-acid residue level.

3.2. Modification conditions for protein footprinting

Labeling a protein in biological relevant settings should be the first step in protein footprinting, and the ability to do so is one of its advantages. The modification experiment usually generates a mixture of unmodified and modified species. To calculate the modification extent, good MS signals for both the unmodified and modified species are essential. Tuning of the modification conditions is required for generating the desired amount of modified species for MS analysis. Ideally, one would aim for a "single hit" in the modification as this would guarantee that the footprinting does not induce unfolding and lead to misleading subsequent modifications. Single-hit modifications are not practical, however, because analysis is difficult if not impossible.

The coupling reaction between the carboxyl group and GEE is initiated by the attachment of the protein to EDC. The next step involves attaching the carboxyl group to form an *O*-acylisourea. The nucleophilic GEE attacks the activated carboxyl group to yield the desired amide and urea. The rate of this coupling reaction depends on the reagent ratio, solvent conditions, and the solvent accessibility of the acid residues of the target protein (Fig. 1).

To test the rate of the GEE coupling reaction, carboxyl groups of CaM were modified with excess amounts of GEE and EDC ([CaM]:[GEE]:[EDC] = 1:20000:500) in PBS buffer at room temperature with different reaction times (Fig. 2). We then monitored the modified protein sample by MS to evaluate the modification extent at different reaction times. For each charge state, the peaks representing the modified protein show mass shifts of 85 Da (amide form) and 57 Da (hydrolyzed form of amide product). With increasing reaction time, multiple peaks for the modified protein (+2GEE, +3GEE, +4GEE, +5GEE) became intense in a sequential manner (Fig. 2b). The results show that the GEE coupling reaction efficiently modified CaM within a timescale of minutes.

The pH of solvent has a major effect on the reaction rate. We examined this effect by modifying CaM at different pHs (from 5.5 to 8.5). The results show that the rate of the coupling reaction can be accelerated by lowering the pH (Fig. 2c). Lowering the pH may induce protein conformational change that affects the modification extent. For CaM, the pH effect on protein conformation can be ignored. A previous study showed that the calcium-bound CaM conformation is effectively constant from pH 7.5 to pH 3.8 [35]. In addition to affecting the coupling reaction, lowering the pH promoted the hydrolysis of the amide product formed in the modification. If the GEE coupling reaction is to be used in probing protein conformational changes that require pH variation, special attention is required to adjust the reaction time. Similarly, lowering the temperature can dramatically reduce the rate of the coupling reaction (Fig. S1).

The rate of the GEE coupling reaction is also affected by protein structure. We chose three proteins, CaM, BlG, and TnC, as models to be modified by carboxyl-group footprinting. Although these three proteins have similar sizes, CaM and TnC have more flexible structures [36–41], whereas BlG has a more compact and rigid structure owing to two pairs of disulfide linkages [42]. At the same reaction times, higher levels of multiply modified species were observed for CaM and TnC samples (up to protein +4GEE) than for the BlG sample (up to protein +2GEE). In the design of a footprinting experiment, protein structural effects on the labeling rate should be considered. The modification conditions that we established for CaM can serve

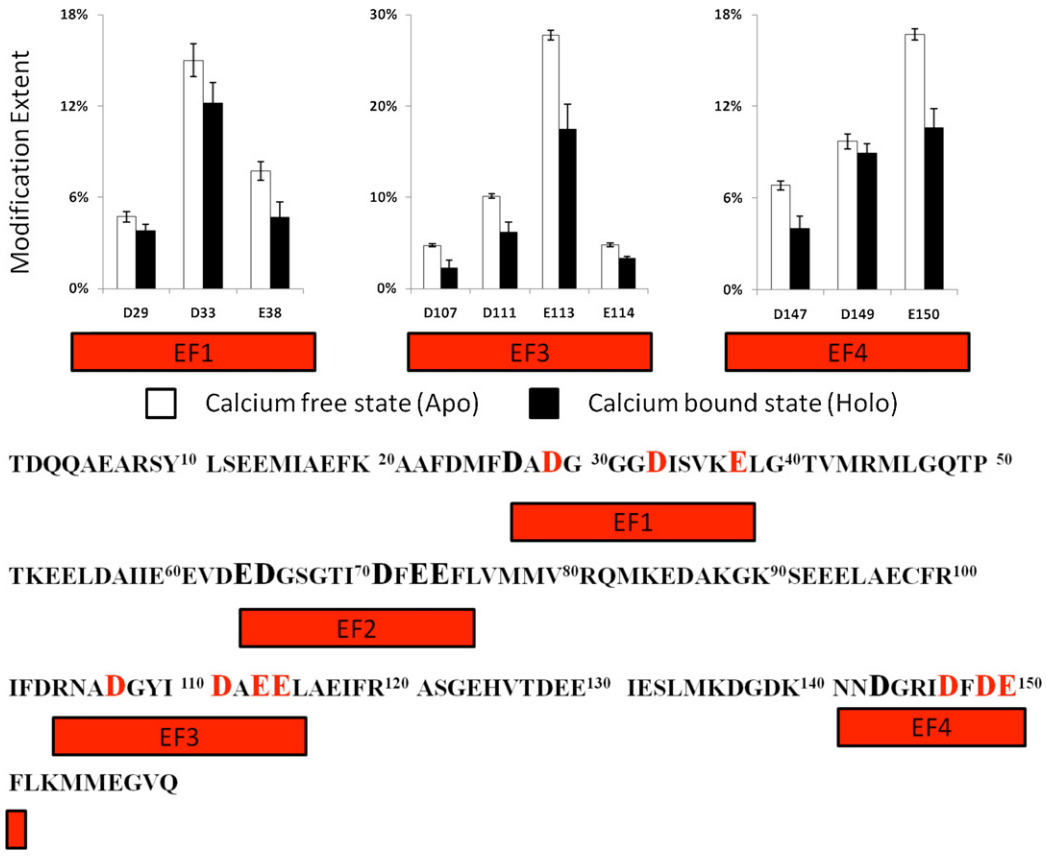

Fig. 7. The modification extents of aspartate and glutamate residues from TnC EF hand regions and TnC sequence. The aspartates and glutamates in the EF hand regions are highlighted in the TnC sequence.

as a good starting point for optimization in other applications of carboxyl-group modification in a biologically relevant setting.

3.3. *Ensuring the protein's structural integrity and preventing over-labeling*

Ensuring a protein's structural integrity is a major concern in labeling-based protein footprinting [7]. Covalent labeling can cause conformational changes by breaking protein noncovalent interactions leading to over-labeling and misleading conclusions about protein conformation. Various approaches can be employed to check the structural integrity following a modification: examples are circular dichroism (CD) [43], fluorescence spectroscopy [44], and activity assays. Another approach measures the reaction kinetics for individual modification sites [45] or the modification pattern at the protein level [46]. The premise is that the modified protein should show the same chemical reactivity as the unmodified version.

CD spectroscopy is the most common check because it is sensitive to variations in protein secondary structure [47]. In the development of carboxyl-group footprinting, we analyzed both native and modified CaM (reaction time 90 s) by CD (Fig. S2). No change occurred in the CD spectrum of modified CaM, and typical α helix CD curves were observed for both samples. The result shows that no significant secondary structural change occurred during modification. Nevertheless, caution is indicated in interpreting CD results. After protein footprinting, the protein sample is a mixture, containing sometimes a majority of unmodified and some modified species. Any conformational change in the small portion of the sample undergoing the modification may be overlooked in the CD spectrum.

Given that MS analysis can identify the unmodified and modified species in the mixture produced for a protein submitted to footprinting, it may be possible to employ a second MS-based footprinting method to check for any conformational changes that occur during the first stage of modification. H/DX is an ideal protein footprinting method for this purpose because the replacement of hydrogen by deuterium induces minimal structural disturbance [48]. Furthermore, the deuterium uptake of each modified protein state, as well as of the unmodified protein state, can be monitored separately by MS. Additionally, the H/DX outcome is directly measured as a mass shift of the protein, allowing any ionization bias induced by the carboxyl-group modification to be ignored.

We analyzed the kinetics of H/DX for three protein samples, CaM, BlG, and TnC, after their carboxylate footprinting (Fig. 3). The unmodified-protein and each modified-protein state share a similar deuterium uptake curve. The results provide additional and more convincing evidence than CD that no major conformational change occurred on the timescale of carboxyl-group footprinting of these proteins. We tried to over-label these proteins to show that H/DX responds to over-labeling, but we were unable to over label the protein. In our recent study of human APE protein, we were able to show, however, that a change in conformation induced by *N*-ethylmaleimide (NEM) labeling can be demonstrated with H/DX [49]. We suggest that the relatively simple design and high sensitivity to each modified protein state make H/DX an attractive check in the field of protein footprinting. With the fast peptic digestion protocol normally used for H/DX, a more detailed check on any local conformational change than that provided by CD is possible, and it should be a powerful control that can be used for the development of other protein footprinting strategies.

3.4. GEE labeling probes conformational changes of CaM and TnC

CaM is a small calcium-binding protein that has two domains; in each domain are two typical EF-hand motifs (helix-loop-helix unit) that bind calcium ions by electrostatic interactions [50]. Calcium-binding EF hand motifs are rich in negatively charged glutamates and aspartates. In the typical EF hand motif, the co-ordination of a calcium ion is by seven ligands arranged in a pentagonal bipyramidal fashion [51]. Among these seven co-ordination sites, five are from the nine-residue loop and two are side chains of amino acids in the helix. In the nine-residue loop, three or four of the coordination sites are aspartate or glutamate carboxyl groups and one is a backbone carbonyl group. It is well known that CaM undergoes conformational changes upon calcium binding, and these changes activate its binding domain for target proteins in downstream signal transduction [52]. Thus, CaM is a good model to test the effectiveness of GEE footprinting.

We used carboxyl-group modification to probe the conformational changes induced by Ca^{2+} binding and found that a total of 30 aspartates or glutamates are modified in the CaM sequence. This coverage enables a thorough comparison of the modification extents of calcium-free and calcium-bound CaM. To see if changes in SASA agree with changes in the modification extents, we show in Fig. 4, a comparison of the calculated SASA of calcium-free vs. calcium-bound CaM. The SASAs of aspartates and glutamates from those EF hands decrease significantly upon calcium binding. Similar trends in the modification extents occur for aspartate and glutamate residues from all the EF hands (Fig. 5), although the differences are often not as large as the relative changes in SASA. The system is in dynamic equilibrium, and we expect that the modification can compete with Ca^{2+} binding, especially if the binding strengths are comparable. Although changes in SASA and in modification extents do not correlate linearly, the decreases induced by calcium binding are clear. For those residues that undergo high modification extents (e.g., EF hand #1), the differences in modification extents between the calcium-free and calcium-bound CaM are statistically significant (99.5% confidence level in *T*-test). Although E104 of EF hand #3 shows no difference in the modification extent between the calcium-free and calcium-bound CaM, the lack of an effect may be masked by experimental error owing to the low modification extent and poor *S*/*N*. The experimental errors from three biological replicates are from 0.5% to 5% (a total of 30 modified residues have an average error ~2%) in modification extent. This exception emphasizes again that adjusting the modification conditions is important for a successful footprinting experiment. For example, to differentiate the change of modification extent at the sites of reactivity, one may be required to adjust the conditions to increase modification extents at certain sites.

Residues E11, D78, D80, E114, E118, E119, and E123 show significant differences between changes in modification extents and calculated SASA. These differences may stem from the dynamics of CaM solution structures. Calcium-free CaM has a flexible structure in solution, and its central linker region can be bent to accommodate different relative positions of the N- and C-terminal domains [53]. Calcium-bound CaM, however, has a relatively rigid solution structure [36]. The central linker forms a seven-turn α helix that connects the two domains. All residues with inconsistent outcomes between modification extents and calculated SASA are from regions of the protein that are close to the central linker region (Fig. 6). The highly flexible properties of CaM in the central linker region could result in inconsistent readouts from different measurements. The extent of carboxyl-group modification is averaged over protein conformational dynamics during the reaction time whereas the calculated SASA results from the different 3D model of protein structures (calcium-free CaM model from NMR spectroscopy [53] and calcium-bound CaM model from X-ray crystallography [36]). The protein model from X-ray crystallography is a "snap shot" of the protein conformation in the crystalline solid state whereas the model from NMR is an integrated picture from solution. Although we find a small difference between Ca^{2+}-bound and Ca^{2+}-free for E139, the calculated SASA shows them to be the same. This may be attributed to the additional carboxyl groups in EF hand #4, some of which in the solid state are not bound to Ca^{2+}.

We looked further by studying the conformational changes induced by calcium binding in another calcium-binding protein, TnC, and detected 22 modified sites of aspartates and glutamates by LC-MS/MS (Fig. S3). Except for EF hand #2, the protection induced by calcium binding on aspartates and glutamates of the EF hand regions is detectable by carboxyl-group footprinting (Fig. 7). The sensitivity of modification to Ca^{2+} binding is lower than that of CaM, which may be due to residual amounts of Ca^{2+} in the putative apo state of TnC. Nevertheless, the trends are in agreement with H/DX results [54].

The conformational effects of calcium binding on the EF-hand regions affect the extent of carboxyl-group modification at the residue level for aspartates and glutamates. Those effects were not seen in an FPOP approach to the same system [32]. Although current H/DX results from our group showed similar conformational variations induced by calcium binding at the EF hands [8,55], those results are at the peptide level, not at the amino-acid residue level. Compared with FPOP and H/DX, carboxyl-group modification does respond to conformational changes of the protein's carboxyl side chains and provides results at the residue level. More importantly, this approach provides protein conformational information that is complementary to that from other approaches.

4. Conclusion

We evaluated carboxyl-group modification by using three model protein systems. A sufficient amount of modified species can be reached in a biologically relevant setting by adjusting the modification conditions. Under these conditions, no major conformational change occurred during the time scale of the carboxyl-group footprinting as determined by H/DX. Thus, GEE footprinting is sensitive to the conformational changes in carboxyl side chains of the protein and can report, for example, the conformational variations induced by calcium binding at the EF hand regions of CaM. Carboxyl-group modifications provide structural information that is complementary not only to traditional structural biology methods (e.g., NMR and X-ray) but also to other protein footprinting approaches (e.g., FPOP and H/DX).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.07.015.

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