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Fast Photochemical Oxidation of Proteins (FPOP) for Epitope Mapping

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Abstract

The growing use of monoclonal antibodies as therapeutics underscores the importance of epitope mapping as an essential step in characterizing antibody-antigen complexes. The use of protein footprinting coupled with mass spectrometry, which is emerging as a tool in structural biology, offers opportunities to map antibody-binding regions of antigens. We report here the use of footprinting via fast photochemical oxidation of proteins (FPOP) with OH radicals to characterize the epitope of the serine-protease thrombin. The data correlate well with previously published results that determined the epitope of thrombin. This study marks the first time oxidative labeling has been used for epitope mapping.

Keywords

Mass spectrometry; oxidation; epitope mapping; protein footprinting

INTRODUCTION

Monoclonal antibodies are increasingly used as therapeutics for a wide range of diseases including multiple sclerosis,[1] autoimmune disorders,[2] and cancer.[3] Their applications in disease treatment stem from their high specificity. The successful use of antibodies and their expansion as therapeutics, however, require the characterization of the antibody-antigen complex. One important means of characterization is mapping the epitope, the part of the antigen recognized by antibodies.

Epitopes are either linear or conformational. A linear epitope consists of a continuous run of amino-acid residues whereas a conformational epitope is made up of a discontinuous sequence that is brought together after the protein has folded to its native structure. X-ray crystallography,[4] peptide scanning,[5] site-directed mutagenesis,[6] epitope excision,[7] and epitope extraction[8] are traditional methods of mapping, and they enjoy good success despite having some limitations. X-ray crystallography requires a relatively large amount of protein, and not every antibody-antigen complex is amenable to crystallization. Peptide scanning is

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SUPPORTING INFORMATION AVAILABLE

Supplementary table S1 is a table that lists the average absolute abundance for each peptide. Supplementary Figure 1 shows the extent of modifications of peptides that did not have significant difference between the apo-thrombin and antibody-bound thrombin. Supplementary Figure 2 shows selected ion chromatograms of modifications on peptide 1-17.

useful for mapping continuous epitopes but not conformational ones. Site-directed mutagenesis is slow, labor-intensive, and inconclusive if the protein's conformation changes owing to the mutation(s). Epitope excision produces a peptide that is larger than the epitope because there is steric hindrance between bound antibody and proteolytic enzyme.

In recent years, the application of hydrogen-deuterium exchange (H/DX) coupled with mass spectrometry (MS) analysis has proven useful for mapping epitopes[9-12] and for determining protein- protein interactions.[13, 14] The advantages of H/DX are its applicability to large proteins, its sensitivity, and its compatibility with proteins in their native buffer conditions. Another type of protein footprinting, oxidative labeling, is also emerging as a powerful tool to analyze protein structure and to elucidate protein-protein and protein-ligand interactions.[15-17] Its advantages relative to H/DX are the high speed of labeling (~ 1 μs), the irreversible nature of the modification, the applicability to a major fraction of the amino-acid residues, and its versatility to accommodate other reagents besides OH.[18] Moreover, oxidative labeling is of side chains, which are the major contributors to epitope recognition, whereas H/DX interrogates backbone amides.

Oxidative labeling of proteins for footprinting was established by Chance and coworkers using a synchrotron to expose solutions to high-energy radiation that ionizes water and forms OH by H^+ loss.[19] Building on this work, Hambly and Gross[20] developed fast photochemical oxidation of proteins (FPOP) and Aye et al.[21] reported at the same time a similar approach. FPOP uses an excimer laser to photolyze hydrogen peroxide, which is added in low concentration to the protein solution, to form hydroxyl radicals.[20, 22] The use of a pulsed laser and a radical scavenger limit radical lifetimes, insuring labeling on a microsecond timescale, faster than most protein unfolding.[23] FPOP has showed success for identifying protein-ligand interactions sites in various protein systems.[22, 24]

In this paper, we describe the use of FPOP to map the conformational epitope of the serine protease thrombin. This marks the first time oxidative labeling has been used for epitope mapping. The FPOP data correlate well with the previous H/DX results that serve as a successful map of the thrombin epitope.[10] In addition, FPOP revealed regions of allostery in the protein not previously observed.

EXPERIMENTAL SECTION

Materials and Reagents

Trypsin, 30% hydrogen peroxide, formic acid, phosphate-buffered saline (PBS), *tris*-(2-carboxyethyl)phosphine (TCEP), iodoacetamide, dithiothreitol (DTT), and HPLC-grade solvents were obtained from Sigma Aldrich (St. Louis, MO). Human thrombin and anti-human thrombin were purchased from Haematologic Technologies, Inc. (Essex Junction, VT), LysC from Roche Applied Science (Indianapolis, IN), and Nu-Tip C-18 zip tips from Glygen Corp. (Columbia, MD).

FPOP of proteins

Thrombin and anti-thrombin were mixed together at a 1:1 molar ratio and incubated for 1 h. Both the apo thrombin and antibody-bound thrombin were mixed with PBS (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4) and glutamine. Hydrogen peroxide was added just prior to infusion in the tubing for oxidative modification by FPOP. The final concentration of thrombin in all samples was 10 μM in 50 uL. The final glutamine and hydrogen peroxide concentrations were 20 and 15 mM, respectively. FPOP was performed as described previously.[20, 23] Samples with a 15% exclusion volume fraction (the volume not irradiated, existing between plugs of the irradiated protein solution) were infused at a rate of 15 uL/min. The excimer laser pulse frequency was 6.0 Hz. For each state, thrombin

alone and thrombin with antibody, three separate samples were made and labeled. In addition, three control samples for each state were made. Hydrogen peroxide was added to the control samples, but they were not subjected to laser irradiation. All collections were in vials containing 10 nM catalase and 30 mM methionine to destroy excess hydrogen peroxide.

Mass spectrometry

Following irradiation, a 20 μL aliquot of each sample was placed in a speed vac and dried. The samples were re-suspended in 20 uL 100 mM Tris, 8 M urea, pH 8.5. The samples were reduced with 10 mM TCEP (2 μL of a 100 mM solution) for 30 min, alkylated with 20 mM iodoacetamide (2.2 μL of a 200 mM solution) in the dark for 30 min, and quenched with 10 mM DTT (2.2 μL of a 200 mM solution) for 15 min. Samples were digested overnight with LysC at 37 °C at an enzyme:protein ratio of 1:100.[25] After digestion, the samples were diluted 1:4 in 100 mM Tris, pH 8.5. The samples were digested with trypsin at 37 °C overnight at an enzyme:protein molar ratio of 1:20. The use of multiple enzymes was shown to increase sequence coverage of proteins.[24] After digestion, samples were acidified with formic acid solution to give a final concentration of 5% and were cleaned up by elution from NuTip C-18 zip tips.

Samples were analyzed on a LTQ Orbitrap XL operated in data-dependent acquisition mode controlled by the Xcalibur 2.0.7 software (Thermo Fisher, San Jose, CA). Samples were loaded onto and eluted from a silica capillary column that was custom-packed with C18 reverse-phase material (Magic, 0.075 mm x 150 mm, 5 μm, 120 Å, Michrom Bioresources, Inc., Auburn, CA) with a pulled tip (Sutter Instruments, Novato, CA); the chromatograph was an Ultra 1D+ UPLC (Eksigent, Dublin, CA). The column was mounted on a Pico View nanospray source (New Objective, Woburn, MA). The gradient was pumped at 260 nL/min from 0-80% solvent B for 60 min, held at 80% B for 10 min, and re-equilibrated to solvent A (water, 0.1% formic acid) for 10 min. Peptide mass spectra were acquired over an *m/z* range of 350-2000 at high mass resolving power (60,000 for ions of *m/z* 400). The six most abundant charge ions, with a charge of at least +2, with a minimum signal intensity of 1000 were subjected to collision-induced dissociation (CID) in the linear ion trap, using data-dependent scanning. Charge-state rejection of +1 ions was employed.

Data analysis

For retention time alignment of shared LC-MS features, the raw MS files were imported into Rosetta Elucidator version 3.3.0.0.220 (Rosetta Biosoftware, Seattle, WA), as previously described.[24] Rosetta Elucidator aligns the chromatograms for all samples, deconvolutes each mass spectral peak to provide peptide masses, and creates a data (dta) file for each product-ion (MS/MS) spectrum. All of the dta files produced by Rosetta Elucidator were merged into a single mascot generic format (mgf) file, and the data were searched for modified and unmodified thrombin tryptic peptides by using MASCOT 2.2.06 (Matrix Science, London, U.K.) and the NCBI 20101215 database. The taxonomy was restricted to *Homo sapiens*, which contains 233,080 sequences. Only the thrombin peptides were searched for modifications. This was made possible by optimizing the chromatography method, minimizing the overlap of all the peptides both from thrombin and the antibody. All known hydroxyl radical side-chain-reaction products[26,27,18] were added to the modifications database for searching as variable modifications. The extents of modification were calculated as described previously,[24] as discussed in detail in the supplementary material. The alkylation of the samples with iodacetamide adds a carbamidomethyl group (MW = 57.0214 Da) to cysteine containing peptides. Additionally, the N-terminus of several peptides are carbamylated (increase in MW of 43.0058 Da) by urea. These modifications were not used for quantitative analysis of oxidative labeling and were subtracted from the

Net Mass Change column in Table S1. In a few cases modified peptide isomers, that is, peptides that differ only in their modification site, co-eluted in the chromatography. In these cases, spectral counting in the MS/MS mode was used to split the single abundance amongst the components in the mixture.[28] For example, if a peptide has a total of 10 product-ion spectra and seven of these are attributed to a Met residue, then 70% of the signal intensity for that peak will be attributed to the Met oxidation. The remaining 30% of the signal will be attributed to the other modification that produces the remaining 3 product-ion spectra.

RESULTS AND DISCUSSION

That the interaction interface of thrombin bound to its antibody was previously described using HDX coupled with MS[10] makes it a good model system for studying the utility of FPOP for epitope mapping. We subjected the protein both by itself and bound to its antibody to FPOP labeling. Trypsin digestion afforded a mixture of at least 34 peptides, some of which contained missed cleavage sites; the sequence coverage was 86% (Figure 1). Of the 34 peptides, 10 had no FPOP modifications. Hence, we could investigate 24 peptides for their extent of modification. The extent of labeling between the apo and holo forms of thrombin was minimal for 15 of these peptides (Supplementary Figure 1). The remaining nine peptides showed, however, significant differences between the two states. The average abundances for each peptide are listed in supplementary table 1.

Regions of Decreased Solvent Accessibility upon Binding

A decrease in modification extent upon antibody binding is apparent in two regions of the protein (Figure 1). The first is represented by peptide 114-119 (Figure 2). The reduced reactivity (solvent accessibility) upon binding strongly suggests that this region is part of the binding site. This result agrees well with that from H/DX, which shows that, upon antibody binding, H-bonding increases (protection occurs) for regions representing the overlapping peptides 97-117 and 113-117.[10] The majority of the protection to H/DX, however, occurred for peptide 113-117, suggesting that antigen binding is in the region between residues 113 and 117.[10] In the FPOP experiment, only the residue M116 was oxidatively modified; therefore, we cannot specify that the binding site involves all the residues 113-117.

A second region that shows decreased modifications for the antibody-bound form of the protein is 130-171. This region is represented by five tryptic peptides, 130-140, 141-159, 141-171, 143-159, and 143-171 (Figure 3). The error bars in two of these peptides, 141-159 and 143-159, are large. This is most likely due to the low intensities of some of the signals used to calculate the extent of modifications of these peptides (Supplementary Table S1). The large size of this predicted binding site, 41 amino acids, is not feasible for a protein the size of thrombin.

To increase the definition of the binding site, we examined the residue-level information. An example product-ion spectrum for peptide 130-140 (Figure 3B) shows an oxidative modification for aspartate 133. Although the product-ion spectrum does not definitively reveal what residue is modified, it does show that the modification occurs either at residue N131, L132, or D133 (The y_7 ion is not modified but the y_{10} ion is). In this case, the modification is -43.9898 Da, a loss of carbon dioxide that is observed to occur from acidic residues.[18,26] Therefore, the modified residue must be aspartate 133. In contrast, the product-ion spectrum for the peptide 130-140 containing a +15.9949 modification shows no change for the y_{10} ion, indicating conclusively that the modification is on residue 130 (data not shown).

For the five tryptic peptides that showed protection upon antibody binding, a total of 14 specific residues were oxidatively modified. Of these, protection in the antibody-bound form

occurred between residues D133 to Y150 (Figure 3C). Our interpretation agrees well with that of the H/DX data taken by Komives and coworkers[10]; these data show protection in the antibody-bound state of thrombin for the region represented by peptide 139-149. The data also highlight one of the advantages of FPOP when conducted in a site-specific way. Even though the peptide-level information indicates modification protection in the antibody-bound state in the region spanning residues 130-171, a closer examination of the residue-specific data indicates that only part of this region is involved in antibody binding. This residue-specific information is difficult to obtain with H/DX because it often relies heavily on overlapping peptides in the pepsin digest. Although electron-capture dissociation (ECD) and electron-transfer dissociation (ETD) can provide residue-specific deuterium uptake,[29-31] these approaches require specialized instrumentation. Because the oxidative modifications of FPOP are irreversible, the protein can be subjected to collision-induced dissociation (CID) without scrambling the modification, thereby providing residue-specific labeling information and, in this case, detailed information about the location of the epitope.

When both regions of decreased solvent accessibility upon binding are mapped onto the thrombin structure, they come together in the fold of the protein even though they are far apart in the primary sequence (Figure 4). This proposed epitope agrees well with that assigned by HDX data.[10]

Regions of Increased Solvent Accessibility upon Binding

There are two regions of thrombin where the extents of modification are increased in the antibody-bound state of the protein (Figure 5). The first region, represented by peptide 126-134, is located in the 99-loop of the protein (Figure 4). The second region, represented by peptides 174-181 and 174-190, is located in the 148-loop of the thrombin. Increases in modification in the antibody-bound form of thrombin are also observed on the residue level, specifically for residues R134, P153, C155, and P157 (Figure 3B); all are located in the loop regions (Figure 4). The increase in modification upon antibody binding may be attributed to allosteric conformational changes in the protein, and, indeed, ligand binding is known to induce allosteric conformational changes in the thrombin structure.[32-36] These changes serve to modulate its catalytic activity and substrate specificity. In particular, the loop regions undergo conformational changes upon ligand binding.[37,38] When structures of thrombin complexed with different substrates and inhibitors were compared, the ability of the 148-loop to adopt a number of conformations was revealed.[39, 40] In addition to these documented ligand-induced allosteric changes for thrombin binding, those of hen egg white lysozyme and human growth hormone, as antigens, also induce allosteric conformational changes with monoclonal antibodies[41] The binding of the anti-thrombin antibody most likely induces conformational changes in the 99- and 148 loop regions that are outside of the antibody binding site.

The increased modification extents for the antibody-bound state of thrombin in the 99- and 148-loops were not observed with HDX. This may be due to the manner in which the conformational change takes place. H/DX monitors the backbone of amides, and its extent is a function of hydrogen bonding as well as solvent accessibility. FPOP, on the other hand, monitors the side chains of amino acids. Some allosteric changes that occur upon antibody binding to thrombin may not involve changes in the hydrogen-bonding pattern of the protein and, therefore, would not be observed by HDX. Rather, the changes may be due to altered side-chain orientations. That these changes occur in the loop regions of the protein (Figure 4), where side-chain rotations are more facile than for α–helix or β–sheet secondary structure, provides further evidence that the side-chain orientation changes upon antibody binding.

CONCLUSIONS

FPOP is a relatively new analytical tool that shows promise for mapping epitope regions of protein antigens. The method utilizes MS to monitor oxidative modification of amino-acid side chains as a function of ligand binding. The irreversible nature of the label allows for long, high resolution chromatography to permit good peptide separation and detailed CID analysis to provide residue-specific information. This method can be used as a stand-alone method or in conjunction with H/DX to elucidate discontinuous binding sites of antibodies and their antigens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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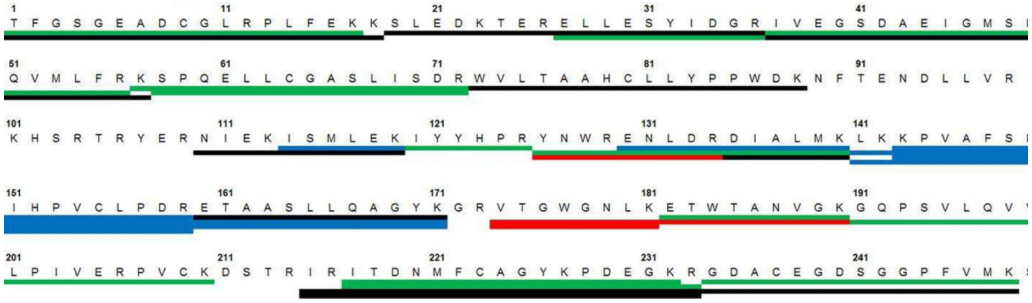

Figure 1.

Coverage map of the tryptic digest of thrombin shows peptides that were not labeled (black lines), showed no change between antibody-bound thrombin and thrombin alone (green bars), showed protection in the antibody-bound form (blue lines), and showed de-protection in the antibody-bound form (red lines)

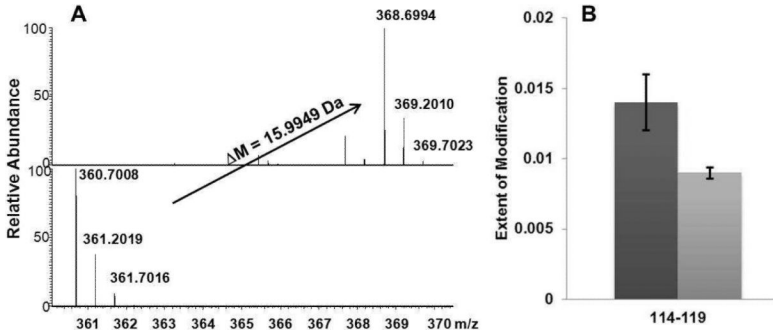

Figure 2.

(a) Mass spectra of peptide 114-119 normalized to 100% unmodified (bottom) and modified (top), (b) extent of modification of peptide 114-119 from apo-thrombin (dark bars) and antibody-bound thrombin (light bars)

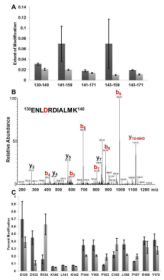

Figure 3.
(a) Extent of modification of the apo-thrombin (dark bars) and antibody-bound thrombin (light bars) of the five peptides that span the 130-171 region. (b) Product-ion (MS/MS) spectrum of peptide 130-140 with the fragment ions that contain the labeled residue highlighted in red. (c) Extent of modification of apo-thrombin (dark bars) compared to antibody-bound thrombin (light bars) at the residue level

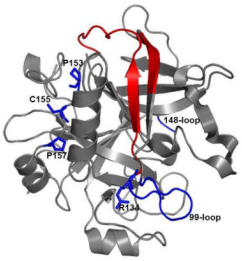

Figure 4.
Structural model of thrombin (pdb file 2AFQ[42]) with the proposed epitope colored red and the loop regions colored blue. The individual residues that show increased modification in antibody-bound thrombin are specified.

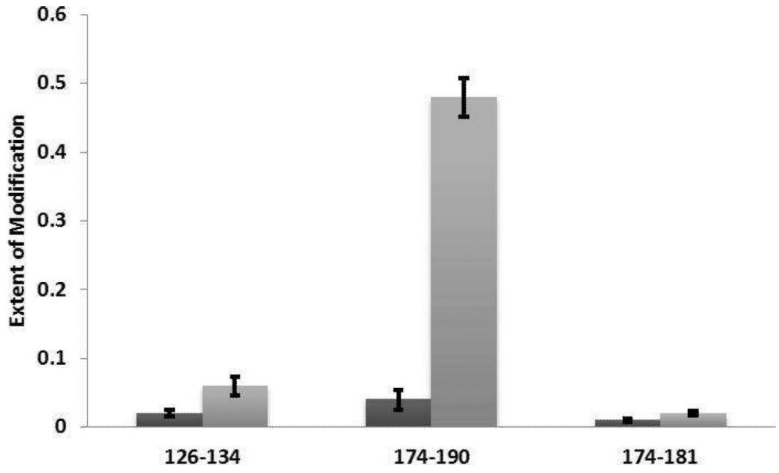

Figure 5.
Extent of modification of peptides from apo-thrombin (dark bars) and antibody-bound thrombin (light bars) showing increased solvent accessibility in the antibody-bound form.