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Site-Specific, Intramolecular Cross-Linking of Pin1 Active Site Residues by the Lipid Electrophile 4-Oxo-2-nonenal

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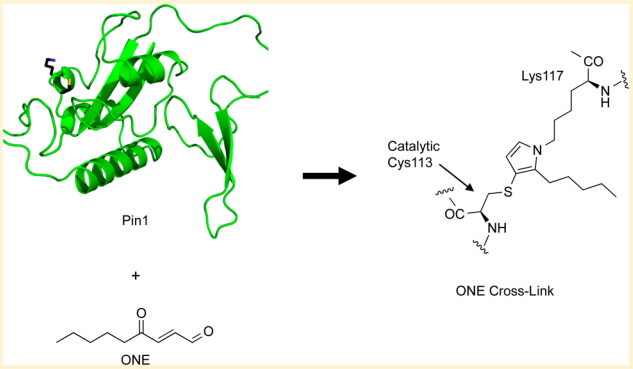
Supporting Information

ABSTRACT: Products of oxidative damage to lipids include 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE), both of which are cytotoxic electrophiles. ONE reacts more rapidly with nucleophilic amino acid side chains, resulting in covalent protein adducts, including residue−residue cross-links. Previously, we demonstrated that peptidylprolyl *cis/trans* isomerase A1 (Pin1) was highly susceptible to adduction by HNE and that the catalytic cysteine (Cys113) was the preferential site of modification. Here, we show that ONE also preferentially adducts Pin1 at the catalytic Cys but results in a profoundly different modification. Results from experiments using purified Pin1 incubated with ONE revealed the principal product to be a Cys-Lys pyrrole-containing cross-link between the side chains of Cys113 and Lys117. *In vitro* competition assays between HNE and ONE demonstrate that ONE reacts more rapidly than HNE with Cys113. Exposure of RKO cells to alkynyl-ONE (aONE) followed by copper-mediated click chemistry and streptavidin purification revealed that Pin1 is also modified by ONE in cells. Analysis of the Pin1 crystal structure reveals that Cys113 and Lys117 are oriented toward each other in the active site, facilitating formation of an ONE cross-link.



INTRODUCTION

Polyunsaturated fatty acids in cellular membranes are major targets for oxidative damage induced by xenobiotics and inflammatory stimuli. The initial oxidation products are fatty acid hydroperoxides, which can be converted to a number of reactive lipid electrophiles. Some of these electrophiles are readily diffusible and can modify proteins and DNA, thereby propagating damage initiated by oxidation.[1,2] This may be an important contributor to diseases associated with environmental exposures or chronic inflammation such as Parkinson's disease, atherosclerosis, diabetes, and cancer.[3,4]

Lipid peroxidation generates a plethora of electrophilic products, varying in length and reactivity; two of considerable interest are 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) (Figure 1). HNE and ONE react rapidly with the side chains of Cys, His, and Lys residues in proteins via Michael addition. HNE and ONE can also form Schiff bases through reaction with Lys residues, while ONE alone is capable of 4-ketoamide formation.[1,5] ONE is >150-fold more reactive than HNE and displays a broader range of reaction products due to differences in its stereoelectronic properties.[6,7] Comprehensive proteomic analyses indicate that HNE and ONE react with many proteins in cells (>1,000) but that they display significant

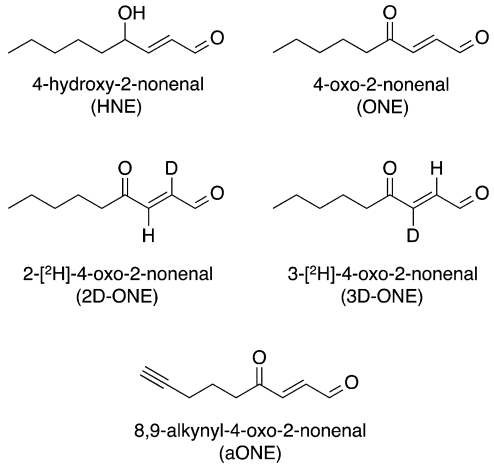


Figure 1. Structures of lipid electrophiles used in these studies.

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differences in protein targets and sites of reactivity;[8−10] few studies have investigated the precise mechanisms responsible for these differences.

We recently reported that HNE reacts with the active site Cys of the peptidyl-prolyl isomerase, Pin1, to form a covalent Michael adduct *in vitro* and in cells exposed to HNE.[11] Pin1 is the only known isomerase to specifically target proline-directed epitopes preceded by a phosphorylated Ser/Thr residue. Pin1 isomerizes this bond from *cis* to *trans*, thereby facilitating dephosphorylation by isomer-specific phosphatases.[12] This unique activity of Pin1 results in the stabilization and/or transactivation of an impressive list of substrates, including p53,[13] β-catenin,[14] Raf,[15] Rb,[16] and tau,[12] among many others. Modification of Pin1 by HNE has been detected by immunochemical analysis of affected regions of brains from Alzheimer's disease patients, and the extent of modification appears to correlate to disease severity.[17,18]

Preliminary investigation of the reaction of ONE with Pin1 indicated that, like HNE, it targets the catalytic Cys. However, detailed analysis indicated that the product of ONE-mediated Pin1 modification is not a simple Michael addition product but rather a stable intramolecular cross-link that forms rapidly and in high yield. We report here the structure of the adduct, a potential mechanism of reactivity, and evidence for the modification of Pin1 by ONE in intact cells. Efficient production of this adduct in Pin1 and in other proteins with similar surface exposed, active site-localized Cys-Lys residues may contribute significantly to the cellular effects of ONE associated with oxidative stress.

MATERIALS AND METHODS

Materials and Reagents. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. ONE, 8,9-alkynyl-ONE (aONE), and UV-cleavable biotin azide were synthesized in the laboratory of Dr. Ned Porter at Vanderbilt University as previously described.[10] Cell culture medium was purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Ft. Collins, CO). Purified Pin1 protein (GWB-523EFE) was purchased from Genway Biosciences (San Diego, CA). Anti-Pin1 antibodies were purchased from Cell Signaling (Danvers, MA), and secondary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). All SDS−PAGE and Western blot supplies were purchased from Bio-Rad (Hercules, CA) unless otherwise noted. Streptavidin Sepharose High Performance beads were purchased from GE Life Sciences (Pittsburgh, PA).

Cell Culture and Treatment. The triple-negative human breast carcinoma MDA-MB-231 cell line was purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI1640 Medium (Gibco) with 10% FBS. Electrophiles dissolved in DMSO or vehicle control were added to the cell culture medium to achieve the desired electrophile concentration while maintaining a DMSO concentration of less than 1%.

Synthesis of Deuterated ONE Analogues. Deuterated ONE analogues were synthesized according to the method of Blair[19] with some modifications. 4-Hydroxy-non-2-ynal diethylacetal was synthesized by the Grignard reaction of hexanal with propiolaldehyde diethylacetal magnesium bromide. The reduction of 4-hydroxy-non-2-ynal diethylacetal with lithium aluminum hydride and workup with deuterium oxide saturated with deuterated ammonium chloride gave 2-[^2H]-4-hydroxy-non-2-enal-diethylacetal. Deprotection of 2-[^2H]-4-hydroxy-non-2-enal-diethylacetal in 1% citric acid gave 2-[^2H]-4-hydroxy-non-2-enal. Finally, Dess−Martin oxidation of 2-[^2H]-4-hydroxy-non-2-enal provided 2-[^2H]-4-oxo-non-2-enal (2D-ONE). For the synthesis of 3-[^2H]-4-oxo-non-2-enal (3D-ONE), 4-hydroxy-non-2-ynal diethylacetal was reduced with lithium aluminum deuteride, and the reaction was quenched by the addition of a saturated solution of ammonium chloride in water to give 3-[^2H]-4-hydroxy-non-2-enal-diethylacetal. Subsequent deprotection under acidic conditions followed by Dess−Martin oxidation resulted in the formation of 3D-ONE.

Click Chemistry. MDA-MB-231 cells were exposed to aONE for 1 h in serum-free medium. Following electrophile exposure, cells were washed with Dulbecco's-modified phosphate-buffered saline (DPBS, Gibco), collected by scraping, and centrifuged for 5 min at 1000*g*. Cell pellets were lysed in NETN buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% Igepal, and mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)). Pellets were sonicated by ten 1 s pulses with a Virsonic Cell Disruptor and cleared by centrifugation at 16,000*g* for 10 min. The bicinchoninic acid assay was used to determine protein concentration (Thermo Scientific, Waltham, MA). Click chemistry and photoelution were performed as previously described.[11]

SDS−PAGE and Western Blotting. Protein samples for SDS−PAGE were mixed 1:1 by volume with 2X Laemmli buffer containing 5% β-mercaptoethanol and boiled for 5 min. A 4−20% gradient Tris-HCl gel was used to separate proteins. Proteins in the gel were transferred onto a 0.45 μm nitrocellulose membrane and blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h. Primary antibodies were incubated (1:1000 for anti-Pin1) with membranes overnight at 4 °C. The following day, blots were washed with TBST three times and incubated with antirabbit secondary antibody (1:5000) for 1 h at room temperature (RT). Blots were washed three times with TBST and developed using luminol-based detection (PerkinElmer, Santa Clara, CA).

In-Solution Modification of Purified Pin1. Purified Pin1 was buffer-exchanged once with DPBS. Protein (2.5 μg, 6.9 μM) was diluted to 20 μL with DPBS and incubated with electrophile at 37 °C as indicated. Reactions were terminated with the addition of $NaBH_4$ at a final concentration of 20 mM for 30 min at RT. Protein samples were dried *in vacuo* and reconstituted in 10 μL of 6 M guanidine hydrochloride for 30 min at RT. Samples were reduced with dithiothreitol (150 μM) for 30 min at 37 °C and alkylated by 750 μM iodoacetamide for 15 min at RT in the dark prior to being diluted to 200 μL with 20 mM NH_4HCO_3. Because of the potential of adducts on Lys residues to result in mis-cleavage by trypsin, samples were digested with 500 ng of chymotrypsin (Promega, Madison, WI) for 24 h at 37 °C. Chymotryptic digests were concentrated and desalted using ZipTips (EMD Millipore, Billerica, MA) and eluted from tips with 60% acetonitrile/0.1% trifluoroacetic acid. Samples were mixed 1:1 by volume with matrix (20 mg/mL α-cyano-hydroxycinnamic acid (CHCA) in 60% acetonitrile) and analyzed by MALDI-TOF MS.

Analysis of Pin1 Peptides via MALDI-TOF and MALDI-TOF/TOF MS. An Autoflex Speed TOF MS or an Ultraflextreme TOF/TOF MS (Bruker Daltonics), both equipped with a Nd:YAG (solid state) laser operating at 355 nm, were used to obtain spectra. All spectra were obtained in positive ion mode. Peptide-CHCA solutions (1 μL) were deposited on 384-spot MALDI target plates and air-dried prior to analysis. Full mass spectra of peptides were obtained in reflectron mode on the Ultraflextreme, using a 500−4500 mass range. Spectra from treated and untreated samples were overlaid to identify peaks corresponding to masses appearing in spectra from ONE-treated Pin1 samples which did not appear in unmodified Pin1 samples. Selected peptide ions were dissociated using LIFT on the TOF/TOF. TOF/TOF fragmentation data were interrogated using FlexAnalysis software and analyzed against a theoretical Pin1 peptide digest using Protein Prospector.

Analysis of Pin1 Peptides via Orbitrap MS/MS. Purified Pin1 was buffer-exchanged once with DPBS. Protein (2 μg, 5.5 μM) was diluted to 20 μL with DPBS and incubated with 25 μM electrophile at 37 °C with agitation. Reactions were terminated with $NaBH_4$ at a final concentration of 20 mM for 30 min at RT. Samples were reduced with 150 μM DTT for 45 min, and available Cys residues were carbamidomethylated with 750 μM iodoacetamide for 45 min. Pin1 was digested with chymotrypsin (10 ng/μL) in 25 mM NH_4HCO_3 for 3 h at 37 °C. The samples were dried by vacuum centrifugation, and the peptides were reconstituted in 0.1% formic acid. Peptides were loaded onto a capillary reversed-phase analytical column (360 μm o.d.

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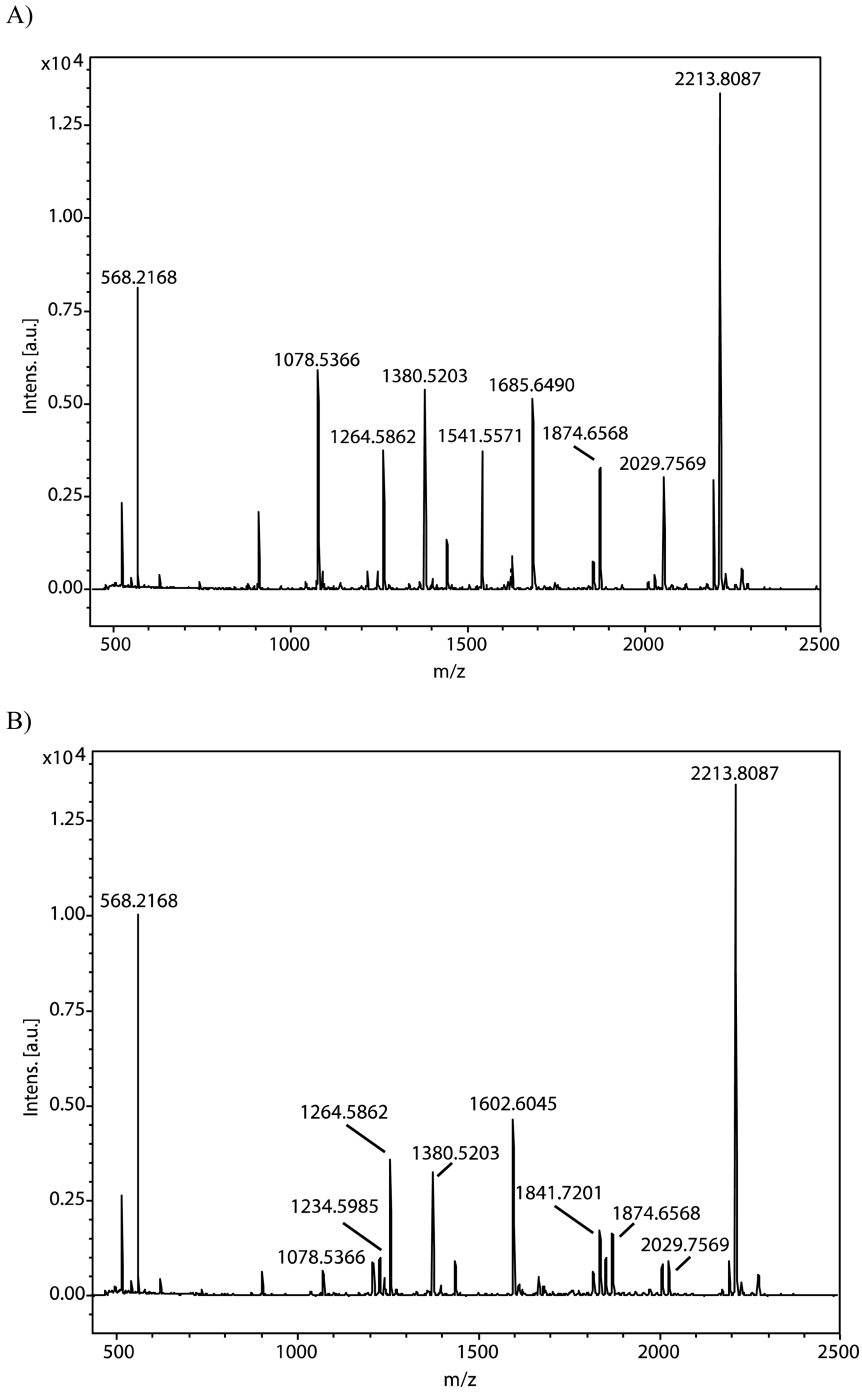

Figure 2. MALDI-TOF spectra of chymotryptic peptides generated from Pin1 treated with (A) vehicle (DMSO) or (B) 300 μM ONE. Treatment with ONE results in complete or nearly complete disappearance of the 1078, 1542, and 1685 m/z peaks and the appearance of 1234, 1603, and 1841 m/z peaks.

× 100 μm i.d.) using an Eksigent NanoLC Ultra HPLC and autosampler. The analytical column was packed with 20 cm of C18 reversed-phase material (Jupiter, 3 μm beads, 300 Å, Phenomenex), directly into a laser-pulled emitter tip. Peptides were gradient-eluted at a flow rate of 500 nL/min, and the mobile phase solvents consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). A 90 min gradient was performed, consisting of the following: 0−10 min, 2% B; 10−50 min, 2−45% B; 50−60 min, 45−90% B; 60−65 min, 95% B; 65−70 min 95−2% B; and 70−90 min, 2% B. Eluting peptides were mass analyzed on an LTQ Orbitrap Velos MS (Thermo Scientific), equipped with a nanoelectrospray ionization source. The instrument was operated using a data-dependent method with dynamic exclusion enabled. Full-scan (m/z 300−2000) spectra were acquired with the Orbitrap (resolution 60,000), and the top 16 most abundant ions in each MS scan were selected for fragmentation in the LTQ. An isolation width of 2 m/z, activation time of 10 ms, and 35% normalized collision energy were used to generate MS^2 spectra. Dynamic exclusion settings allowed for a repeat count of 2 within a repeat duration of 10 s, and the exclusion duration time was set to 15 s. For identification of Pin1 peptides, tandem mass spectra were searched with Sequest (Thermo Scientific) against a human subset database created from the UniprotKB protein database (www.uniprot.org). Variable modifications of +57.0214 on Cys (carbamidomethylation), +15.9949 on Met

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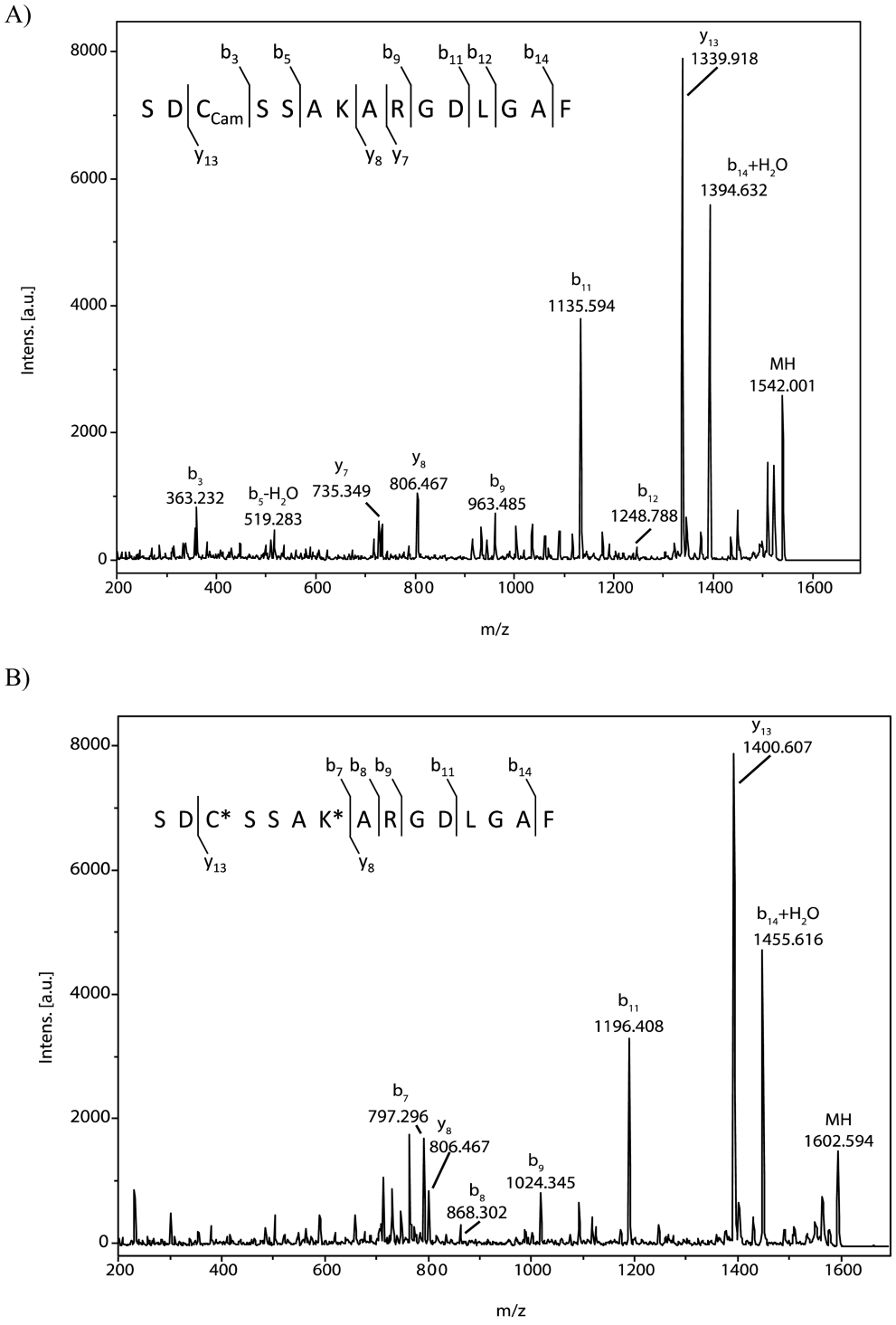

Figure 3. TOF/TOF spectra of the chymotryptic peptide containing the Pin1 active site Cys (SDCSSAKARGDLGAF) following (A) carbamidomethylation (Cam) with a parent ion at 1542 *m*/*z* and (B) ONE-treatment, resulting in a cross-link between Cys and Lys with a parent ion at 1603 *m*/*z*. In the cross-linked peptide fragmentation, the ions C-terminal to the Lys match the indicated peptide when an additional mass of +61 *m*/*z* relative to the carbamidomethylated peptide (57 Da + 61 Da = 118 Da) is considered on either the Cys or the Lys.

(oxidation), +141.1279 on Lys and Arg (corresponding to reduced Schiff base), +158.1306 on Cys, Lys, and His residues (corresponding to reduced ONE modification), +156.1150 on Lys (corresponding to the 4-ketoamide), and +118.0783 on Cys or Lys (corresponding to the pyrrole cross-link) were included for database searching. Search results were assembled using Scaffold 3.0 (Proteome Software). Spectra acquired of Pin1 peptides of interest were then inspected using Xcalibur 2.1 Qual Browser software (Thermo Scientific). The 4-ONE-cross-linked Pin1 peptide SDCSSAKARGDLGAF was confirmed following manual examination of the corresponding MS^1 and MS^2 spectra. For analysis of sample sets including 2D and 3D ONE treatments, Pin-1 was similarly digested with chymotrypsin, and peptides were subsequently analyzed using a targeted LC-MS/MS method on the LTQ Orbitrap Velos. A 90 min gradient was performed, consisting of the following: 0–14 min, 2–5% B; 14–70 min, 5–40% B; 70–78 min, 40–92% B; 78–79 min, 92–2% B; and 79–90 min, 2% B. For analysis of deuterium-containing cross-linked peptides, the LTQ Orbitrap Velos was operated using a combination

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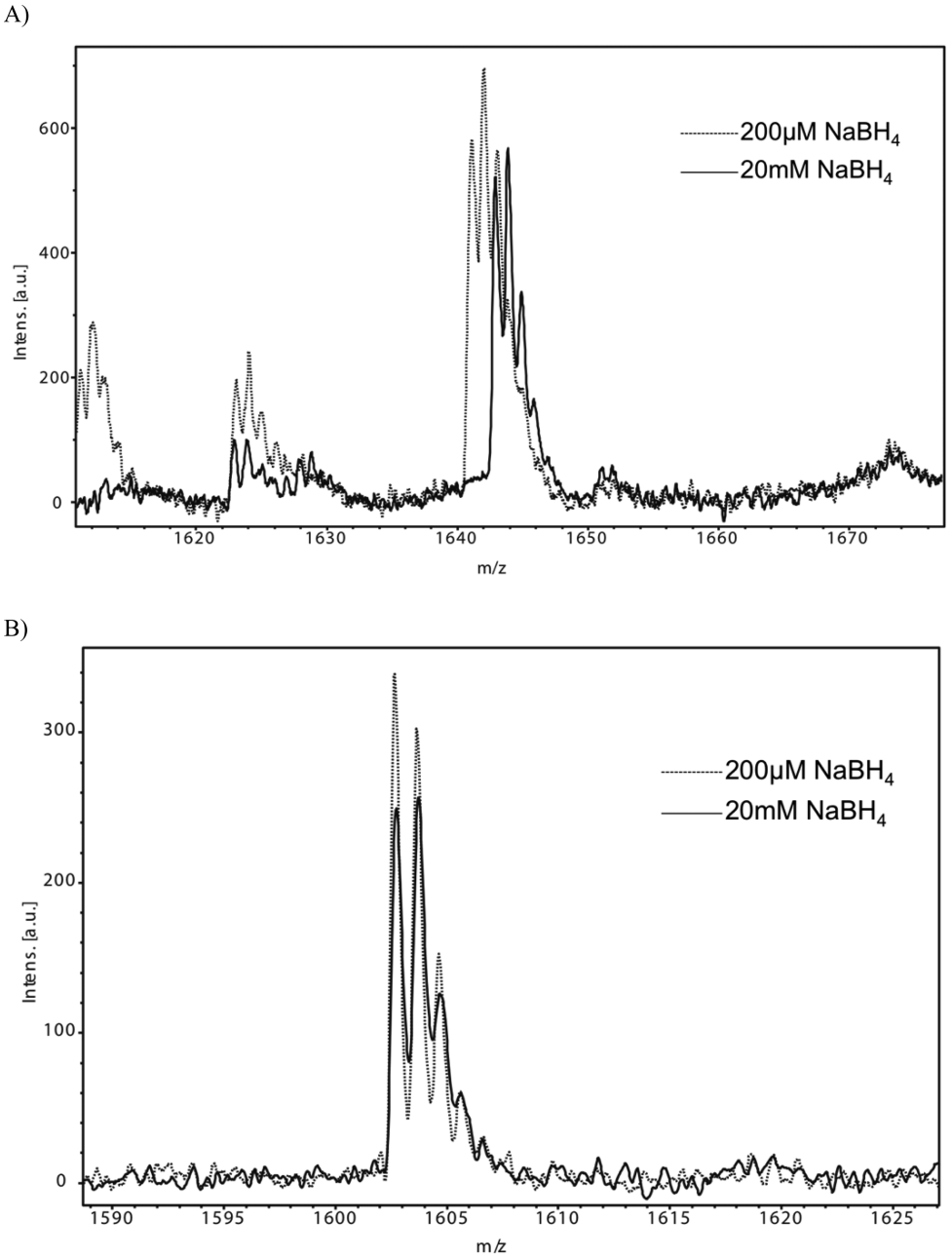

Figure 4. Analysis of the effects of $NaBH_4$ reduction on the Cys113-containing peptide treated with HNE or ONE. Cys113-HNE and the suspected Cys113-Lys117 pyrrole ONE adduct. (A) Pin1 exposed to HNE and treated with (solid line) or without (dashed line) $NaBH_4$. (B) Pin1 exposed to ONE with (solid line) or without (dashed line) $NaBH_4$.

method of data-dependent and targeted scan events. Targets were of specific m/z values corresponding to the 4-ONE cross-linked peptide, SDCSSAKRGDLGAF, and m/z values included those that would correspond to nondeuterated as well as deuterated cross-link forms. For these targeted scan events, MS2 spectra were acquired using the Orbitrap as the mass analyzer such that data were collected at higher resolution. Specifically, mass resolution of 15,000 was employed, and target AGC values were increased to $2e^5$ with a maximum ion time of 250 ms. All high-resolution MS2 data were analyzed by manual interrogation of unprocessed spectra.

RESULTS

Pin1 Modification by ONE Results in a Cys-Lys Pyrrole-Containing Cross-Link in the Active Site. To investigate the adduct chemistry of ONE-adducted Pin1, adducted peptides were examined for ions that were not present in an unmodified Pin1 digest; three new adduct ions were detected in the ONE-treated Pin1 sample. Our previous study identified the ion appearing at 1542 m/z to be the Cys113-containing peptide.[11] Although this peak was again present in the control Pin1 digest (Figure 2A), it was completely absent in the ONE-treated sample and was replaced by a peak at 1603 m/z (Figure 2B). The TOF/TOF spectrum of m/z 1603 (Figure 3B) was identified as the peptide containing Cys113, as evidenced by the most intense ions matching the theoretical peptide spectrum, but with a mass shift of +61 m/z relative to the carbamidomethylated peptide (Figure 3A). This mass shift represents a total mass shift of +118 m/z relative to the unmodified, noncarbamidomethylated

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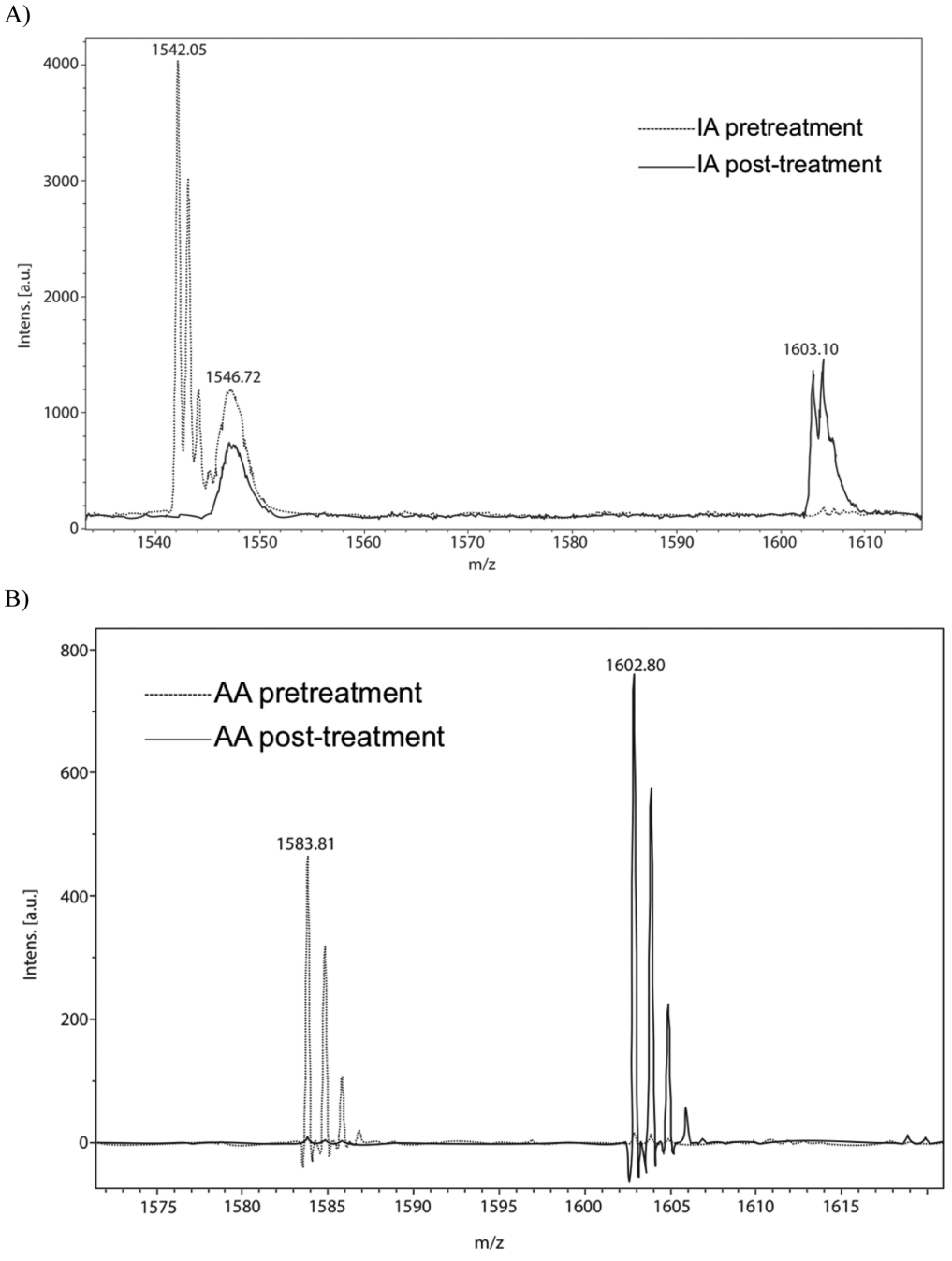

Figure 5. Effect of iodoacetamide and acetic anhydride on ONE-dependent Pin-1 adduction. (A) Pin1 was treated with 750 μM iodoacetamide (IA) prior to (dashed line) or after (solid line) exposure to ONE. (B) Pin1 was treated with 5 mM acetic anhydride (AA) prior to (dashed line) or after (solid line) exposure to ONE. Pretreatment of Pin1 with either IA or AA prior to ONE prevents the formation of the ONE adduct (1603 m/z), while treatment of Pin1 with either reagent after ONE exposure had no effect on adduct formation, suggesting a requirement for both Cys and Lys in adduct formation.

peptide. A previous study on the reaction of ONE with Histone H4 reported a +118 m/z mass shift corresponding to a His-Lys pyrrole-containing interpeptide cross-link.[20] Therefore, we interrogated the possibility of an active site cross-link resulting from the ONE reaction with Pin1.

All of the most intense ions in the TOF/TOF fragmentation spectrum of 1603 m/z were identified as fragment masses of SDC_{Cam}SSAKARGDLGAF N-terminal to Ala-118 with a variable mass shift of +61 m/z placed on either the Cys or Lys (Figure 3B). Reduction of ONE-modified Pin1 with $NaBH_4$ did not result in a mass shift, as evidenced by MALDI-TOF MS, suggesting the absence of a reducible carbonyl group (Figure 4B). By contrast, treatment of HNE-modified Pin1 with $NaBH_4$ resulted in a shift of +2 Da resulting from the reduction of the aldehyde group in the Cys113 Michael adduct to the corresponding alcohol (Figure 4A). The isotopic distribution of 1603 m/z in the ONE-Pin1 spectrum indicates a +1 charge state (data not shown), minimizing the possibility of a multiply charged interpeptide cross-link.

To determine the requirement of Cys113 for cross-link formation, Pin1 was pretreated with iodoacetamide to block Cys residues prior to ONE treatment. Under these conditions,

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the 1603 m/z peptide was eliminated; however, treatment with iodoacetamide after Pin1 modification by ONE did not interfere with the formation of the 1603 m/z peptide (Figure 5A). Similarly, to assess the requirement of Lys117, Pin1 was preteated with acetic anhydride, resulting in Lys acetylation, to block accessible Lys residues prior to ONE treatment. These conditions prevented the appearance of the 1603 m/z ion, whereas acetic anhydride treatment after ONE modification did not (Figure 5B). These data are supportive of an intrapeptide pyrrole-containing cross-link of +118 m/z resulting from the reaction of ONE with Cys113 and Lys117.

Cys-Lys Pyrrole Cross-Link in the Active Site of Pin1 Forms More Rapidly than Other Observed ONE-Modifications. Two additional peaks with m/z values corresponding to Pin1 peptides containing ONE-modifications were also identified (Figure 2B), although both were present in low abundance relative to the ion of the Cys113-Lys117 cross-link. Peptide masses of 1234 m/z and 1842 m/z in the spectrum of ONE-treated Pin1 corresponded to the addition of +156 m/z to SRGQMQKPFEDSAF and ADEEKLP-PGWEKRM, respectively. This mass shift is suggestive of reduced 4-ketoamide adducts derived from ONE modification of Lys residues.[5,21] Because of the relatively low ion intensities of these adducts formed upon Pin1 reaction with ONE, MALDI-TOF/TOF fragmentation resulted in rather low quality spectra; therefore, to further verify the sites of these adducts using a more sensitive approach, we analyzed these peptides using LC-coupled tandem mass spectrometry (LC-MS/MS). LC-MS/MS analysis of Pin1 treated with ONE identified 3 total adducts: the suspected cross-link and one each on Lys residues contained in the suspected peptides from the MALDI experiment (SRGQMQKPFEDSAF and ADEEKLP-PGWEKRM). The fragmentation of 1234 m/z indicates a ketoamide at Lys132 (Figure S1A in the Supporting Information), which was previously identified as a site for Michael addition on Pin1 by HNE.[11] Because the ADEEKLP-PGWEKRM peptide contains two Lys residues (Lys6 and Lys13), fragmentation of the ion was necessary to identify the specific amino acid site of modification. LC-MS/MS fragmentation spectra of peptides from Pin1 treated with ONE identified Lys13, not Lys6, as the site of adduction on this peptide (Figure S1B in the Supporting Information).

Since more than one adduct was identified in ONE-treated Pin1, we examined their relative rates of formation using MALDI-TOF MS. After proteolysis, peaks corresponding to unmodified and modified Pin1 peptides are detectable simultaneously in the MALDI-TOF spectra, so the relative rates of modification of the individual sites can be deduced.[22] Pin1 was incubated with either a fixed concentration of ONE for varying times or with varying concentrations of ONE for a fixed time. As shown in Figure 6A, the Cys-Lys pyrrole cross-link is formed very rapidly and at the lowest concentration of ONE. In contrast, the formation of ketoamide adducts at Lys132 or Lys13 requires high ONE concentrations (Figure 6B) and long reaction times. Comparison of the modification of Cys113 by equivalent concentrations of ONE and HNE indicated high reactivity with ONE but no reaction with HNE (Figure 7).

Reaction of Pin1 with Specifically Deuterated ONE. To determine the mechanism of cross-link formation and further elucidate the possible structure, deuterated ONE analogues were synthesized to contain a deuterium at C2 or at C3, designated 2D-ONE and 3D-ONE (Figure 1), respectively.

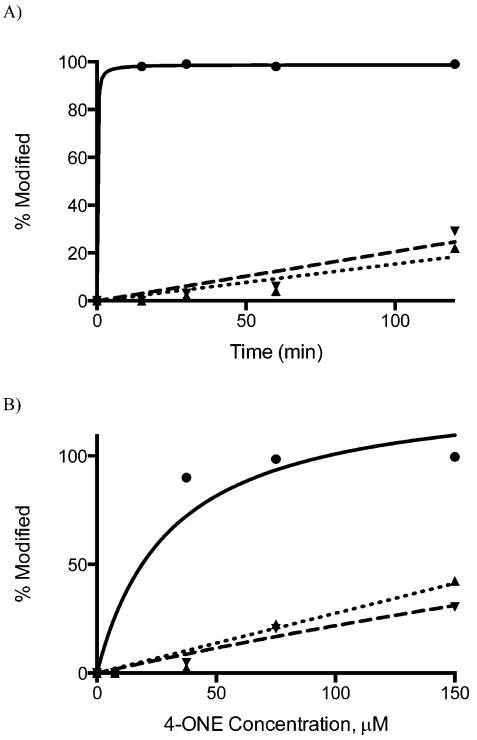

Figure 6. Relative reactivity of recovered Pin1 adducted peptides as a function of (A) time of exposure to 200 μM ONE and (B) ONE concentration for 1 h. % Modified was calculated using the ion intensity of the formed adduct divided by the sum of the intensities of the adduct ion and the corresponding unadducted ion. The Cys113-Lys117 pyrrole adduct (solid line) outcompetes the other two adducts (Lys13 (dashed line), Lys132 (dotted line)) observed.

Recombinant Pin1 was incubated with vehicle control, ONE, 2D-ONE, or 3D-ONE (25 μM) for 1 h, carbamidomethylated, and then digested with chymotrypsin. Under these conditions, the Cys113-containing peptide ($SDC_{Cam}SSAKARGDLGAF$), is observed as parent ion with a +2 charge at 771.35 m/z (Figure S2A in the Supporting Information). Treatment with ONE results in a mass shift of +61 (m/z 30.5), producing a parent ion at 801.88 m/z, consistent with the formation of the cross-link (Figure S2B in the Supporting Information). The 2D-ONE-treated sample has a major +2 parent ion at 802.38 m/z. This observed ion has a mass error of 1.2 ppm relative to the theoretical mass of the peptide containing a deuterium within the cross-link, thereby verifying the presence of deuterium at the C2 position in the cross-link. Additionally, fragmentation of 802.38 m/z showed a shift in the observed b-series ions corresponding to the presence of deuterium in fragment ions containing the cross-linked portion of the peptide (Figure S2C in the Supporting Information). Interestingly, the 801.88 m/z peak is still present in the isotopic distribution of SDCSSAK-ARGDLGAF with the 2D-ONE, indicative that some cross-linked peptides do not contain the deuterium. The spectrum for the 3D-ONE sample shows the dominant +2 parent ion at 801.88 m/z, 0.4 ppm relative to the theoretical mass of the nondeuterated cross-link. Fragmentation of this peptide shows a spectrum identical to that of the undeuterated ONE sample, further indicating that the deuterium is not present in the cross-link (Figure S2D in the Supporting Information). These data indicate that the first step in cross-link formation, Michael addition of Cys113, occurs through nucleophilic attack at C3 of

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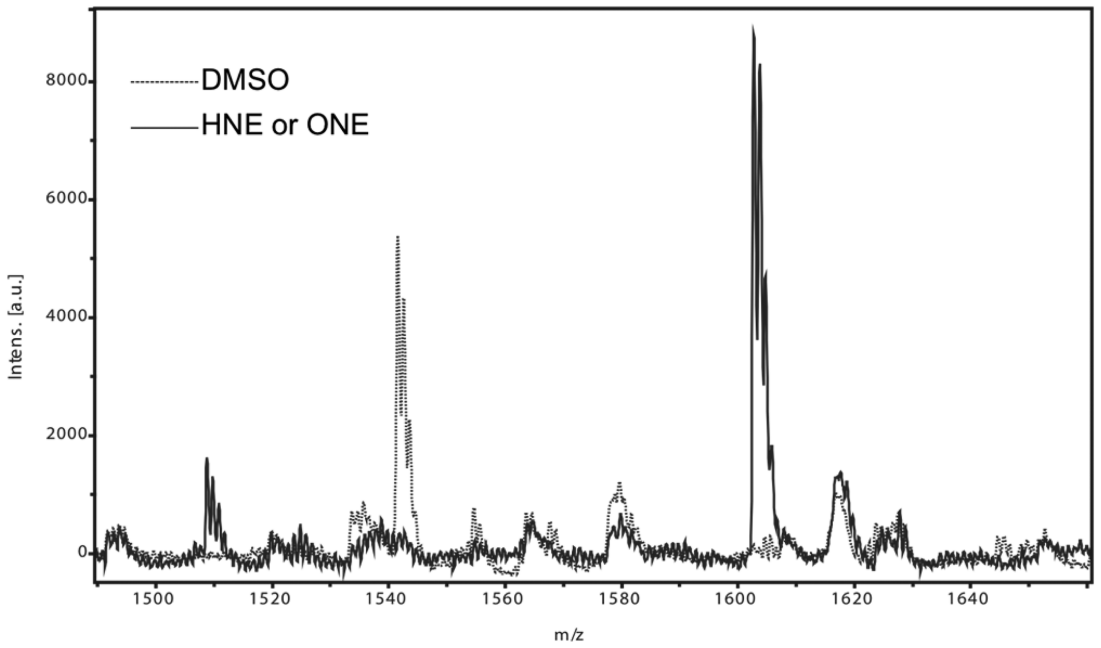

Figure 7. Competition of HNE versus ONE for the active site cysteine (Cys113). Pin1 was incubated with DMSO (dashed line) or a 50:50 mixture of 150 μM each HNE/ONE (solid line), digested with chymotrypsin, and analyzed by MADLI-TOF mass spectrometry for the presence of the Cys113-HNE Michael adduct (1643 m/z) and the Cys113-Lys117 ONE cross-link (1603 m/z).

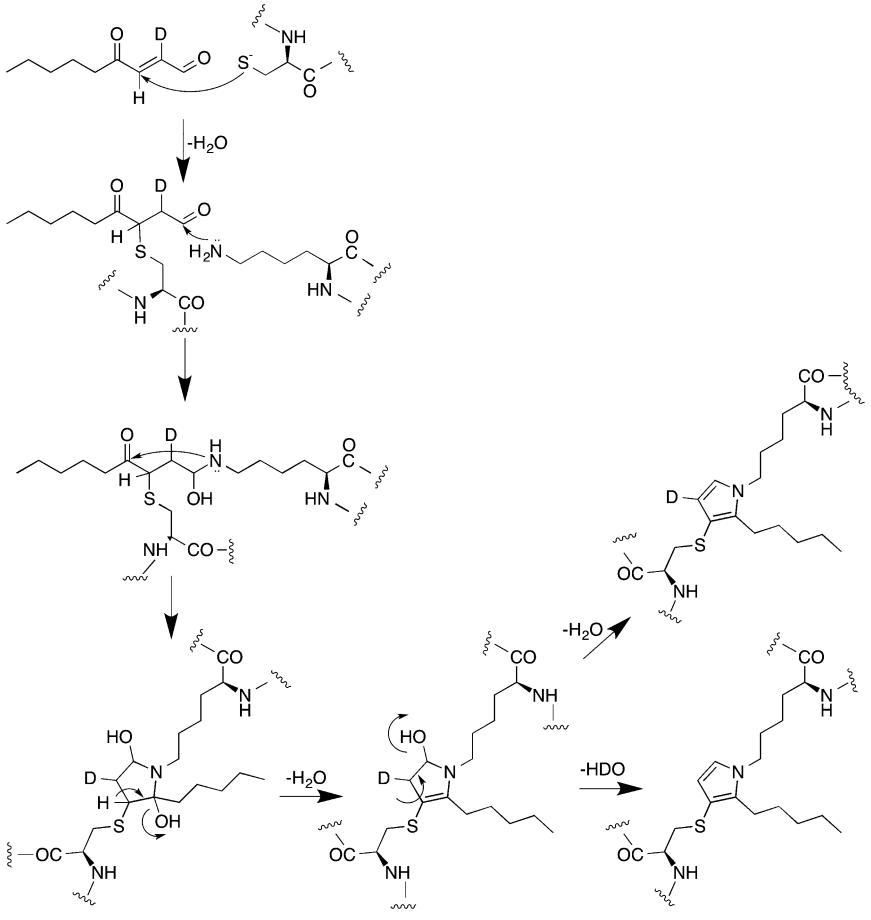

Figure 8. Proposed mechanism of cross-link formation. Cross-link formation is initiated by nucleophilic attack of the thiolate of Cys to C3 of ONE. The ε-amine of lysine then attacks C1 of ONE to form the carbinolamine, followed by an additional attack to C4 of ONE to form the pyrrolidine. Two dehydration reactions result in the formation of the pyrrole-containing cross-link.

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ONE, resulting in the loss of the deuterium in that position (Figure 8).

Pin1 Is a Target of ONE in MDA-MB-231 Cells. To assess the susceptibility of Pin1 to modification by ONE in a cellular setting, MDA-MB-231 cells were treated with varying concentrations of aONE (Figure 1) for 1 h. Following click chemistry, streptavidin pull-down, and cleavage of the photocleavable biotin linker, Pin1 Western blotting was conducted. As shown in Figure 9, Pin1 is susceptible to modification by

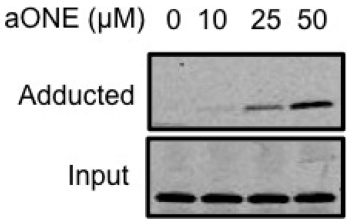

Figure 9. Western blot of adducted Pin1 from aONE-exposed MDA-MB-231 cells. Click chemistry was performed on total cells lysates with increasing concentrations on aONE. Following photoelution of aONE-modified proteins, eluates (adducted) and total cell lysates (input) were separated by SDS−PAGE and subjected to Western blot with anti-Pin1 antibody.

aONE at 10 μM, which is within the pathological range of electrophiles.[1,23,24] Given the high efficiency of formation of the Cys-Lys cross-link *in vitro*, it is likely that this is the identity of the modification in the intact cells.

DISCUSSION

Many previous studies on the reactivity of lipid electrophiles with proteins have focused on HNE, as it has long been considered a major lipid hydroperoxide-derived electrophile. However, the discovery of ONE as another important electrophilic product of lipid peroxidation has generated interest in the relative reactivity of ONE with DNA and proteins, as compared to that of HNE. Relative to ONE, HNE-protein adducts are relatively straightforward to investigate, mainly because the principal reaction of HNE with proteins is the formation of Michael adducts to Cys or His residues. In contrast, despite a difference in structure of only two hydrogen atoms, ONE-derived modifications to proteins can be profoundly more difficult to characterize, largely due to the rapid reactivity with Cys and Lys residues, the potential to cross-link between two residues, and the ability to generate adducts with multiple chemical structures depending on the microenvironment.[5,21,25,26] Therefore, the physiological spectrum of potential adducts arising from ONE is far more complicated than that of HNE.[26] Additionally, some ONE adducts, including the ketoamide and possibly the cross-link, are irreversible, making these adducts significantly more stable and inherently longer lived.[21] The more persistent effects of ONE modifications makes them a pivotal area of research.

Because of the fact that ONE generates various structural modifications, MS-based analysis of ONE-treated proteins likely provides the most information in elucidating site-specific protein adducts. Incubation of purified Pin1 with ONE revealed mass shifts of +156 m/z and +118 m/z relative to Pin1 chymotryptic peptides. Through multiple independent experiments, our data support the identity of an ONE adduct to Pin1 as a Cys-Lys pyrrole-containing cross-link in the active site of the protein (Figure 8). Blockage of either Cys113 or Lys117 by iodoacetamide and acetic anhydride, respectively, prevented the formation of the cross-link by ONE. Furthermore, reduction via $NaBH_4$ did not result in an additional mass shift. These data suggest that the resulting adduct does not contain a carbonyl functionality, further indicating the pyrrole adduct, which lacks a carbonyl. This adduct was formed at lower concentrations of ONE and shorter incubation times, relative to other ONE adducts, and its formation also completely outcompeted the formation of the Cys-HNE Michael adduct, indicating that this reaction proceeds with considerable efficiency relative to those of many other electrophile-protein modifications.

A Cys-Lys pyrrole-containing cross-link derived from ONE was detected by Zhu et al.[27] in the reaction of oxidized linoleic acid with β-lactoglobulin, but it represented a small fraction of the total ONE adduct burden; this contrasts with the present findings with Pin1, which indicate that the pyrrole-containing cross-link forms rapidly and in high yield. Examination of the crystal structure of Pin1 provides insights into the possible reasons for the high reactivity of Pin1 with regard to the formation of this adduct (Figure 10). Cys113 sits in the active

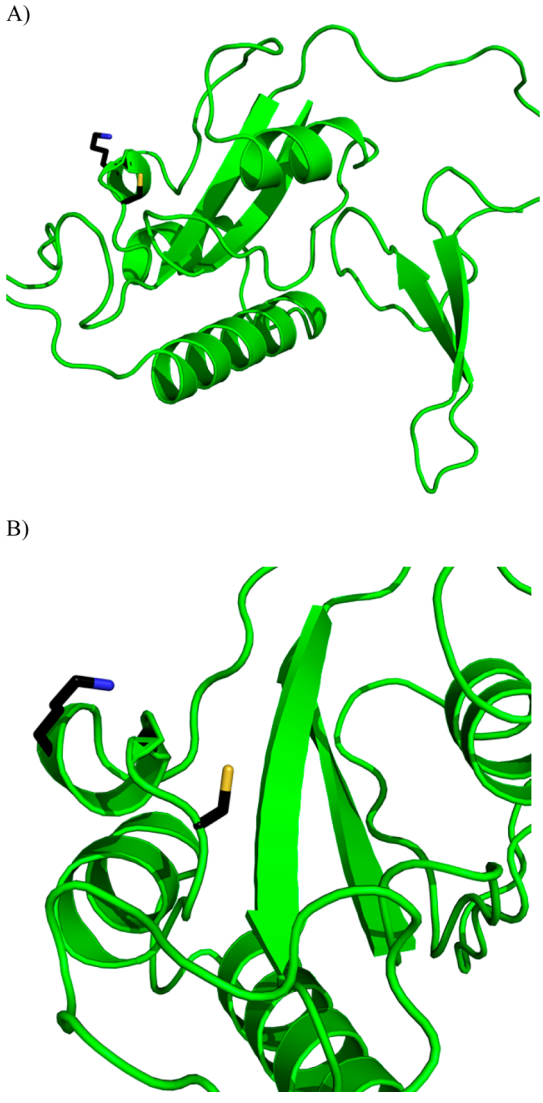

Figure 10. Pin1 crystal structure. (A) Crystal structure of Pin1 highlighting Lys117 and Cys113 (black). (B) Cys113 side chain orientation relative to Lys117.

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site of the enzyme and is separated from Lys117 by only 6.5 Å. Lys117 is located on the turn of a short α helix (5 amino acids) C-terminal to Cys113. The Lys117 side chain is directed toward Cys113, facilitating the reaction of the amino group with the initial Michael adduct formed by the reaction of ONE with the catalytic Cys (Figure 8). Since Cys113 participates as a nucleophile in peptidyl cis−trans isomerization, it has enhanced nucleophilicity compared to those of other nucleophilic sites in the protein, allowing it to trap ONE and position the carbonyl groups of the Michael adduct adjacent to Lys117 for condensation. Oe et al.[20] have reported the formation of a His-Lys pyrrole-containing cross-link on the reaction of ONE with Histone H4 between His75 and Lys77, and indicated that −HAK− amino acid sequences in proteins may represent a primary sequence target motif for the formation of the pyrrole adduct resulting from ONE. This adduct could also likely be formed between a Cys/His residue distant from a Lys residue based on primary sequence but spatially close based on the three-dimensional structure of the protein. The Cys-Lys epitope modified by ONE in Pin1 is both spatially close and separated by only four amino acids, supporting the feasibility of the reaction for this specific protein. The studies with deuterated ONE analogues further support the mechanism of pyrrole cross-link formation predicted by Oe et al.[20] (Figure 8).

Treatment of cells with aONE followed by click chemistry conjugation to biotin revealed that Pin1 is modified by aONE as a function of concentration. The results support that aONE does not alter the total level of Pin1, but rather modifies the existing pool of protein. Interestingly, oxidative modification of Pin1 has been observed in the brains of Alzheimer's disease patients, and the modification leads to inhibition of Pin1 isomerase activity.[28] Pin1 inhibition has been suggested to underlie the formation of neurofibrillary tangles in the AD brain, thereby catalyzing disease pathogenesis.[29] Isomer-specific antibodies of tau, a Pin1 substrate, display increased *cis*-tau labeling in the AD brain compared to that in the control brain, indicating a Pin1 inhibitory event.[30] Because of the rapid formation of the Cys-Lys pyrrole adduct in our *in vitro* experiments, we expect the same modification to occur in cells exposed to ONE. Furthermore, Miyashita et al.[31] demonstrated that adduction of a pyrrole onto Lys residues increases protein surface electronegativity, resulting in the formation of a damage-associated molecular pattern capable of triggering an autoimmune response.

cis-Isomers of proline-containing peptide bonds occur with a frequency of 5−6%, and many of these bonds are present at bend, coil, or turn conformations, which are surface exposed.[32,33] Phosphorylation of serine or threonine preceding a proline in peptide bonds renders this motif resistant to isomerization by conventional PPIases, except Pin1. Pin1 binds protein substrates through a conserved WW-binding domain, followed by isomerization of the peptide bond by the PPIase domain. Some protein substrates of Pin1 contain multiple pSer-Pro or pThr-Pro motifs, and the overall three-dimensional structure and therefore protein activity can be dictated by whether these bonds are in cis or trans.[34] Because modifications to Pin1 can adversely affect the network of proteins it controls, elucidation of potential oxidative adducts to this protein is of high importance, particularly considering that oxidative stress and Pin1 dysfunction coexist in some diseases.[35] The ONE adduct to Pin1 may be a particularly important contributor to cellular dysfunction associated with oxidative stress because it forms rapidly and in high yield, completely blocks the active site, and is irreversible. The biological implications of Pin1 cross-linking by ONE are currently being explored by our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Figures of tandem mass spectra of ketoamide adducts and the cross-linked peptide with deuterated ONE. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Pin1, peptidylprolyl *cis/trans*-isomerase A1; HNE, 4-hydroxy-2-nonenal; ONE, 4-oxo-2-nonenal; AD, Alzheimer's disease; PPIase, peptidyl-prolyl *cis/trans*-isomerase; ATCC, American Type Culture Collection; aONE, alkynyl-4-oxo-2-nonenal; 2D-ONE, 2-[^2H]-4-oxo-2-nonenal; 3D-ONE, 3-[^2H]-4-oxo-2-nonenal; RT, room temperature; TBST, tris-buffered saline plus Tween-20; CHCA, α- cyano-4-hydroxycinnamic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; IA, iodoacetamide; AA, acetic anhydride

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