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Carbonylation of Mitochondrial Aconitase with 4-Hydroxy-2-(*E*)-nonenal: Localization and Relative Reactivity of Addition Sites

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Abstract

Mass spectrometry was used to investigate the effects of exposing mitochondrial aconitase (ACO2) to the membrane lipid peroxidation product, 4-hydroxy-2-(*E*)-nonenal (HNE). ACO2 was selected for this study because (1) it is known to be inactivated by HNE, (2) elevated concentrations of HNE-adducted ACO2 have been associated with disease states, (3) extensive structural information is available, and (4) the iron-sulfur cluster in ACO2 offers a critical target for HNE adduction. The aim of this study was to relate the inactivation of ACO2 by HNE to structural features. Initially, western blotting and an enzyme activity assay were used to assess aggregate effects and then gel electrophoresis, in-gel digestion, and tandem mass spectrometry were used to identify HNE addition sites. HNE addition reaction rates were determined for the most significant sites using the iTRAQ approach. The most reactive sites were Cys³⁵⁸, Cys⁴²¹, and Cys⁴²⁴, the three iron-sulfur cluster-coordinating cysteines, Cys⁹⁹, the closest non-ligated cysteine to the cluster, and Cys⁵⁶⁵, which is located in the cleft leading to the active site. Interestingly, both enzyme activity assay and iTRAQ relative abundance plots appeared to be trending toward horizontal asymptotes, rather than completion.

Keywords

mitochondrial aconitase; 4-hydroxy-2-(*E*)-nonenal; protein carbonylation; iTRAQ labeling; mass spectrometry

Introduction

The , -unsaturated aldehyde, 4-hydroxy-2-(*E*)-nonenal (HNE), which is generated by membrane lipid peroxidation [1,2], has come to be associated with numerous diseases [3,4]. While adduction of HNE to DNA is important [5], here we focus on the reactions of HNE with proteins. Elevated concentrations of HNE-adducted proteins have been known to be correlated with Parkinson's [6] and Alzheimer's [7] diseases since the mid-1990s. Antibody- [8] and absorbance-based [9] assays exist for the estimation of total protein HNE-addition load, but these methods alone provide an incomplete picture since modification impact would be expected to be closely related to addition site. Mass spectrometry (MS) is well-suited to addition site localization [10]. Furthermore, with carefully constructed experiments, relative site reactivity can be estimated [11–13]. Since HNE production is a radical-induced process, HNE flux would be expected to be especially high from mitochondrial membranes. While numerous mitochondrial proteins must be impacted, mitochondrial aconitase (ACO2) has been selected for this investigation because (1) reduced activity and elevated HNE-modification levels have been reported in the brains of

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Alzheimer's disease patients [14], (2) ACO2 activity has separately been found to be reduced on exposure to HNE in cell culture [15], (3) extensive structural information is available, and (4) the iron-sulfur cluster may make it particularly sensitive to HNE adduction.

ACO2 is an important citric acid cycle enzyme, containing a cubane $[4\text{Fe-4S}]^{2+}$ cluster, that catalyzes the stereo-specific interconversion of citrate to isocitrate via *cis*-aconitate [16,17]. In addition to the inorganic sulfides, three of the iron-sulfur complex irons coordinate cysteines, while the fourth (Fe) coordinates substrate [18–21]. ACO2 is inherently sensitive to oxidation of the solvent-exposed $[4\text{Fe-4S}]^{2+}$ cluster, which results in decomposition to an inactive $[3\text{Fe-4S}]^+$ form (the Fe is lost) [22–25]. Inactivation by superoxide [26,27], hydrogen peroxide [28], and peroxynitrite [29] in this manner has been reported. However, damage to the cluster is not the only conceivable pathway to enzyme inactivation. ACO2 exposure to superoxide has been shown to result in the generation of hydroxyl radicals [30], and exposure to peroxynitrite has been shown to result in tyrosine nitration and cysteine oxidation to cysteic acid [31]. Large HNE adducts would be expected to have an effect on addition not only to active site amino acid side chains [17], but also to nearby reactive residues. Furthermore, since the relative stability of HNE when compared to radical species allows diffusion over greater distances [2], oxidative damage in non-membrane proteins such as ACO2 may be biased away from radicals and towards species like HNE.

HNE concentrations have been reported to be circa 0.1 μM in normal human blood plasma [32], but up to 100 μM is conceivable in and around the membranes where lipid peroxidation is occurring [2]. Protein modification by HNE occurs most frequently through Michael additions to the side chains of cysteine, histidine, and lysine, to create protein carbonyls. Michael adducts at cysteine and histidine are relatively stable, while those at lysine are less so [33,34]. MS has previously been applied to HNE-exposed hemoglobin [35], -lactoglobulin B [35], apomyoglobin [36,37], cytochrome *c* [38,39], albumin [11–13,40,41], cytochrome *c* oxidase [42], and rat brain mitochondrial proteins [43]. None of these proteins offers an iron-sulfur cluster, where a single cysteine modification could eliminate enzyme activity. In this work, HNE addition to porcine ACO2 was first investigated by western blotting. The impact of HNE addition on enzyme activity was then assayed. Finally, MS was used to locate HNE addition sites and to follow addition reaction progression over time, using the iTRAQ approach [44]. A commercially available porcine heart aconitase preparation was used for these experiments. Gel electrophoresis and in-gel digestion were employed to improve sensitivity because ACO2 was present at low concentration in this preparation. In a two-step approach, HNE addition sites were first identified and then those sites that appeared to be most reactive were targeted for reaction rate measurements.

Experimental Procedures

Western Blotting Assay

Commercial porcine heart aconitase was supplied by Sigma (St. Louis, MO) under catalog number A5384. HNE was obtained from Cayman Chemical (Ann Arbor, MI). Aconitase preparations were exposed to 0, 12, 60, and 120 μM HNE for 2 h at 37°C with gentle shaking. The impure protein preparation was present at 1 $\mu\text{g}/\mu\text{L}$ in the reaction mixture and the buffer used was PBS (pH 7.4). After the incubation period was complete, the procedure divided into two branches. In the first, excess HNE was immediately removed by repeated washes using 50 kDa molecular weight cut-off (MWCO) centrifugal filter devices (Millipore; Billerica, MA). In the second, incubation for 60 min at room temperature with NaBH_4 , added to 5 mM, was used to quench the HNE adduction reaction before buffer exchange using the 50 kDa MWCO centrifugal filter devices. The BCA assay

(ThermoFisher Scientific; Rockford, IL) was then used to determine total protein concentrations.

Aliquots of each sample containing 40 µg of protein were mixed with equal volumes of 2× Laemmli buffer (Bio-Rad; Hercules, CA), heated at 95°C for 5 min, and then separated by SDS-PAGE on a homemade gel (4% stacking, 8% resolving). A ladder of protein molecular weight markers was also loaded. When not proceeding to transfer, the gel was fixed and stained using Imperial Protein Stain (ThermoFisher Scientific). Otherwise, proteins were transferred to a PVDF membrane. Blocking was achieved by incubation at room temperature for 1 h with 5% non-fat dry milk in tris-buffered saline containing 0.05% Tween 20 (TBST). After TBST washes, the blocked membrane was incubated overnight at 4°C with the mouse anti-HNE primary antibody (NHEJ2; Japan Institute for the Control of Aging; Fukuroi, Japan) diluted 1:1000 in 5% bovine serum albumin (BSA) in TBST. Subsequently, following TBST washes, the membrane was incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-mouse secondary antibody diluted 1:5000 in 5% BSA in TBST. Finally, following TBST washes, the signal was developed using an enhanced chemiluminescence visualization kit (GE Healthcare; Pittsburgh, PA).

Enzymatic Activity Assay

Changes in aconitase activity were assayed in response to reaction time (0, 1, 2, and 6 h) at fixed HNE concentration (120 µM) and to HNE concentration (0, 12, 120, and 600 µM) at fixed reaction time (2 h) using kits obtained from Cayman Chemical. In all cases, the crude aconitase preparation was present at 1 µg/µL and reactions were performed at 37°C in 100 mM HEPES buffer (pH 7.4) with gentle shaking. Reactions were terminated by protein precipitation on ice with 80% ammonium sulfate. Following 80% ammonium sulfate washes and resuspension in 50 mM tris buffer (pH 7.4), protein concentration was determined using the BCA assay. Enzyme activity was then measured according to the kit manufacturer's instructions. Briefly, aconitase preparations were first incubated on ice for 1 h with an activation solution composed of 50 mM cysteine and 1 mM ferrous ammonium sulfate in 50 mM tris buffer (pH 7.4). For enzyme activity determination, solutions of activated aconitase, NADP⁺, isocitrate dehydrogenase, and citrate, were made up in triplicate both with and without an inhibitor (oxalomalate) present. After incubation at room temperature for 30 min, the NADPH generation rate was measured by recording absorbance at 340 nm every 2 min for 30 min.

Mass Spectrometric Assay

First, modification sites were surveyed by exposing crude aconitase to 12, 24, 120, and 600 µM HNE for 2 h at 37°C with gentle shaking. Second, using the iTRAQ relative quantitation approach (Applied Biosystems; Foster City, CA), reaction progression was monitored by exposing aconitase to 120 µM HNE for 0, 1, 2, and 6 h at 37°C with gentle shaking. In all cases, the aconitase preparation was present at 1 µg/µL and the buffer used was PBS (pH 7.4). After the initial exposure was complete, excess HNE was consumed by adding dithiothreitol (DTT) to 10× the HNE concentration and then incubating at room temperature for 30 min. Proteins were denatured and HNE modification-associated cross-linking was reduced by adding urea to 6 M and then incubating at room temperature for 30 min. Finally, adducted HNE was stabilized by adding NaBH₄ to 5 mM and incubating for 60 min at room temperature. Repeated washes employing 50 kDa MWCO centrifugal filter devices were used to remove excess reagents and concentrate samples for SDS-PAGE.

Samples were readied for SDS-PAGE by mixing with an equal volume of 2× Laemmli buffer and heating at 95°C for 5 min. Electrophoresis was performed on a homemade gel (4% stacking, 8% resolving), with samples split and loaded to multiple wells. A protein

molecular weight ladder was also loaded and, once the separation was complete, the gel was fixed and stained using Imperial Protein Stain. Gel bands containing aconitase were carefully excised and subjected to an in-gel digestion procedure. Briefly, after destaining, gel slices were repeatedly washed with 100 mM ammonium bicarbonate (aq) and then dehydrated with acetonitrile. Fixed proteins were reduced with DTT and then alkylated with iodoacetamide before the gel slices were again subjected to washing and dehydration. Digestion, using sequencing grade trypsin (Promega; Madison, WI) or chymotrypsin (Roche Diagnostics; Indianapolis, IN), was carried out overnight at 37°C. Both trypsin and chymotrypsin were used for the modification site survey (varying HNE concentration), but only trypsin was used for the reaction progression experiment (varying reaction time). Peptides were extracted from the gel using 0.1% formic acid in 95/5 water/acetonitrile followed by 0.1% formic acid in 50/50 water/acetonitrile. Aliquots were then combined and the resulting peptide solutions were dried in a centrifugal evaporator. For modification site survey samples, dried peptides were stored at -20°C until liquid chromatography (LC)-MS analysis could be performed. The whole sample was then reconstituted in a minimal volume of equilibration mobile phase and consumed in a single run.

For reaction progression experiment samples, intended for relative quantitation measurements using the iTRAQ system, peptide mixtures were reconstituted and then desalted using Nest Group (Southboro, MA) MicroSpin C₁₈ solid phase extraction columns according to the manufacturer's suggested protocol. Eluate was dried in a centrifugal evaporator and then reconstituted to allow peptide solution concentrations to be determined using the BCA assay. Aliquots of each sample containing 25 µg of peptide were dried in a centrifugal evaporator and then reconstituted in 25 µL 100 mM triethylammonium bicarbonate (aq) for labeling with the iTRAQ reagents. The 0 h sample was labeled with the 114 reagent, 1 h with 115, 2 h with 116, and 6 h with 117. After incubation at room temperature for 2 h with gentle shaking, the peptide mixtures were combined and then peptides were recovered from excess reagents using a Waters (Milford, MA) Oasis MCX solid phase extraction cartridge. The final sample was dried using a centrifugal evaporator and then resuspended in the appropriate LC-MS equilibration mobile phase. Peptide solutions were then stored at -20°C until LC-MS analysis could be performed.

Ion Trap MS—Modification site survey samples were analyzed using a micro-scale LC-MS system centered on a Thermo (San Jose, CA) LTQ XL linear ion trap mass spectrometer. This instrument was not used with iTRAQ reagent-labeled, reaction progression experiment samples. The LTQ XL was interfaced with a Thermo Surveyor LC system. Peptides were separated on a Column Technology (Fremont, CA) CTCAP5150100 reversed-phase, C₁₈ column (5 µm particles, 150 µm × 100 mm, 300 Å pores) at a flow rate of ~0.5 µL min⁻¹ using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in methanol as mobile phase B. Approximately 2 µg of peptides were injected and a Michrom (Auburn, CA) CapTrap trapping column was used to reduce sample loading time. Equilibration mobile phase was 2% B. The gradient program began with a rapid increase to 15% B over 5 min, which was followed by slower increases to 80% B over 70 min and then to 95% B over 15 min. Eluting peptides were introduced into the mass spectrometer through a nanospray ion source operating at a spray voltage of 2.1 kV and an inlet capillary held at 200°C. The instrument was programmed to cycle between collecting survey spectra, which were used to locate precursor ions for fragmentation, and tandem mass spectrometry (MS/MS) spectra, which were used for peptide identification. The four most abundant precursor ions from each survey spectrum (300 to 2000 *m/z* ratio range) were subjected to collision-induced dissociation (CID) MS/MS. Precursor ions picked for fragmentation twice within a 30 s window were excluded from further analysis for 180 s.

Orbitrap MS—Modification site survey and reaction progression experiment samples were analyzed using a micro-scale LC–MS system centered on a Thermo LTQ Orbitrap Velos mass spectrometer. Separations were performed on a Waters nanoACQUITY reversed-phase, C₁₈ column (1.7 μ m particles, 100 μ m $\times$ 100 mm). Elution was achieved using a gradient of 0.1% formic acid in acetonitrile (B) versus 0.1% formic acid in water (A) at a flow rate of 0.4 μ L min⁻¹. Approximately 2 μ g of peptides were injected, with the equilibration mobile phase being 1% B. The linear gradient initially ran to 15% B over 25 min, then to 25% B over 35 min, then to 35% B over 40 min, and finally to 85% B over 20 min. Eluting peptides flowed into a nanospray ion source that was operated at 3.5 kV. The Orbitrap-based system was operated in a similar manner to the linear ion trap-based system, cycling between survey and MS/MS spectra, but instead of CID fragmentation, higher-energy C-trap dissociation (HCD) was employed [45]. Parameters included normalized collision energy set to 40 and activation time at 0.1 ms. For modification site survey samples, MS/MS spectra were recorded for the ten most intense ions in each survey spectrum; ions selected twice for fragmentation within a 30 s window were excluded for 180 s. For reaction progression experiment samples, where iTRAQ reporter ion measurements were required, a targeted mass list was used and MS/MS spectra were recorded for the eight most intense targeted ions in each survey spectrum.

Peptide Identification—Peptides were identified using the BioWorks version 3.3.1, SP1 implementation of Sequest (Thermo) supplemented, for LTQ Orbitrap Velos raw data files, with a standalone version of ExtractMSn version 5.0 (Thermo). Scan grouping was not performed in preparing peak lists for database searching. The protein sequence database was constructed on February 8, 2012, using the NCBI Reference Sequence version of the complete pig proteome (24575 proteins) and 112 common contaminant proteins (Global Proteome Machine, common Repository of Adventitious Proteins; current version at <ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta>). The sequence for ACO2 (NCBI Reference Sequence NP_999119.1) was modified by deleting the 27-amino acid mitochondrial targeting peptide to allow detection of the N-terminal peptide of the mature protein. Reversed versions of all sequences were included for false discovery rate estimation [46]. Trypsin was defined as cutting to the C-terminal side of lysine and arginine and chymotrypsin was defined as cutting to the C-terminal side of phenylalanine, tryptophan, tyrosine, methionine, and leucine; for both enzymes, cleavage was not permitted when the residue to the C-terminal side of the proposed break was proline. Only peptides fully conforming to these rules were considered in the search and up to three missed cleavage sites were allowed.

Precursor ion tolerances were ± 2 Da for linear ion trap measurements and ± 15 ppm for Orbitrap measurements. Fragment ion tolerances were ± 1 Da for CID spectra (linear ion trap) and ± 0.05 Da for HCD spectra (Orbitrap). Monoisotopic masses were used in all cases except for linear ion trap precursor ion measurements, where average masses were used. Fixed mass shifts were applied for alkylated cysteines (+57.021464 Da) while differential mass shifts were incorporated for oxidized methionines (+15.994915 Da), NaBH₄-reduced Schiff base adducts at lysine (+140.120115 Da), and NaBH₄-reduced Michael adducts at cysteine (+101.109216 Da when the fixed mass shift is considered), histidine, and lysine (both +158.130680 Da). Each peptide was permitted a maximum of four variable modifications. When the iTRAQ labels were present, fixed mass shifts of +144.103991 Da were added at lysine residues and the peptide N-terminus, which resulted in the differential mass shifts associated with HNE modification at lysine being changed to +14.026689 Da (Michael adducts) and -3.983876 Da (Schiff base adducts). Raw Sequest output was refined using version 4.5.2 of the Trans-Proteomic Pipeline (TPP) software package (Institute for Systems Biology; Seattle, WA). Specifically, a PeptideProphet [47] scoring threshold was used for identification acceptance. MS/MS spectra for modified peptides were manually examined and any found to be inconsistent with the proposed identification were rejected.

Relative Quantitation Measurements—Reporter ion intensities were extracted and isotopic corrections were applied using the TPP Libra module. For each accepted spectrum, all four reporter ions intensities, excepting the 0 h time point for modified peptides, were required to have intensity 100. For each peptide, linear regression through the origin was used to calculate consensus relative abundance ratios [48]. Reporter ion intensities at each time point were plotted either against the 0 h-intensity for unmodified peptides or against the 6 h-intensity for modified peptides. Calculated ratios were corrected using an ACO2 peptide in which both peptide termini resulted from trypsin-conforming cuts to the C-terminal side of arginine and where the remaining peptide sequence did not contain any potential modification sites (WVIGDENYEGSSR). The errors given are linear regression standard errors multiplied by 95% confidence *t*-values (two-tailed test). In cases where data for more than one unmodified peptide was available for an addition site, the most frequently-detected peptide was used.

Results

HNE mainly reacts with proteins through Michael addition to the nucleophilic amino acid side chains of cysteine, histidine, and lysine; for lysine, Schiff base formation is also possible [2–4]. Michael adducts at cysteine and histidine are effectively stable while those at lysine are more prone to disassociation; Schiff base adducts at lysine are even less stable than Michael adducts at lysine [33,34]. As indicated in Scheme 1, Michael addition results in equilibrium between a form containing an aldehyde functional group and a hemiacetal isomer. Scheme 1 also shows the results of reduction with NaBH₄, which was required to stabilize adducts and prevent further reaction through the later stages of the experimental procedure. Reduction also results in simplified CID MS/MS spectra: non-reduced HNE-adducted peptide CID MS/MS spectra are dominated by HNE neutral loss peaks, which can be used as an analytical strategy [42,43,49], but the neutral loss is not observed for reduced adducts [40,50,51]. While electron capture dissociation MS/MS for non-reduced HNE-adducted peptides has also been shown to result in spectra where neutral losses are not problematic [37,49,51], we decided to follow the reduction and CID approach due to the advantage of preventing additional reactions at the aldehyde site during sample preparation. Once the 27-amino acid mitochondrial targeting sequence has been removed, porcine ACO2 is an 83 kDa protein with 12 cysteine, 25 histidine, and 53 lysine residues. There are no disulfide bridges, but the side chains of Cys³⁵⁸, Cys⁴²¹, and Cys⁴²⁴ are coordinated with the iron-sulfur cluster as ligands [18–21]. HNE has the potential to modulate ACO2 activity by addition to amino acid side chains in the active site region of the enzyme.

Western Blotting Assay

To confirm that addition of the α,β -unsaturated aldehyde to ACO2 had occurred and to estimate the relative degree of modification at different HNE concentrations, western blotting with a mouse monoclonal anti-HNE antibody was performed. The primary antibody used has been described as being particularly sensitive to modified histidine sites [8]. From initial test runs, where the gel was stained instead of being subjected to transfer, it became clear that the crude aconitase preparation contained numerous proteins (see Supplementary Data Figure S1a). While both NaBH₄-reduced and non-reduced samples were analyzed, HNE-modified proteins were only visualized for the non-reduced samples (see Supplementary Data Figure S1b). Clearly, the antibody is effective only for the non-reduced, hemiacetal form of the HNE adduct. For the non-reduced samples, numerous bands were observed and staining became more intense with increased HNE concentration. The band position of aconitase could only be determined roughly because the anti-aconitase antibody used was only weakly responsive in this study (data not shown).

Enzymatic Activity Assay

Aconitase activity was measured using an established indirect assay where NADPH generation, resulting from the conversion of isocitrate to α -ketoglutarate by the enzyme isocitrate dehydrogenase, was monitored by measuring absorbance at 340 nm [23,52]. Two comparisons were made: in the first, incubation time was kept constant (2 h) while HNE concentration was varied (0, 12, 120, and 600 μ M); in the second, HNE concentration was fixed (120 μ M) while the incubation period was changed (0, 1, 2, and 6 h). Each sample was analyzed in triplicate, both with and without an aconitase inhibitor (oxalomalate). Rates found in the presence of the inhibitor were subtracted from those found without the inhibitor. Figure 1 shows the impact of (a) increasing HNE concentration at fixed reaction time and (b) increasing reaction time at fixed HNE concentration for the crude aconitase preparation. For both comparisons, a gradual loss of activity with increasing HNE concentration was observed, as expected, but it appeared that activity was declining toward somewhere in the region of half the original value rather than toward zero. Large errors preclude a more detailed discussion of these results. Higher HNE concentrations and longer incubation periods could not be examined due to extensive protein precipitation, presumably caused by HNE-associated cross-linking. The data used to construct Figure 1 is also available in tabular form as Supplementary Data Table S1.

Mass Spectrometric Assay

While enzyme activity assays and anti-HNE western blotting can provide aggregate information for the whole protein, and while NMR spectroscopy and X-ray crystallography can offer detailed structural information when pure samples are available, MS is able to generate useful data with complex mixtures of differentially modified proteins, even for sites where the degree of modification is low. Of the two mass spectrometers used in this work, the LTQ XL linear ion trap system can be regarded as the standard instrument. The LTQ Orbitrap Velos system adds higher mass measurement accuracy and offers HCD, where high m/z ratio fragment ions can be collected simultaneously with low m/z ratio iTRAQ reporter ions, in addition to CID MS/MS. High mass measurement accuracy can be particularly valuable in improving identification confidence. For example, NaBH_4 -reduced Michael adducts have a close mass coincidence with the isobaric amino acid residue pairs, alanine and serine, and glycine and threonine ($m = 0.06$ Da).

HNE Addition Site Identification—Addition sites were located by identifying the modified peptides that resulted from the in-gel digestion of HNE-exposed ACO2. Concentration-dependence was investigated by exposing the crude aconitase preparation to HNE at 12, 24, 120, and 600 μ M. Identified modification sites for the trypsin and chymotrypsin digestions are presented together in Table 1. For both digestion enzymes, the experiment was repeated four times at each of the HNE concentrations. In each case, three repeats were analyzed using linear ion trap CID MS/MS while one was investigated using Orbitrap HCD MS/MS. Only one LC-MS run was recorded for each sample preparation due to the limited amount of sample obtained from in-gel digestion. The numbers presented in Table 1 are peptide-spectrum match (PSM) counts (often referred to as spectrum counts), which can provide an approximate measure of relative abundance [53]. PSM counts for all peptides supporting the presence of each modification site (trypsin and chymotrypsin data combined) were summed to construct Table 1. However, linear ion trap and Orbitrap data were summed separately. Since many factors influence the likelihood of obtaining a PSM, count magnitudes should chiefly be compared between different conditions for the same site, rather than between different sites. All modified peptide identifications were manually validated (see Supplementary Data). Modification sites detected using both instrumental platforms were regarded as high confidence while those found using only one system were

assigned medium confidence. These data are also presented in terms of modified peptide as Supplementary Data Table S2.

PeptideProphet score thresholds for PSM acceptance were 0.90 for tryptic and 0.98 for chymotryptic peptides. These thresholds were chosen to obtain false discovery rates (FDRs), calculated as twice the reversed database PSM count divided by the sum of the real and reversed database PSM counts [54], of less than 1%. For trypsin, twelve linear ion trap/CID runs gave 47735 PSMs at 0.80% FDR and four Orbitrap/HCD runs gave 9427 PSMs at 0.87% FDR; ACO2 peptides constituted 55.8% of linear ion trap and 53.3% of Orbitrap PSMs. For chymotrypsin, twelve linear ion trap/CID runs gave 8734 PSMs at 0.87% FDR and four Orbitrap/HCD runs gave 4395 PSMs at 0.73% FDR; ACO2 peptides constituted 66.9% of linear ion trap and 74.3% of Orbitrap PSMs. Combined ACO2 sequence coverage for all experiments was 94.2%, with only three histidine (His²⁷⁴, His⁶⁴⁶, and His⁶⁵³) and three lysine (Lys²⁷⁷, Lys²⁷⁸, and Lys⁶⁰¹) residues remaining inaccessible (see Supplementary Data Figure S2). Combined sequence coverage for all trypsin digests was 68.0% while that for chymotrypsin was 88.5%. Trypsin was relatively ineffective because the ACO2 sequence contains regions poor in conforming cleavage sites, resulting in large peptides that are difficult to identify using this approach (Lys¹³³ to Lys¹⁹⁸ results in a 65-, Lys²⁹⁶ to Lys³⁴³ results in a 47-, and Lys²³¹ to Arg²⁷⁵ results in a 44-amino acid peptide).

From Table 1 it can clearly be seen that more modification sites were identified as the applied HNE concentration was increased. Counts of modification sites identified at high confidence were 4, 6, 9, and 15 at HNE concentrations of 12, 24, 120, and 600 μ M, respectively. High-confidence identifications at one or more HNE concentration levels were obtained for 16 sites. Medium-confidence counts were 4, 6, 15, and 14 sites at HNE concentrations of 12, 24, 120, and 600 μ M, respectively. The total number of sites only identified at medium confidence was 17. Cysteine sites dominate at the lowest HNE concentration tested (the four high-confidence hits at 12 μ M are Cys⁹⁹, Cys³⁵⁸, Cys⁴²⁴, and Cys⁵⁶⁵); however, nine cysteine, fifteen histidine, and nine lysine sites were found when all investigated concentrations were considered (ACO2 contains 12 cysteines, 25 histidines, and 53 lysines). Four sites were identified using both digestion enzymes while 18 were found only in tryptic and 11 were found only in chymotryptic peptides (see Supplementary Data Table S2). All identified modification sites were Michael additions; no Schiff bases were detected.

In general, the expected pattern of modification site identifications becoming stronger with increasing HNE concentration was observed. However, a reduction in PSM count was observed on going from 120 to 600 μ M HNE for a number of the sites. This decline can be correlated with the detrimental sample precipitation that was observed on incubation at higher applied HNE concentrations. Cross-linking, caused by two residues reacting with the same HNE molecule [55,56], could be responsible for this aggregation, resulting in precipitates that are not amenable to enzymatic digestion. Cross-linking will also lead to branched peptides that cannot be identified using our Sequest approach. While those steps added to the sample preparation procedure to reduce cross-linking were clearly beneficial, as evidenced by sharper SDS-PAGE bands, they were clearly not sufficient to eliminate this phenomenon. Furthermore, unlike with the iTRAQ approach, no obvious means of correction exists.

Five of the eight modification sites that were identified at the lowest HNE concentrations were cysteines. Three of these, Cys³⁵⁸, Cys⁴²¹, and Cys⁴²⁴, are in the active site and involved in the iron-sulfur cluster. This result matches previous research that showed that cysteine is the most reactive Michael addition site in proteins [4], but it is noteworthy that the iron-sulfur cluster is available for modification by HNE. Also evident in the Orbitrap

HCD MS/MS spectra were peaks at $m/z = 268.2$, consistent with the generation of reduced, modified histidine immonium ions. As the amount of HNE increases, first more modified histidines appear, then more lysines. Overall, HNE addition was observed at 75% of cysteine, 60% of histidine, and 17% of lysine residues. However, effects other than concentration can have a major impact on the ability to identify modifications. Some care needs to be employed in analyzing data from these types of experiments. Therefore, a more direct quantitation method, such as iTRAQ reagent-labeling, is needed for ranking reactivities.

Modification Reaction Rate Estimation—The iTRAQ approach was used to quantify the accumulation of modified and the concomitant depletion of related unmodified peptides resulting from exposure of the crude aconitase preparation to 120 μM HNE for 0, 1, 2, and 6 h. However, relative abundance measurements rely on the detection of low- m/z ratio reporter ions. Due to a fundamental limitation of resonance excitation CID, which is the standard fragmentation method used in the linear ion trap mass spectrometer, fragment ions can only be detected that have m/z ratios greater than approximately one third of that of the precursor ion. Switching to pulsed q -CID [57] allows reporter ions to be detected with the linear ion trap instrument, but we have previously found reporter ions to be of lower intensity and the resulting ratios to be less precise when compared with those obtained using LTQ Orbitrap Velos HCD MS/MS [13].

Variance in extracted reporter ion ratios for spectra supporting the same peptide, even when using HCD MS/MS, was often substantial, requiring large numbers of repeat measurements for adequate accuracy. Therefore, expected peptide m/z ratios were targeted and only tryptic peptides were considered, since PSM counts were higher for trypsin in the initial survey. While the increased number of measurements obtained makes trypsin the best choice in general, chymotrypsin might seem like a better choice for the lysine sites (since a modified lysine would be expected to lead to a missed cleavage); however, nearly all modified lysine PSMs in the initial survey were identified from tryptic peptides (see Supplementary Data Table S2). For the anticipated peptides, all singly, doubly, and triply charged precursor ions in the m/z ratio range from 300 to 2000 Th were targeted. Table 2 lists the modified peptides that were used to cover 17 modification sites. When more than one site was present on the same peptide, all combinations of absence and presence of the modifications were included. Unmodified versions of the listed peptides were also targeted; for unmodified peptides that contained a trypsin cleavage site, all possible peptides were included. A peptide bounded by arginine tryptic cleavage sites and containing no HNE addition sites (WVVIGDENYGEGR) was also added to the list to act as an internal standard. Naturally, following this strategy, it was not practical to monitor all possible HNE addition locations. The most significant sites from the initial survey were chosen along with a selection of other locations designed to allow relative cysteine, histidine, and lysine reactivity to be compared. His⁵¹⁹ was included because peptides for this site, although they did not score above threshold in the initial survey, scored well in pilot iTRAQ runs performed without targeting (the addition of iTRAQ tags can significantly impact peptide detectability). Only Michael additions were considered as no Schiff bases had been detected in the initial survey.

The data presented here are the result of three replicate LC–MS runs performed on a single sample preparation at each time point. Longer reaction times and higher applied HNE concentrations were not possible in this study due to poor protein solubility under such experimental conditions. Even under the conditions investigated, a significant amount of material was lost. The internal standard peptide, WVVIGDENYGEGR, for which 611 PSMs with all four reporter ions having intensity 100 were obtained, gave a 0 : 1 : 2 : 6 h ratio of 1 : 0.79 : 0.74 : 0.66. This decline in signal intensity can clearly be seen in

Supplementary Data Figure S3. WVVIGDENYGEGSSR was used to correct all modified and unmodified peptides.

Results for iTRAQ relative abundance ratio measurements are presented as Figure 2. (These results are also provided in tabular form as Supplementary Data Table S3 and individually for each peptide as Supplementary Data Figures S4-S28.) Cysteines (with associated histidines that share the same peptides), histidines, and lysines are displayed on separate axes. For the depletion plots, the 0 h time point is scaled to one, while the 6 h time point is scaled to one for the accumulation plots; however, this does not imply that the reaction has gone to completion at 6 h. Generally, depletion plots are easier to compare because the starting point is well defined—time zero gives 100%. In contrast, for the modified peptides, there is no way to know when 100% conversion has been reached (standards are not available). Therefore, relative intensity for the modified peptides cannot readily be related to concentration. Furthermore, the modified peptides often give low signal intensities, sometimes due to low concentration and sometimes due to poor ionization characteristics, which would be expected to result in greater random error.

Quantitative information was obtained for all five targeted cysteine HNE addition sites. However, relative abundance ratio measurements were only obtained for the unmodified peptide associated with Cys⁴²¹ and Cys⁴²⁴ (Figure 2a). Direct detection of Cys⁴²¹ and Cys⁴²⁴ is difficult because very high quality MS/MS spectra are often required to unambiguously localize isobaric modifications on the same peptide. While both sites were detected in the initial survey, the addition of iTRAQ tags probably deleteriously altered peptide fragmentation patterns. Another possibility is that the addition of the iTRAQ tags brought the chromatographic peaks for the two isobaric peptides closer, resulting in both molecules being fragmented together, which would result in MS/MS spectra that would be rejected by the scoring algorithm because both possible explanations would have very similar scores. Cys⁹⁹ and Cys⁵⁶⁵ were found both alone and in combination with other modifications. Since Cys⁹⁹ and Cys⁵⁶⁵ share peptides with other modification sites, the contribution of these sites to depletion of the associated unmodified peptides is not certain. Cys³⁵⁸ is the only targeted cysteine isolated from other targeted modification sites; furthermore, the supporting peptide is bounded by arginine tryptic cleavage sites and contains no other potential modification sites. Therefore, depletion of the Cys³⁵⁸-associated unmodified peptide should provide a clear picture of reactivity at this site.

Beginning with 800 µg of the crude aconitase preparation yielded 30 µg of peptides after in-gel digestion for the 0 h sample, where losses due to HNE-associated cross-linking are not expected. This provides an initial estimate of the ACO2 concentration in the crude preparation of 3.75% by mass. Using the proportion of PSMs that indicated ACO2 peptides in the initial survey would tend to lower while a consideration of sample preparation-associated losses would tend to increase this percentage. While we cannot currently provide an accurate measurement, it seems reasonable to assume that the crude preparation was less than 10% ACO2 by mass. Therefore, the ACO2 concentration used throughout these experiments would have been at most circa 1.2 µM. Given the applied HNE concentration was 120 µM, pseudo-first order methodology can be used to estimate reaction rates (only relative protein concentrations are required for reaction rate calculation by this method, so knowledge of the exact ACO2 concentration is not essential). For Cys³⁵⁸, using depletion of the unmodified peptide and excluding the 6 h data point, a reaction rate of $0.3 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$ can be calculated. Similarly, a combined rate of $0.9 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$ can be obtained from the unmodified peptide containing the Cys⁴²¹ and Cys⁴²⁴ sites. Because both are part of the iron-sulfur cluster, distinguishing between them is not as critical. For comparison, values for the reaction of HNE with N-acetylated cysteamine and RRWWCR-amide of 1.21 ± 0.03 and $6.8 \pm 0.6 \text{ M}^{-1} \text{ s}^{-1}$, respectively, have been reported [58], while that given for the reaction of

HNE with the only non-bridging cysteine in human serum albumin, Cys³⁴, was circa 30 M⁻¹ s⁻¹ [41].

The cysteines that share peptides with histidine modification sites are more difficult to analyze. Depletion of the His⁹⁸, Cys⁹⁹, and His¹⁰¹-associated unmodified peptide suggests a rate of $0.6 \pm 0.3 \text{ M}^{-1} \text{ s}^{-1}$, while that for the Cys⁵⁶⁵ and His⁵⁶⁹ peptide is $0.6 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$. However, while they probably made the largest relative contributions, depletion of the unmodified peptides alone cannot show that the cysteines were the most reactive sites. For the accumulation plots, since the end point is not well defined, curve shape is the best guide to reactivity. The expected ratio for a very rapidly reacting site would be 0 : 1 : 1 : 1, that is, the reaction is complete or has reached equilibrium before the 1 h time point. For the least reactive sites, a curve-shape close to a straight line would be expected because one is measuring essentially initial velocities with little change in substrate concentration. For the first case (Figure 2b), not all possible combinations were detected, but the modified peptide containing Cys⁹⁹ alone clearly saturates at the earliest time. The observed decrease in relative abundance at the longest reaction time is probably due to addition of a second HNE molecule to the peptide. For the peptide containing Cys⁵⁶⁵ and His⁵⁶⁹, all possible combinations were detected: Cys⁵⁶⁵ appears fastest, followed by His⁵⁶⁹ and then the combined Cys⁵⁶⁵ and His⁵⁶⁹ modified peptide (Figure 2b). It is likely that the histidines represent only a small fraction of the depletion at early times and play a greater role late in the kinetic studies. This assumption is supported by the rate constants estimated below for individual histidines. As a result, it is reasonable to assume that depletion of the parent peptides of Cys⁵⁶⁵ and, especially, Cys⁹⁹ is mainly caused by cysteine modifications at the 6 h time point.

At least some accumulation information was obtained for all targeted histidines, but a majority of the addition sites were only detected in combination with other modifications. For the histidines, reasonable depletion curve shapes (Figure 2c) are seen for His⁴⁶ and His⁵¹⁹, resulting in rate estimations of $0.16 \pm 0.06 \text{ M}^{-1} \text{ s}^{-1}$ and $0.13 \pm 0.04 \text{ M}^{-1} \text{ s}^{-1}$, respectively. As expected, the histidines react more slowly than the cysteines. For His⁴⁶⁰, an unusual curve shape is seen; depletion reverses at longer reaction times and the calculated errors are particularly large. Therefore, any rate estimated for His⁴⁶⁰ is likely to be misleading. Considering the accumulation plots for the isolated histidines, the curve shapes are all similar and indicate relatively low reactivity. The histidines that share peptides with cysteines were discussed above, leaving only His⁹ and His¹³. These sites were only detected together, with the accumulation curve shape suggesting low reactivity. Depletion of the unmodified peptide suggests a rate of $0.24 \pm 0.03 \text{ M}^{-1} \text{ s}^{-1}$, which is in line with the rates seen for single modification sites. Overall, for the histidine addition sites, only modest depletion is observed and the accumulation plots (Figure 2d) indicate that most are far from reaching completion at 6 h; they still retain nearly peak reaction velocities.

Evaluation of the lysines is easier since all are isolated from both other modification sites and each other. However, Lys⁴⁹⁰ was not detected and the other sites show very limited depletion (see Figure 2e). In fact, the depletion plots appear to be indicating no change over the time period monitored, indicating that the addition of HNE to lysine sites is inefficient. Furthermore, in the accumulation plots (Figure 2f), significant background modification is seen at time zero. This might perhaps represent a small concentration of the modification present in the starting aconitase or be background noise in the iTRAQ ion region of the spectrum. In either case, the plots in Figure 2e indicate that lysine modifications are not significant under these conditions and only reach concentrations suitable for detection, but not quantification. However, these data do support the expected relative reactivity relationship between the cysteine, histidine and lysine addition sites.

Discussion

After performing the initial western blotting assays, it became clear that the crude aconitase preparation was a protein mixture of some complexity. Using protein concentration and MS measurements, the proportion of ACO2 in the commercial preparation was estimated to less than 10% by mass. This low relative concentration necessitated the adoption of a SDS-PAGE band-cutting and in-gel digestion approach. One of the greatest practical problems in this work was sample precipitation and poor data quality at higher applied HNE concentrations. Processing of these samples resulted in smeared gel bands and few MS/MS identifications. HNE-associated cross-linking was assumed to be the cause (two different residues can form bonds with the same HNE molecule [55,56]), with intra-molecular linkages being responsible for the smearing. Cross-linking would be expected to impact MS/MS performance through the production of branched peptides not amenable to our Sequest-based identification strategy.

Therefore, since some part of the cross-linking is known to be prone to dissociation [56], a procedure was developed that did succeed in improving data quality at higher applied HNE concentrations. DTT was first added to consume excess HNE and then urea was used to denature modified proteins. Incubation under these conditions is expected to be effective in pushing the equilibrium of Schiff base formation backwards, with the denaturing conditions improving the entropic favorability of breaking linkages. Since dissociation of Michael adducts at lysine would also be favored by these conditions, the impact of this procedure was investigated by exposing crude aconitase to HNE at 120 μ M, a concentration at which precipitation was not a significant problem, and then testing the DTT and urea treatment against proceeding immediately to reduction with NaBH₄. Similar modifications were observed for treated and untreated samples. However, comparison is hindered by the detection of relatively few lysine adducts at an applied HNE concentration of 120 μ M. Moreover, the obvious drawback of this strategy is that the ability to detect Schiff base adducts is at least greatly reduced and possibly completely lost (none were found).

Relative Amino Acid Reactivity

ACO2 contains twelve cysteines, three of which are ligated to the iron-sulfur cluster, but does not contain any disulfide bridges [18–21]. The ligated cysteines (Cys³⁵⁸, Cys⁴²¹, and Cys⁴²⁴), along with Cys⁹⁹, the closest non-ligated cysteine to the cluster, and Cys⁵⁶⁵, which is located in the cleft leading to the active site, were found to be among the most reactive sites in the initial survey and were targeted to obtain kinetic information using the iTRAQ approach. Approximate reaction rates with HNE were obtained for all five targeted cysteines, using the depletion of the associated modified peptides, but all except Cys³⁵⁸ are located on tryptic peptides containing other modification sites. However, modified peptide accumulation curve shapes (see Figure 2), where available, support assigning the majority of the unmodified peptide depletion rate to the cysteine addition sites, resulting in the following order of reactivity: Cys⁴²¹ & Cys⁴²⁴ > Cys⁹⁹ $\approx$ Cys⁵⁶⁵ > Cys³⁵⁸. Naturally, since Cys⁴²¹ and Cys⁴²⁴ share the same peptide, only a combined rate could be calculated. Accumulation curves cannot be used directly for rate calculations because the final degree of modification is not known.

Accumulation curves for Cys⁴²¹ and Cys⁴²⁴ would be expected to provide a rough guide to apportioning the combined rate, but quantitative data was not obtained for either site. However, detections from the initial survey would seem to support similar reactivity at these sites, indicating that the combined rate could be split evenly. Since Cys⁵⁶⁵ was identified in previous studies as being the most reactive with electrophiles [59,60], it is somewhat surprising that the rate for Cys⁹⁹ is similar. However, while the Cys⁵⁶⁵-associated peptide contains only one histidine addition site, the Cys⁹⁹-associated peptide contains two, possibly

indicating that Cys⁵⁶⁵ is slightly more reactive than Cys⁹⁹. Overall, the data indicate that the cysteines are more reactive than the histidines, and that the lysines have very limited reactivity. This is consistent with earlier work.

Residue accessibility to the solvent is one possible explanation for the differences in observed reactivity. To test this hypothesis, and to provide some insight into the environments of the reactive residues, solvent-accessible surface areas (SASAs) were calculated using GETAREA software (<http://curie.utmb.edu/getarea.html>) [61]. The input crystal structure was Protein Data Bank identifier 7ACN, which contains the cubane iron-sulfur cluster and isocitrate [21]. Plots of side chain and backbone SASAs for ACO2 cysteines and histidines are provided as Figure 3. Calculated side chain SASA values indicate that all cysteines are buried, but that some histidines are exposed to the solvent (His⁹, His¹³, His²⁷⁴, and His³⁰⁶). To annotate residues as buried, the side chain SASA must be under 20% of the random coil value for the amino acid in question. Exposed sites are defined as those having side chain SASAs of more than half this value. The random coil value is effectively maximum side chain SASA and is equal to 102.3 for cysteine and 154.6 Å² for histidine. From the plots, there seems to be no relationship between SASA and reactivity for either the cysteines or histidines. Accessibility may play a role at some locations, but it clearly is not the most important factor. In contrast, there does appear to be a clear correlation between site reactivity and proximity to the active site region of the protein. Finally, lysines were not considered because no experimental relative reactivity information was available.

Correlation between HNE Modifications and Enzyme Activity

Exposure of ACO2 to peroxynitrite has been reported to result in the oxidation of Cys³⁵⁸ to cysteic acid [31], a modification that would clearly impact enzyme function. The presence of HNE adducts in the active site region would also be expected to reduce enzyme activity. Naturally, additions to the iron-sulfur cluster-ligated cysteines (Cys³⁵⁸, Cys⁴²¹, and Cys⁴²⁴) and to the key active site histidines (His¹⁰¹, His¹⁴⁷, and His¹⁶⁷) [17] would be most significant, but sites where additions might block access to or alter the conformation of the active site region could also be important. For example, additions to Cys⁵⁶⁵ are reported to reduce activity by restricting substrate access to the active site cavity [59,60].

Western blot staining was observed to darken with increased applied HNE concentration, but limited dynamic range precluded quantitative measurements. Conversely, the enzymatic assay allowed quantitation of reduced ACO2 activity with increasing applied HNE concentration. However, both assays can only generate aggregate information, which is not sufficient to disprove alternative hypotheses such as modifications distant from the active site region resulting in changes in protein conformation that alter enzymatic activity. For example, the fourth domain of the closely-related protein cytoplasmic aconitase is postulated to swing out to allow RNA binding [17,62]; an addition in the hinge region that caused the fourth domain to swing out would represent a change distant from the active site that would be expected to impact aconitase activity. The MS approach selected for this study allowed HNE addition sites to be identified and, using iTRAQ labeling, provided approximate rates of reaction at targeted sites. The addition sites identified as being most reactive (Cys⁹⁹, Cys³⁵⁸, Cys⁴²¹, Cys⁴²⁴, and Cys⁵⁶⁵) are all associated with the active site: Cys³⁵⁸, Cys⁴²¹, and Cys⁴²⁴ are ligated to the iron-sulfur cluster, Cys⁹⁹ is the closest of the non-ligated cysteines to the Fe of the cluster (residue C to Fe distance is circa 12 Å), and adducts at Cys⁵⁶⁵, as mentioned above, are known to restrict access to the catalytic center. While some of the reactive histidines can be explained by proximity to the active site (His⁹⁸ and His¹⁰¹), in general they are more widely dispersed than the cysteines. Nevertheless, it would still seem reasonable to conclude that the observed reduction in enzymatic activity chiefly resulted from HNE additions to the key active site region cysteines.

The enzymatic assays indicate that approximately half the initial activity remains despite high HNE concentrations and long incubation periods. While there is not sufficient information from these studies to elucidate the cause, a possible explanation is that some HNE modifications do not eliminate the potential functionality of the aconitase, but do alter the structure of aconitase in such a way that further HNE addition is inhibited. This may involve a reversible or irreversible change in folding induced by the HNE. Further investigation or speculation is beyond the scope of this study. Finally, it could be proposed that features of the active site are directing HNE modifications to adjacent residues. Active site components might be catalyzing the addition reactions, which would make aconitase very sensitive to HNE modification. While the most reactive sites in human serum albumin were previously also found not to be strongly correlated with the most exposed locations, no clustering of modifications to a particular region of the protein was observed [13]. In contrast, the most reactive sites in ACO2 are clearly clustered to the active site region of the protein.

Conclusion

HNE addition to ACO2 was found to exhibit relatively high selectivity. While higher applied HNE concentrations were shown to result in greater adduct accumulation by western blotting and a reduction in aconitase activity using the enzymatic assay, MS was required to locate modification sites. Using the iTRAQ approach, approximate rates of reaction were obtained at targeted addition sites. The sites found to be most reactive were all cysteines closely associated with the active site of the enzyme (Cys⁹⁹, Cys³⁵⁸, Cys⁴²¹, Cys⁴²⁴, and Cys⁵⁶⁵). These sites could not be distinguished by calculated solvent accessibility (all twelve ACO2 cysteines are buried). Reactive histidines, which were less reactive, were more widely distributed throughout the protein; however, no correlation between reactivity and accessibility was found for the histidines either, even though exposed histidines are present. Clearly, the most reactive sites are located near the active site, raising the possibility that active site components could be catalyzing the addition reaction. Finally, both the enzymatic activity assay and the iTRAQ depletion curves have similar profiles, but appear to be approaching horizontal asymptotes, rather than going to completion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACO2	mitochondrial aconitase
CID	collision-induced dissociation
DTT	dithiothreitol
FDR	false discovery rate
HCD	higher-energy C-trap dissociation
HNE	4-hydroxy-2-(<i>E</i>)-nonenal

LC	liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MWCO	molecular weight cut-off
PSM	peptide-spectrum match
SASA	solvent-accessible surface area
TBST	tris-buffered saline containing 0.05% Tween 20
TPP	Trans-Proteomic Pipeline

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Figure 1. Changes in aconitase activity, measured indirectly using isocitrate dehydrogenase NADPH generation rates, (a) in response to varying HNE concentration (0, 12, 120, and 600 μM) at fixed reaction time (2 h) and (b) in response to varying reaction time (0, 1, 2, and 6 h) at fixed HNE concentration (120 μM). Activities were corrected using duplicate samples to which an inhibitor had been added. Each comparison was repeated three times; the error bars plotted are standard deviations. The crude aconitase preparation was present in each case at 1 $\mu\text{g}/\mu\text{L}$.

Figure 2.

Progression of the HNE addition reaction, measured using the iTRAQ approach, displayed as accumulation curves for the targeted (b) cysteines, (d) histidines, and (f) lysines and as depletion curves for the corresponding (a) cysteine-, (c) histidine-, and (e) lysine-containing unmodified peptides. His⁹⁸, His¹⁰¹, and His⁵⁶⁹ are displayed with the cysteines since these sites share tryptic peptides with targeted cysteines. 0 h is defined as 1 for the depletion curves and 6 h is defined as 1 for the accumulation curves; however, this does not imply that the reaction has gone to completion at 6 h. When more than one unmodified peptide was associated with a modification site, the most frequently detected peptide was used. The error bars represent 95% confidence limits applied to the standard error.

Figure 3. Solvent-accessible surface areas (SASAs) for all (a) cysteines and (b) histidines in ACO2. SASAs, which were generated using GETAREA software from Protein Data Bank crystal structure 7ACN, provide a measure of amino acid residue exposure to the solvent. Asterisks indicate those cysteines and histidines that were found to be particularly reactive in this study. Clearly, reactivity with HNE is not well-correlated with SASA.

Scheme 1.

Reaction products for (a) Michael addition of HNE to the nucleophilic side chains of cysteine, histidine, and lysine residues and (b) Schiff base formation with lysine residues. Michael addition results in an equilibrium between a form containing an aldehyde functional group and a hemiacetal isomer. “Nuc” (nucleophile) represents the cysteine thiol sulfur, the histidine imidazole N, and the lysine N. The results of adduct stabilization with NaBH₄ are also shown.

Table 1

List of the 33 modification sites identified (manual evaluation of MS/MS spectra performed for all accepted peptides) in the initial survey of HNE addition to porcine ACO2; only Michael additions were detected. Summed PSM counts, which can be used as an approximate measure of relative abundance (compare magnitudes between conditions but not between sites), for all peptides supporting each site are presented. PSM counts obtained using the linear ion trap-based and Orbitrap-based systems are given separately. Active site residues are bolded.

Modification Site ^a	PSM Count at 12 μ M HNE ^b	PSM Count at 24 μ M HNE ^b	PSM Count at 120 μ M HNE ^b	PSM Count at 600 μ M HNE ^b
Cys ⁹⁹	12/6	26/11	34/17	30/11
Cys ¹⁷⁸	–/–	1/–	14/–	1/–
Cys ²⁵⁰	–/–	–/–	1/–	–/–
Cys ³⁰⁵	–/–	3/–	3/–	–/–
Cys³⁵⁸	50/8	96/19	151/28	58/28
Cys⁴²¹	3/–	3/1	24/6	6/–
Cys⁴²⁴	2/7	2/11	14/20	5/7
Cys ⁵⁶⁵	24/12	36/11	64/21	47/15
Cys ⁷¹³	–/–	–/–	1/–	–/–
His ⁹	–/–	2/–	8/–	27/20
His ¹³	–/–	–/–	12/1	38/20
His ⁴⁶	–/–	4/–	74/4	72/23
His ⁹⁸	2/–	1/–	9/–	24/–
His¹⁰¹	–/–	–/–	13/–	29/–
His¹⁴⁷	–/–	–/–	–/–	2/–
His ³²¹	–/–	–/–	2/–	3/–
His ³³²	–/–	2/–	33/–	26/18
His ³⁷⁹	–/–	–/–	–/1	1/3
His ⁴⁶⁰	2/–	3/4	78/17	102/23
His ⁵⁶⁹	–/–	–/–	18/–	21/1
His ⁵⁸²	–/–	–/–	1/–	2/–
His ⁶⁶⁸	–/–	–/–	–/2	–/4
His ⁶⁹²	–/–	–/–	1/–	–/–
His ⁷²⁹	–/–	–/–	–/–	–/7
Lys ²³	–/–	–/–	–/–	6/3
Lys ⁵⁹	–/–	–/–	–/–	1/–
Lys ¹¹⁷	1/–	–/–	7/1	16/4
Lys ²¹⁸	–/–	–/–	–/–	1/1
Lys ³⁸⁴	–/–	–/–	–/–	1/–
Lys ⁴⁹⁰	–/–	–/–	–/–	1/–

Modification Site ^a	PSM Count at 12 μ M HNE ^b	PSM Count at 24 μ M HNE ^b	PSM Count at 120 μ M HNE ^b	PSM Count at 600 μ M HNE ^b
Lys ⁴⁹⁶	–/–	–/–	1/–	4/–
Lys ⁶⁶²	–/–	–/–	–/–	–/1
Lys ⁶⁷⁴	–/–	–/–	–/–	6/8

^aModification site numbering is for porcine ACO2 after removal of the mitochondrial targeting peptide—add 27 for the translated sequence

^bSummed PSM counts for measurements made using the linear ion trap-based system/Orbitrap-based system; hyphen indicates not detected

Table 2

List of targeted modified peptides with modification sites indicated; all singly, doubly, and triply charged precursor ions in the m/z ratio range from 300 to 2000 Th were anticipated.

Modified Peptide ^a	Modification Site ^b
VAVPSTIH#CDHLIEAQLGGEK	His ⁹⁸
VAVPSTIHC#DHLIEAQLGGEK	Cys ⁹⁹
VAVPSTIHC#DHLIEAQLGGEK	His ¹⁰¹
VAVPSTIH#C#DHLIEAQLGGEK	His ⁹⁸ & Cys ⁹⁹
VAVPSTIH#CDH#LIEAQLGGEK	His ⁹⁸ & His ¹⁰¹
VAVPSTIHC#DH#LIEAQLGGEK	Cys ⁹⁹ & His ¹⁰¹
VAVPSTIH#C#DH#LIEAQLGGEK	His ⁹⁸ , Cys ⁹⁹ , & His ¹⁰¹
VGLIGSC#TNSSYEDMGR	Cys ³⁵⁸
VGLIGSC#TNSSYEDM*GR	Cys ³⁵⁸
DVGGIVLANAC#GPCIGQWDR	Cys ⁴²¹
DVGGIVLANACGPC#IGQWDR	Cys ⁴²⁴
DVGGIVLANAC#GPC#IGQWDR	Cys ⁴²¹ & Cys ⁴²⁴
C#TTDHISAAGPWLK	Cys ⁵⁶⁵
CTTDH#ISAAGPWLK	His ⁵⁶⁹
C#TTDH#ISAAGPWLK	Cys ⁵⁶⁵ & His ⁵⁶⁹
VAMSH#FEPHEYIR	His ⁹
VAM*SH#FEPHEYIR	His ⁹
VAMSHFEPH#EYIR	His ¹³
VAM*SHFEPH#EYIR	His ¹³
VAMSH#FEPH#EYIR	His ⁹ & His ¹³
VAM*SH#FEPH#EYIR	His ⁹ & His ¹³
IVYGH#LDDPANQEIER	His ⁴⁶
NDANPETH#AFVTSPEIVTALAAGTLK	His ⁴⁶⁰
AEFDPGQDTYQH#PPK	His ⁵¹⁹
AK#DINQEVYNFLATAGAK	Lys ¹¹⁷
FNPETDFTLGK#DGK	Lys ⁴⁹⁰
FK#LEAPDADELPR	Lys ⁴⁹⁶
K#QGLLPLTFADPADYNK	Lys ⁶⁷⁴

^aUnmodified versions of the listed modified peptides were also targeted. For addition sites at lysine, peptides resulting from cleavage at the modification site were also included. Peptide WVVIGDENYEGSSR, which is bounded by arginine cleavage sites and contains no potential HNE addition sites, was also targeted to provide an internal standard; M* indicates methionine sulfoxide and # indicates the HNE addition site.

^bModification site numbering is for ACO2 after removal of the mitochondrial targeting peptide—add 27 for the translated sequence