Identification of Bovine Heart Cytochrome *c* Oxidase Subunits Modified by the Lipid Peroxidation Product 4-Hydroxy-2-nonenal[†,‡]

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ABSTRACT: Bovine heart cytochrome *c* oxidase (CcO) was inactivated by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) in a time- and concentration-dependent manner with pseudo-first-order kinetics. Cytochrome *c* oxidase electron transport activity decreased by as much as 50% when the enzyme was incubated for 2 h at room temperature with excess HNE (300−500 μM). HNE-modified CcO subunits were identified by two mass spectrometric methods: electrospray ionization mass spectrometry (ESI/MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). All of the experimentally determined molecular masses were in excellent agreement with published sequence values with an accuracy of ∼1 part per 10000 mass units for subunits smaller than 20 kDa and ∼1 part per 1000 mass units for the three subunits larger than 20 kDa. Both MS methods detected six CcO subunits with an increased mass of 156 Da after reaction with HNE (subunits II, IV, Vb, VIIa, VIIc, and VIII); this result indicates a single Michael-type reaction site on either a lysine or histidine residue within each subunit. Reaction of HNE with either subunit VIIc or subunit VIII (modified ∼30% and 50−75%, respectively) must be responsible for CcO inhibition. None of the other subunits were modified more than 5% and could not account for the observed loss of activity. Reaction of HNE with His-36 of subunit VIII is most consistent with the ∼50% inhibition of CcO: (1) subunit VIII is modified more than any other subunit by HNE; (2) the time dependence of subunit VIII modification is consistent with the percent inhibition of CcO; (3) His-36 was identified as the HNE-modified amino acid residue within subunit VIII by tandem MS analysis.

Cytochrome *c* oxidase (CcO)[1] (EC 1.9.3.1) is the terminal complex of the respiratory chain in mitochondria and bacteria. It functions to catalyze electron transfer from cytochrome *c* to molecular oxygen and to generate a proton gradient required for ATP synthesis. The mammalian enzyme consists of 13 dissimilar subunits per monomer (*1*, *2*). The three largest subunits, I−III, are encoded by mitochondrial genes and constitute the functional core of the enzyme. The ten smaller subunits are encoded by the nuclear genome and surround this central core. The role of the nuclearly encoded subunits is still a matter of debate. Some are known to stabilize the dimeric form of oxidase and bind structurally important cardiolipin (*3*, *4*). Others probably have a regulatory function since they bind allosteric effectors such as nucleotides and/or hormones (*5*−*8*).

The coupling of electron transfer to oxidative phosphorylation within the mitochondrial inner membrane is imperfect and results in the "leakage" of electrons. This leakage generates superoxide anions. Therefore, mitochondrial electron transport is recognized as a major intracellular source of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, which oxidize and damage proteins, nucleic acids, and phospholipids (*9*−*12*). Especially vulnerable to oxidative damage are the highly unsaturated acyl groups of phospholipids, resulting in the production of reactive and highly toxic lipid products, e.g., malondialdehyde and 4-hydroxy-2-nonenal (HNE) (*13*, *14*). During oxidative stress, these reactive aldehydes reach high local concentration (∼1 mM), sufficient to react with several amino acid side chains, including those of lysine (*15*), histidine (*16*), and cysteine (*17*). Such chemical modification of enzymes by HNE may cause inactivation or disruption of structure. CcO is a potential target of HNE and other lipid peroxidation products since it is in close proximity to the sources of ROS, primarily complexes I, II, and III. CcO is also in intimate contact with cardiolipin, a highly unsaturated phospholipid that is very susceptible to oxidative damage.

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[1] Abbreviations: CcO, bovine heart cytochrome *c* oxidase; HNE, 4-hydroxy-2-nonenal; ROS, reactive oxygen species; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ESI/MS, electrospray ionization mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CID, collision-induced dissociation.

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Therefore, to gain a better understanding of the molecular basis of oxidative damage, we have examined the functional and structural consequences of incubating purified, detergent-solubilized bovine heart CcO with micromolar to millimolar concentrations of HNE.

EXPERIMENTAL PROCEDURES

Materials. Dodecyl maltoside was purchased from Anatrace Inc. Sodium cholate, human recombinant insulin, horse apomyoglobin, yeast enolase, and bovine pancreas α-chymotrypsin were purchased from Sigma Chemical Co. Thioredoxin, sequencing grade-modified trypsin, and sequencing grade v8 protease were from Promega. 4-Hydroxy-2 nonenal dimethylacetal was purchased from Oxis International, Inc. Other chemicals were of analytical grade.

Cytochrome c Oxidase. Cytochrome *c* oxidase was isolated from Keilin−Hartree bovine heart muscle particles as previously described (*18*). The final oxidase pellet was solubilized in 0.1 M NaH_2PO_4, pH 7.4, buffer, containing 23 mM sodium cholate and 1.0 mM EDTA. Purified enzyme (20−30 mg/mL) was quickly frozen in liquid nitrogen and stored at −60 °C. The isolated complex contained 9.5−10 nmol of heme *a*/mg of protein. The purified enzyme had a molecular activity of 350−390 s^{-1}, when assayed spectrophotometrically at 25 °C with 30 μM ferrocytochrome *c* as substrate in 25 mM phosphate buffer, pH 7.2, containing 2 mM dodecyl maltoside. Molecular activities were calculated from the first-order rate of ferrocytochrome *c* oxidation as described previously (*18*).

4-Hydroxy-2-nonenal. HNE was generated by acid treatment (1 mM HCl) of 4-hydroxy-2 nonenal dimethylacetal. The concentration of HNE was determined spectrophotometrically using $\epsilon_{224} = 13\ 750\ M^{-1}\ cm^{-1}$.

Reaction of Cytochrome c Oxidase with HNE. Isolated CcO (5 μM) in 1−2 mM sodium cholate was solubilized in 20 mM Tris−SO_4 buffer, pH 7.4, containing 2 mM dodecyl maltoside and dialyzed versus two changes of the same buffer containing 0.2 mM dodecyl maltoside for 24 h at 4 °C to remove the cholate. CcO was reacted with 5−500 μM HNE for 2 h at room temperature and the reaction stopped by dilution. The resulting product was subsequently analyzed for changes in activity and modification of subunits.

Reversed-Phase High-Performance Liquid Chromatography. Quantitative RP-HPLC analysis of CcO subunit content was done using gradient elution from a Vydac C_{18} reversed-phase column (5 μm, 0.46 × 25 cm, 300 Å pore size) and a Waters/Millipore liquid chromatography system (*21*). The gradient was made from mixtures of solvent A (0.2% TFA in water) and solvent B (0.2% TFA in acetonitrile). The column was equilibrated with solvent A, and subunits eluted at 1 mL/min with a linear gradient from 25% to 50% solvent B in 50 min, followed by a linear gradient from 50% to 85% solvent B in 17.5 min. Elution was monitored at 214 nm after 0.2−2.0 nmol of unmodified or HNE-modified CcO was loaded. The percent yield of each subunit was based upon quantitative integration of peak areas as compared with unmodified, purified CcO (*21*). Fractions (0.5 mL) were also collected for analysis of unmodified and modified subunits by either SDS−PAGE or mass spectrometry.

Mass Spectrometry. Electrospray ionization mass spectra were acquired on a Finnigan LCQ ion trap mass spectrometer adapted for microspray ionization sample introduction. An electrospray voltage of 2.75 kV was employed. HPLC separations were accomplished with a Michrom BioResources MAGIC 2002 micro HPLC fitted with a Michrom precolumn flow splitter. HPLC conditions were as follows: injected sample, 2 pmol; column, New Objective PicoFrit (75 μm i.d.; 5 μm tip); column packing, Vydac C_{18} (218MSB5; 5 μm; 300 Å; packed to 7 cm length); mobile phase (solvent A, 0.5% acetic acid/0.005% trifluoroacetic acid in water; solvent B, 90% acetonitrile/0.5% acetic acid/0.005% trifluoroacetic acid/9.5% water) linear gradient of 2% solvent B to 72% solvent B in 20 min; flow rate, 0.4 μL/min. For analysis of RP-HPLC-purified subunits of CcO (prepared as above) the 0.5 mL fractions were concentrated 2-fold and mixed with acetonitrile/acetic acid to yield ∼2 pmol/μL of protein in 50% acetonitrile/0.5% acetic acid.

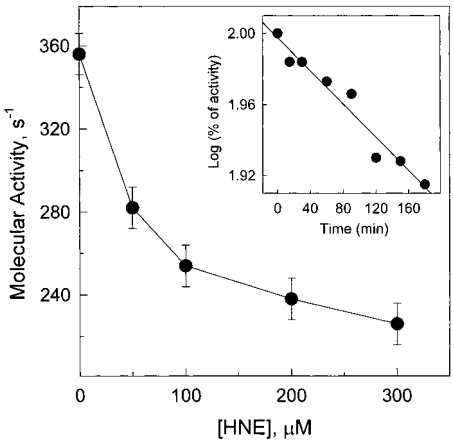

FIGURE 1: Inactivation of electron transport activity of bovine heart cytochrome *c* oxidase by HNE. Cytochrome *c* oxidase (0.25 μM) solubilized in 25 mM phosphate buffer, pH 7.2, containing 2 mM dodecyl maltoside was incubated for 2 h at room temperature with different concentrations of HNE. The reaction was stopped by dilution and the activity measured spectrophotometrically as described in Experimental Procedures. The error in determination of molecular activity was ±5%. Inset: Semilogarithmic plot of cytochrome *c* oxidase activity versus incubation time with 100 μM HNE.

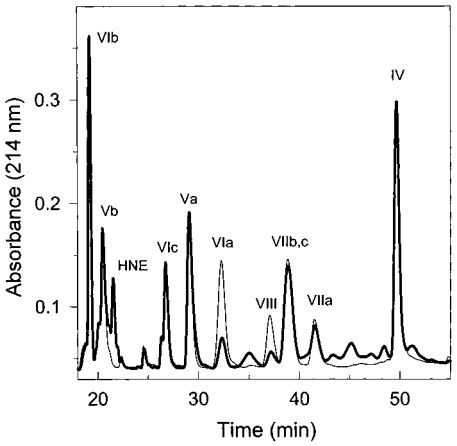

FIGURE 2: Analysis of CcO (thin line) and HNE-modified CcO (thick line) by C_{18} reversed-phase HPLC. Cytochrome *c* oxidase (5 μM) was incubated with and without HNE (100 μM) for 2 h at room temperature. In both cases 0.5 nmol of cytochrome *c* oxidase was injected onto the HPLC column. Gradient elution of the subunits was as described in Experimental Procedures. The peak eluting at about 22.5 min observed for HNE-modified CcO did not contain protein and was due to residual HNE.

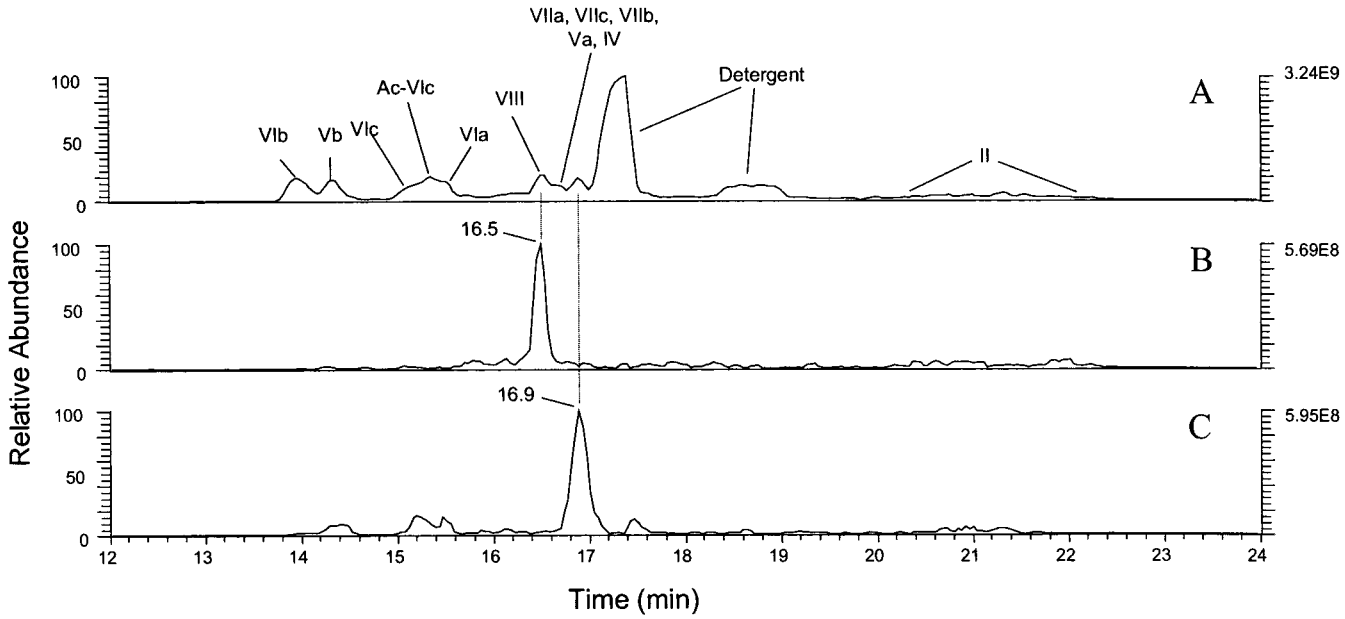

FIGURE 3: HPLC−ESI/MS analysis of cytochrome *c* oxidase: (A) reconstructed base peak chromatogram of CcO subunits after incubation of enzyme (5 μM) with 100 μM HNE for 2 h at room temperature; (B) selected ion retrieval trace for the 6+ ion (*m*/*z* 827.8) of unmodified subunit VIII; (C) selected ion retrieval trace for the 6+ ion (*m*/*z* 853.9) of HNE-modified subunit VIII. Peaks eluting at 17.5 and 18.5 min did not contain protein and were due to residual dodecyl maltoside.

Samples were infused into the electrospray interface through a New Objective PicoTip (10 μm tip) at a rate of 0.4 μL/min. Full-scan mass spectra were acquired over a range of *m*/*z* 300−2000. Ions for each charge state were then isolated in the ion trap (isolation width 3) and fragmented at 35% relative energy to produce MS/MS spectra. MS^3 spectra were obtained after isolation and collision-induced dissociation of appropriate MS/MS fragment ions. MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager DE-STR. For each sample, 1 μL of a solution containing 2 μM CcO and 20 mM dodecyl maltoside was spotted on the MALDI target. After partial drying, 1 μL of a saturated solution of sinapinic acid in 50% acetonitrile/0.1% TFA was added to the spot, and the solution was allowed to dry at room temperature under a gentle stream of air. The spectra represent the average of 100 laser shots. Mass assignments were made by the "close external calibration" method, using insulin, thioredoxin, apomyoglobin, and enolase as references. Noise reduction and smoothing algorithms were applied to each spectrum. Peak heights used for determination of the percent of subunit modification were obtained from the peak data table associated with each spectrum.

Table 1: Molecular Masses of Subunits of Bovine Heart Cytochrome *c* Oxidase

subunit	reported mass[a]	ESI/MS[o]	MALDI-TOF/MS[q]
I	57032[b]	ND[p]	57056
II	26022[b,c]	26043	26030
III	29919[b]	ND	29793
IV	17153[d,e]	17151	17142
Va	12436[f]	12436	12434
Vb	10670[g]	10669	10668
VIa	9533[h]	9533	9531
AcVIb	10067[i]	10063	10063
AcVIc	8521[j]	8520	8521
VIIa	6674[k]	6672	6676
VIIb	6357[l]	6356	6359
VIIc	5441[m]	5440	5446
VIII	4962[n]	4960	4964

[a] Molecular masses (Da) were determined either from direct amino acid sequence analysis or by DNA sequence analysis. [b] Reference *22*. [c] Reference *23*. [d] Reference *24*. [e] Reference *25*. [f] Reference *26*. [g] Reference *27*. [h] Reference *28*. [i] Reference *29*. [j] Reference *30*. [k] Reference *31*. [l] Reference *32*. [m] Reference *33*. [n] Reference *34*. [o] Experimentally determined mass (Da) by ESI/MS. [p] ND, not detected. [q] Experimentally determined mass (Da) by MALDI-TOF/MS.

RESULTS

HNE Inhibition of Cytochrome c Oxidase. The electron transport activity of CcO decreased progressively with increasing HNE concentration. For example, activity was approximately two-thirds of the control after reaction with 300 μM HNE for 2 h at room temperature (Figure 1, main panel). Maximum activity loss varied somewhat with different enzyme preparations but usually decreased 30−50% with 300−500 μM HNE. The activity loss was time-dependent and followed pseudo-first-order kinetics (Figure 1, inset).

Many inhibitors of CcO, e.g., cyanide or azide, bind ferric cytochrome a_3 and/or Cu_B and cause spectral changes in the Soret band (*19*, *20*). However, incubation of 5 μM CcO with even 500 μM HNE did not measurably alter the visible spectrum. Clearly, HNE inhibition of CcO is not caused by significant alterations in the heme environment. Inhibition by HNE also does not appear to be due to its reaction with one of the three large core CcO subunits since HNE does not inhibit *Rhodobacter sphaeroides* CcO (data not shown). This enzyme contains the three large core subunits and all of the redox centers but does not have subunits corresponding to the 10 nuclearly encoded subunits of bovine CcO. If HNE modification of one of the large core CcO subunits were responsible for inhibition of the bovine enzyme, we would expect that the *Rb. sphaeroides* enzyme would also be inhibited by HNE. Therefore, HNE almost certainly inhibits the bovine heart enzyme by reacting with one of the smaller nuclearly encoded subunits.

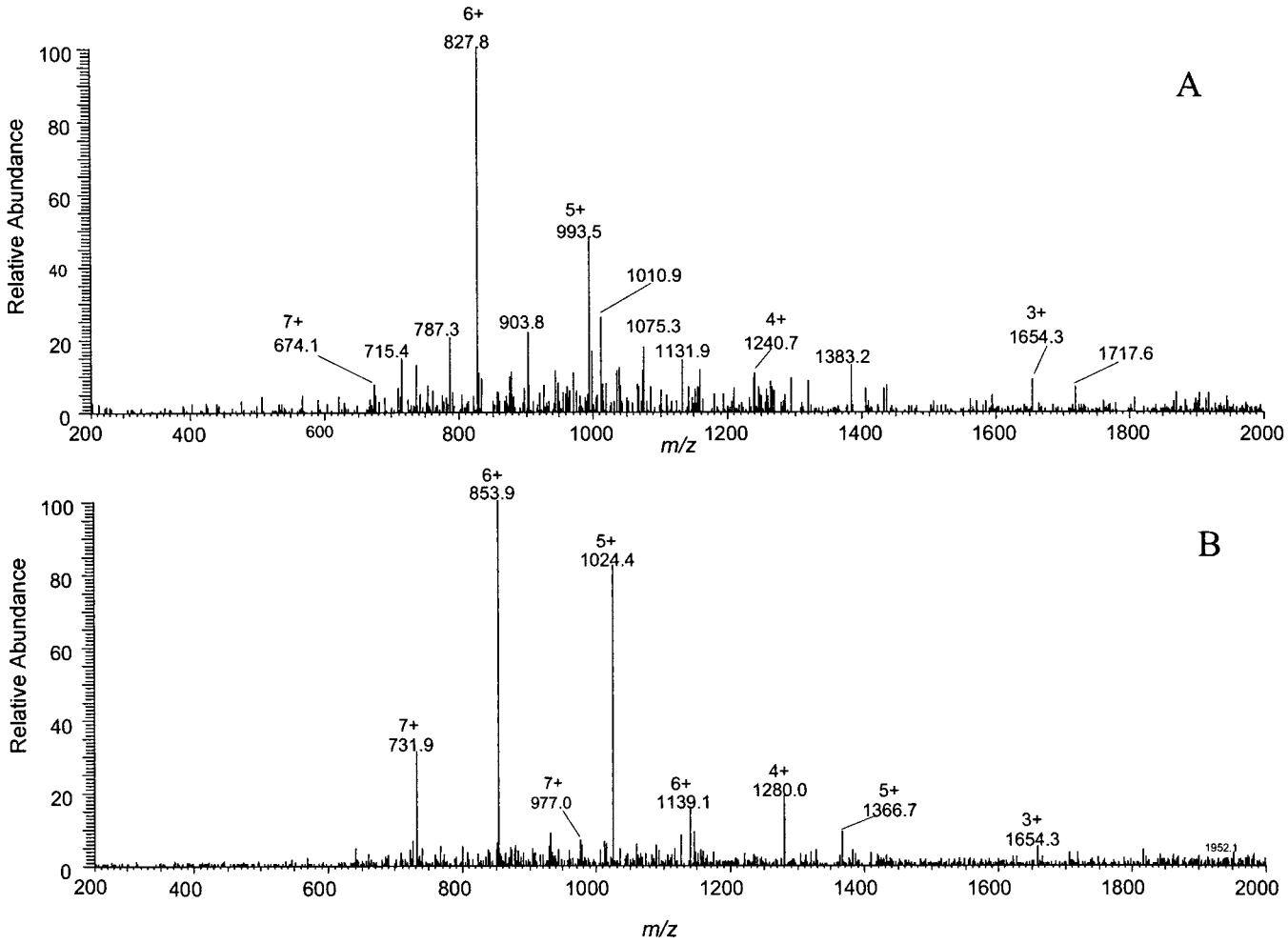

FIGURE 4: Electrospray ionization mass spectra of unmodified and HNE-modified CcO subunit VIII: (A) unmodified subunit VIII (eluting at 16.5 min as indicated in Figure 3B); (B) HNE adduct of subunit VIII (eluting at 16.9 min as indicated in Figure 3C). Conditions for mass spectrometry analysis are given in Experimental Procedures.

Reversed-Phase High-Performance Liquid Chromatography of HNE-Treated CcO. All 10 nuclearly encoded subunits of CcO can be separated on C_{18} reversed-phase HPLC (*4*, *21*). Therefore, we utilized this approach to detect HNE-modified subunits, which would be expected to have altered elution positions. CcO (5 μM), incubated for 2 h at room temperature with 100 μM HNE, exhibited a different reversed-phase HPLC elution profile than untreated CcO. The peaks corresponding to subunits VIa and VIII each decreased in area by 60−80%, and new elution peaks appeared (Figure 2). Small changes were also observed for two peaks corresponding to subunits VIIb + VIIc and VIIa.

Electrospray Ionization Mass Spectrometry of CcO Subunits. Eleven of the 13 CcO subunits elute from reversed-phase HPLC and, therefore, can be analyzed by HPLC ESI/MS (Figure 3A). The two most hydrophobic core subunits, I and III, precipitate on the reversed-phase column and could not be analyzed by this method (*21*). The masses of the 11 subunits were determined, and each agreed well with the value calculated from its amino acid sequence (Table 1). Agreement was within 1 part in 10000 for the smaller, nuclearly encoded subunits and within 7 parts in 10000 for subunit II. Two different approaches were used to identify HNE-modified subunits by HPLC ESI/MS: (1) CcO was incubated with HNE and introduced by on-line HPLC without further purification or removal of HNE, and (2) CcO was incubated with HNE, subunits were separated on C_{18} RP-HPLC, and their masses were determined individually by ESI/MS. Both procedures detected six HNE-modified CcO subunits: II, IV, Vb, VIIa, VIIc, and VIII. Each of these subunits had an increased mass of +156 Da, corresponding to the addition of a single molecule of HNE. Subunit VIII had the highest percent modification (Figures 3B,C and 4), a result that is in agreement with the reversed-phase HPLC analysis (Figure 2). For HNE-modified subunit VIII, the resulting ESI mass spectrum corresponded to an increased mass of 156 Da (Figure 4B). This suggests addition of a single HNE modification to one of the seven basic groups in subunit VIII, i.e., either a lysine, the histidine, or the α-amino group. However, in contrast to the reversed-phase HPLC results, which suggested significant modification of subunit VIa, only a very small percent of the HNE adduct was detected.

Analysis by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. More complete and informative data were obtained from MALDI-TOF mass spectrometry. All 13 subunits were detected by this method, and the measured molecular masses were in excellent agreement with values calculated from the known sequences and also with previous ESI/MS values (Figure 5 and Table 1). All of the MALDI-TOF/MS values are within 6 parts in 10000 of the calculated masses with the exception of subunit

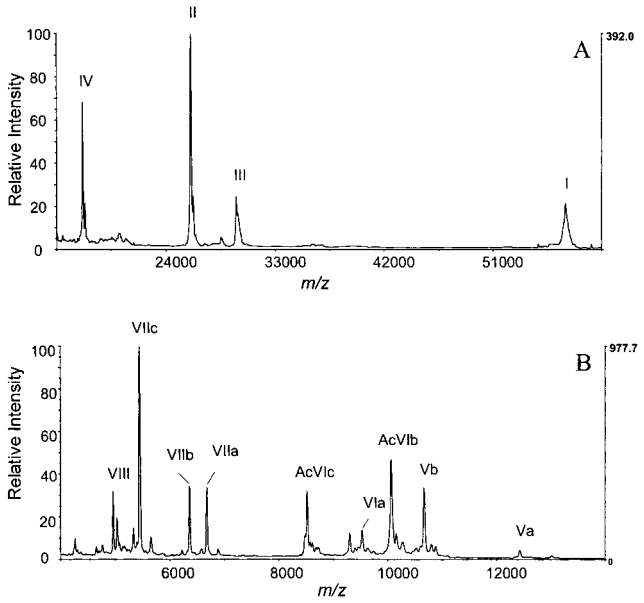

FIGURE 5: MALDI-TOF mass spectra of CcO subunits. (A) Analysis of subunits with molecular mass greater than 15 kDa. (B) Analysis of subunits with molecular mass less than 14 kDa. CcO (5 μM) was solubilized in 20 mM Tris−SO_4 buffer, pH 7.4, containing 2 mM dodecyl maltoside and dialyzed versus two changes of the same buffer containing 0.2 mM dodecyl maltoside for 24 h at 4 °C. Conditions for sample dilution and mass spectrometry analysis are given in Experimental Procedures.

III. Our value of 29793 Da for subunit III is 126 Da lower than the value calculated from the known sequence. This is probably due to a missing N-terminal methionyl residue (*35*).

The MALDI-TOF/MS data for HNE-modified CcO were fully consistent with the HPLC-ESI/MS results with one exception. The small amount of HNE-modified subunit VIa was not detected by MALDI-TOF/MS. However, HNE-modified subunits II, IV, Vb, VIIa, VIIc, and VIII were found, and each had the mass difference expected for a Michael addition product, i.e., +156 Da. One advantage of the MALDI-TOF/MS method is that it is much easier to compare the relative amounts of each subunit or adduct in the prepared samples. We used this to our advantage to determine the extent of HNE modification as a function of HNE concentration. CcO (5 μM) was incubated at room temperature for 2 h with 0, 25, 50, 75, and 100 μM HNE, the reaction was stopped by dilution, and the mixture was analyzed by MALDI-TOF/MS (Figure 6). Subunits VIII, VIIa, and VIIc were modified to the greatest extent and permitted an evaluation of adduct formation as a function of HNE concentration. With each of these subunits, the ion intensity increase for the modified subunits was plotted as percent modification versus HNE concentration (Figure 7). Modification of subunits VIII, VIIc, and VIIa progressed in a concentration-dependent manner with a maximum of 50%, 30%, and 5% HNE−adduct formation, respectively. We conclude that HNE modification of either subunit VIII or VIIc must be responsible for the HNE-induced decrease in enzymatic activity. These are the only HNE-modified subunits detected in sufficient yield to account for the 30−50% decreases in enzymatic activity.

Identification of the HNE Reaction Site in Subunit VIII. Localization of the covalent attachment site of HNE within subunit VIII was accomplished by tandem ESI/MS analysis.

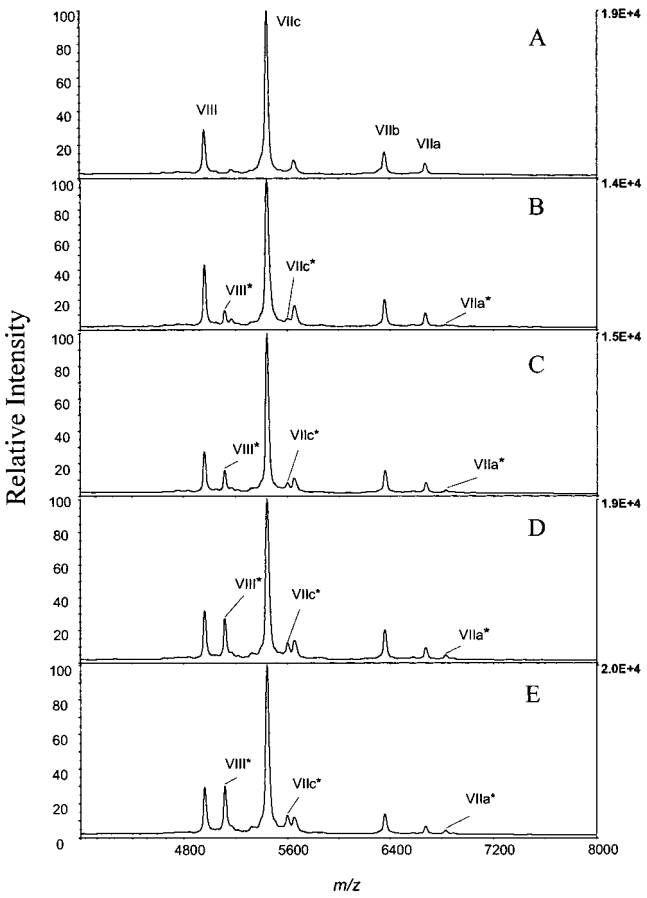

FIGURE 6: Effect of HNE concentration on formation of HNE-modified CcO subunits as detected by MALDI-TOF/MS. Cytochrome *c* oxidase (5 μM) was incubated with various concentrations of HNE for 2 h at room temperature and the reaction stopped by dilution. In each case 1 μL of solution containing 2 μM CcO was spotted on the MALDI target. Conditions: (A) no NHE; (B) 25 μM HNE; (C) 50 μM HNE; (D) 75 μM HNE; (E) 100 μM HNE. HNE-modified subunits are identified with an asterisk.

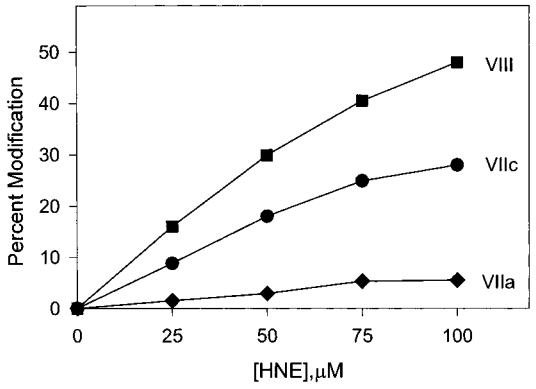

FIGURE 7: Concentration dependence of HNE adducts of CcO subunits VIII (■), VIIc (●), and VIIa (◆). Percent peak heights of $[MH]^+$ for HNE Michael addition products, as detected in the MALDI-TOF mass spectra (Figure 6), are plotted versus HNE concentration.

Tandem MS to obtain sequence information from intact proteins was first described by the Smith group using in-source CID in conjunction with a triple quadrupole mass spectrometer (*36*, *37*). It was later extended to FT-ICR/MS by McLafferty and co-workers (*38*, *39*). The mass spectra of subunit VIII from control and HNE-treated CcO exhibited the expected distribution of charge state ions when HPLC-

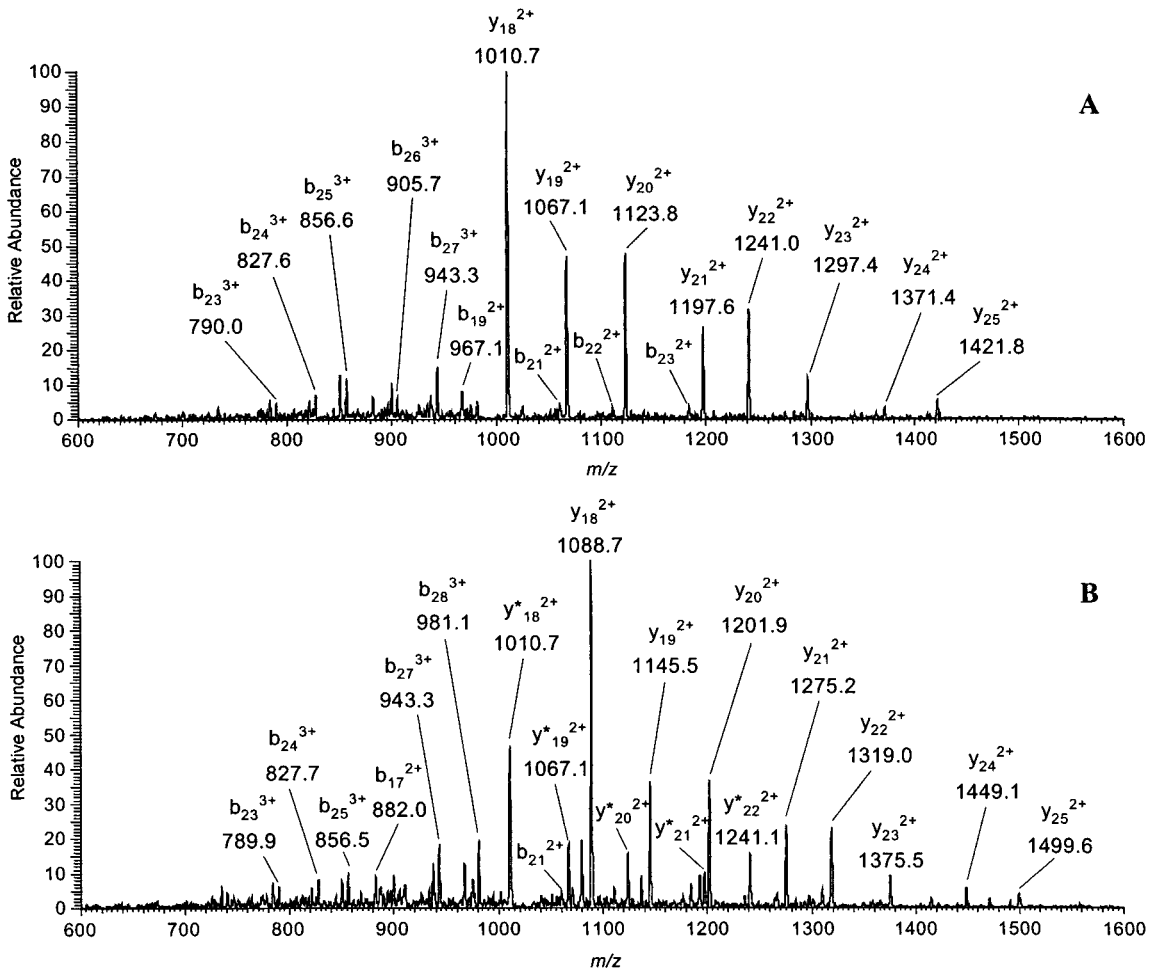

FIGURE 8: Electrospray MS/MS spectra of control (A) and HNE-treated (B) cytochrome *c* oxidase subunit VIII. Samples were isolated by RP-HPLC and aliquots infused into the LCQ ion trap mass spectrometer as described in Experimental Procedures. Precursor ions (5+) were as follows: control, *m*/*z* 992.8; HNE-treated, *m*/*z* 1024.1. Spectra were acquired in profile mode and represent the average of five scans. Assignments were made by comparison with *in silico* digests for control and HNE-His-36 CcO subunit VIII generated by the MS-Product component of Protein Prospector (http://prospector.ucsf.edu/ucsfhtml4.0u/msprod.htm). Asterisks indicate fragments produced by loss of HNE from the corresponding 2+ ion.

purified samples were infused into the ion trap mass spectrometer (spectra not shown). Collision-induced dissociation of each ion was performed to produce a series of MS/MS spectra for both samples. Comparison of the MS/MS spectra of the 5+ charge states for control (*m*/*z* 992.8) and HNE-treated (*m*/*z* 1024.1) CcO was particularly informative (Figure 8). The prominent, doubly charged, y-ion series from y_{18}^{2+} to y_{25}^{2+} clearly indicated that HNE was attached to a residue on the C-terminal half of subunit VIII. Results obtained from MS/MS analysis of all other charge state ions were in complete agreement with this conclusion. Identification of the specific amino acid that had reacted with HNE was made by subsequent CID (MS^3) of the corresponding y_{18}^{2+} ions (sequence, PAGWVLYHLDNYKKSSAA; control, *m*/*z* 1010.3; HNE-treated, *m*/*z* 1088.8) as shown in Figure 9 and Table 2. The fact that ions y_5, y_6, and y_8 are the same in both spectra (Figure 9) rules out the possibility of the HNE being attached to either of the Lys residues in this fragment. Furthermore, the prominent b-series ions which result from charge retention on the N-terminal Pro (*40*) unambiguously locate the HNE on His-36 (numbering for the bovine heart enzyme), which is N-terminal residue 8 in y_{18}^{2+}. As for the MS/MS analyses, all MS^3 spectra that were examined for the other ions concurred with the results described above.

DISCUSSION

One of the major products formed by lipid peroxidation is 4-hydroxy-2-nonenal (HNE). This aldehyde is more stable than the initial ROS, and its concentration can reach the high micromolar range in certain cell compartments under pathophysiological conditions (for review, see ref *14*). HNE either forms Michael addition adducts with nucleophilic lysyl, sulfhydryl, or histidyl residues or forms Schiff base adducts with primary amino groups (*14*−*17*). Such chemical modification of enzymes could easily cause inactivation and help to explain the mechanism by which ROS play an important role in a number of diseases caused by oxidative stress.

Reaction of HNE with detergent-solubilized CcO causes up to a 50% loss of electron transport activity with 300−500 μM HNE (refer to Figure 1). These results are similar to the inhibition that occurs when HNE reacts with either purified rat liver CcO or isolated rat liver mitochondria (*41*, *42*). A similar inhibition of CcO by HNE was not observed by Humphries and Szweda (*43*). They used isolated mito-

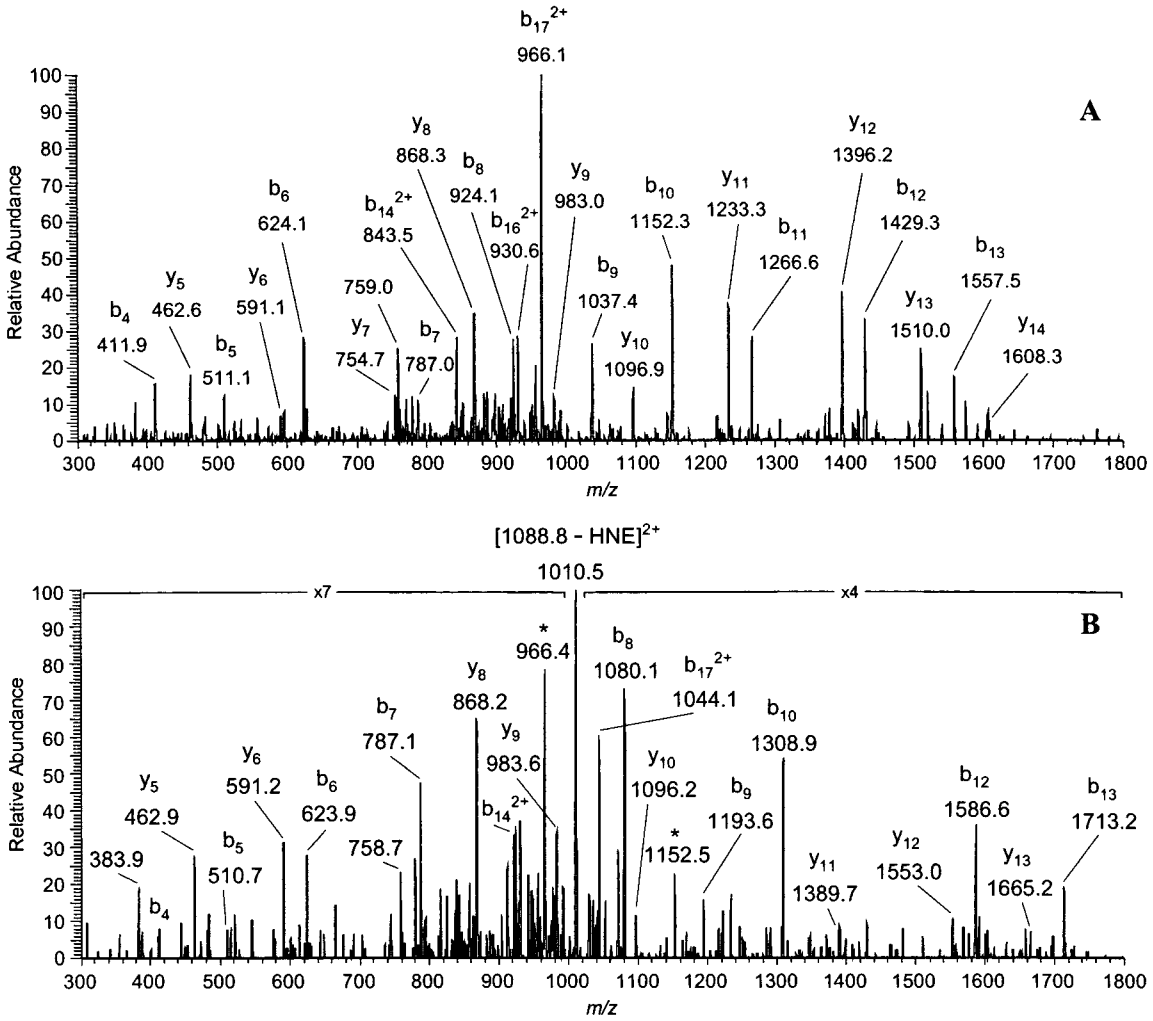

FIGURE 9: Electrospray MS^3 spectra of control (A) and HNE-treated (B) cytochrome *c* oxidase subunit VIII. The y_{18}^{2+} generated in the MS/MS spectra shown in Figure 8 were isolated in the ion trap and further fragmented by CID. The spectra were acquired as described in the caption to Figure 8. Asterisks indicate fragments produced by loss of HNE from the corresponding 2+ ion.

chondria rather than purified CcO, much lower HNE concentrations, and shorter incubation times, i.e., 50 μM HNE for 5 min. Under those conditions, it is probable that any small decrease of CcO activity would not be statistically significant (compare Figure 1 with Table 1 in ref *43*).

Neither perturbation of the heme environments nor HNE reaction with one of the three large core CcO subunits appears to be responsible for CcO inactivation. The small percentage of subunit II adduct cannot account for the observed loss in activity. Rather, reaction of HNE with one of the nuclearly encoded subunits almost certainly causes enzyme inhibition. Supporting evidence for a mechanism involving only the nuclearly encoded subunits is the inability of HNE to inhibit *Rb. sphaeroides* CcO. This bacterial enzyme contains all of the redox centers, together with only the three large core subunits, but does not contain any subunits homologous to the eukaryotic nuclearly encoded subunits. Therefore, *Rb. sphaeroides* CcO lacks the HNE reaction site that is responsible for inhibition of the bovine heart enzyme.

Incubation of bovine heart CcO with HNE decreases the size of several HPLC peaks corresponding to nuclearly encoded subunits and concomitantly produces new elution peaks. The most significant changes occur with subunits VIa and VIII which decrease ~75%. The decreased amount of subunit VIII is almost certainly due to its reaction with HNE. Subunit VIII is also the major site of HNE modification that was detected by ESI/MS and MALDI-TOF/MS. Furthermore, it is unlikely that the decrease in the amount of subunit VIII is due to its dissociation from CcO. Subunit VIII is bound very tightly to CcO and does not dissociate from the core subunits even after incubation with 6 M urea (Sedlák and Robinson, unpublished data). On the other hand, the decrease in subunit VIa is probably due to its dissociation from the HNE-modified complex and is not due to production of an HNE adduct. HNE-modified subunit VIa is barely detectable by ESI/MS and not detected at all by MALDI-TOF/MS. Strong precedent for this explanation exists since subunit VIa often dissociates from detergent-solubilized CcO. Twenty to thirty percent is routinely lost during isolation of CcO from mitochondria while 20−100% can be lost during anion-exchange chromatography (*21*). Furthermore, no subunit VIa remains bound to CcO after complete removal of the structurally important phospholipid, cardiolipin (*4*). In each of these cases, dissociation of subunit VIa has essentially no effect upon CcO activity. Therefore, dissociation of subunit VIa cannot be the causal event for CcO inactivation by HNE. It is more likely that reaction of HNE with another

Table 2: MS^3 Fragmentation of the y_{18}^{2+} Ion Generated in the MS/MS Spectrum of the 5+ Charge State Ion of Control and HNE-Treated Cytochrome *c* Oxidase Subunit VIII

C-terminal fragments (*m*/*z*)[a]						N-terminal fragments (*m*/*z*)[a]				
control[b]		+HNE[c]				control[b]		+HNE[c]	
calcd	obsd	calcd[d]	obsd		residue[e]		calcd	obsd	calcd[d]	obsd
					P_{29}	b_1	98.1		98.1	
1923.0		2079.1		y_{17}	A_{30}	b_2	169.1		169.1	
1851.9		2008.1		y_{16}	G_{31}	b_3	226.1		226.1	
1794.9		1951.0		y_{15}	W_{32}	b_4	412.2	411.9	412.2	411.6
1608.8	1608.3	1765.0		y_{14}	V_{33}	b_5	511.3	511.5	511.3	510.7
1509.8	1510.0	1665.9	1665.2	y_{13}	L_{34}	b_6	624.4	624.1	624.4	623.9
1396.7	1396.2	1552.8	1553.0	y_{12}	Y_{35}	b_7	787.4	787.0	787.4	787.0
1233.6	1233.3	1389.7	1389.7	y_{11}	*H*$_{36}$	b_8	924.5	924.1	1080.6	1080.1
1096.6	1096.9	1096.6	1096.2	y_{10}	L_{37}	b_9	1037.6	1037.4	1193.7	1193.6
983.5	983.0	983.5	983.6	y_9	D_{38}	b_{10}	1152.6	1152.3	1308.7	1308.9
868.5	868.3	868.5	868.2	y_8	N_{39}	b_{11}	1266.6	1266.6	1422.7	
754.4	754.7	754.4		y_7	Y_{40}	b_{12}	1429.7	1429.3	1585.8	1586.6
591.3	591.1	591.3	591.2	y_6	K_{41}	b_{13}	1557.8	1557.5	1713.9	1713.2
463.3	462.6	463.3	462.9	y_5	K_{42}	b_{14}^{2+}	843.4	843.5	921.5	921.4
335.2		335.2		y_4	S_{43}	b_{15}^{2+}	887.0		965.0	
248.1		248.1		y_3	S_{44}	b_{16}^{2+}	930.5	930.6	1008.5	1008.4
161.1		161.1		y_2	A_{45}	b_{17}^{2+}	966.0	966.1	1044.1	1044.1
90.1		90.1		y_1	A_{46}	b_{18}^{2+}	1001.5		1079.6	

[a] Absence of an entry in the observed column indicates ion not detected; numbering of b and y ions is for the internal fragment; for clarity, some detected ions are not labeled on the corresponding spectra. [b] MS^3 fragmentation: *m*/*z* 992.8 → *m*/*z* 1010.3 →. [c] MS^3 fragmentation: *m*/*z* 1024.1 → *m*/*z* 1088.8 →. [d] Fragment ions calculated for covalent attachment of HNE to His. [e] Numbering is based on amino acid residue position in unfragmented CcO subunit VIII.

subunit of CcO triggers a conformational change that leads to a weakened association of subunit VIa with the remainder of the enzyme.

Direct identification of HNE-modified subunits was obtained using two mass spectrometric methods: ESI and MALDI-TOF. A total of six subunits were identified as HNE adducts: subunits II, IV, Vb, VIIa, VIIc, and VIII. However, the only subunits found to be modified more than 5% were subunits VIIc and VIII, which were modified 30% and 50%, respectively. HNE modification of one or both of these subunits must be responsible for the 30−50% loss of CcO activity upon exposure to HNE.

The most likely HNE reactive sites within subunits VIII and VIIc would be expected to be lysine, histidine, or cysteine. Cysteine can be ruled out for both subunits since they do not contain this amino acid. Subunits VIII and VIIc contain five and four lysyl residues and one and two histidyl residues, respectively. Lysyl groups are more numerous but are far more likely to be protonated and, therefore, less reactive at pH 7.5. We attempted to digest control and HNE-modified subunit VIII with chymotrypsin, trypsin, and v8 protease so that the HNE reactive site could be more clearly defined; however, subunit VIII proved to be resistant to digestion, and this approach was not fruitful.

Direct analysis using ion trap tandem mass spectrometry permitted identification of His-36 as the primary reaction site for HNE within subunit VIII. Subunit VIII is a transmembrane subunit that is located 28.5 Å from heme *a* and 41 Å from heme a_3 (*3*). Although, subunit VIII is peripherally located and has no known role in either electron transport or proton translocation, dissociation of this subunit by urea causes a complete loss of enzymatic activity (Sedlak and Robinson, unpublished). Subunit VIII is in close proximity to both helices I and XII of subunit I (*3*). Helix XII of subunit I has been proposed as a possible channel for proton pumping (*3*); therefore, conformational changes of subunit VIII may control enzymatic activity via indirect effects on subunit I. Reaction of HNE with CcO certainly leads to significant conformational perturbation of CcO since it causes 75% of subunit VIa to dissociate from the remainder of the complex. Dissociation of VIa, of itself, cannot be responsible for the loss of enzymatic activity since this subunit can be completely removed with almost no change in electron transport. We conclude that HNE modification of His-36 of subunit VIII must cause loss of activity via an indirect, conformation-related mechanism.

To date, very little is known about the physiological function of subunit VIII. Subunit VIII has been suggested to participate in thermogenesis (*44*) and, therefore, regulate enzymatic activity. Subunits VIII and VIIc, which are both modified 30−50% by HNE, also participate in binding one of the functionally important cardiolipins (*4*; Sedlak et al., unpublished). Removal of cardiolipin from the subunit VIIc−subunit VIII site causes a 50% loss of CcO electron transport activity (*4*) due to slowing of the electron-transfer rate between cytochrome *a* and a_3, a decrease similar to that observed in the present study. Peroxidation of cardiolipin, possibly that bound to subunit VIIc, also causes a loss of CcO activity (*45*). The mechanism by which each of these structural perturbations near subunits VIIa and VIII alters electron-transfer rates within CcO has not been explained, but as discussed previously, they all may involve a similar perturbation of the environment of one of the redox centers of CcO.

Lastly, as part of this study, we were able to verify the molecular mass of each of the 13 CcO subunits using ESI/MS and/or MALDI-TOF/MS methods. All experimental values agreed within experimental error of mass values calculated from the known sequences (refer to Table 1). The only apparent exception is the mass of 29793 Da obtained for subunit III. This value is 126 Da lower than the mass calculated from the known DNA sequence. However, a

significant proportion of subunit III was missing its N-terminal methionine when it was sequenced (*35*). Our MALDI-TOF/MS value for subunit III is within experimental error of the value based upon a sequence missing the N-terminal methionine. On the basis of this correction, all of our values are within 6 parts in 10000 of the sequence values. The close agreement not only illustrates the extremely high precision of the mass spectrometry methods but also verifies the mass spectrometry approach for detecting HNE adducts of CcO.

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