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# Oxidatively damaged proteins of heart mitochondrial electron transport complexes

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**Abstract**

Protein modifications, such as carbonylation, nitration and formation of lipid peroxidation adducts, e.g. 4-hydroxynonenal (HNE), are products of oxidative damage attributed to reactive oxygen species (ROS). The mitochondrial respiratory chain Complexes I and III have been shown to be a major source of ROS in vitro. Additionally, modifications of the respiratory chain Complexes (I–V) by nitration, carbonylation and HNE adduct decrease their enzymatic activity in vitro. However, modification of these respiratory chain complex proteins due to in vivo basal level ROS generation has not been investigated. In this study, we show a basal level of oxidative damage to specific proteins of adult bovine heart submitochondrial particle (SMP) complexes, and find that most of these proteins are localized in the mitochondrial matrix. We postulate that electron leakage from respiratory chain complexes and subsequent ROS formation may cause damage to specific complex subunits and contribute to long-term accumulation of mitochondrial dysfunction.


*Keywords:* Oxidative stress; Mitochondrial dysfunction; Carbonylation; Nitration; 4-hydroxynonenal; Lipid peroxidation adduct

## 1. Introduction

Mitochondria are a major source of oxidative stress [5]. This stress is generated by oxidative phosphorylation processes carried out by the mitochondria and the production of reactive oxygen species (ROS) by electron leakage from respiratory chain complexes. Mitochondrially generated ROS are difficult to measure directly both in vitro and in vivo. The production of stable free radicals in Complex I and Complex III has been estimated by electron paramagnetic resonance [12,16,35]; however, the short half-life of these radicals make their accurate measurement difficult. In contrast, the products of ROS reaction with nucleic acids, proteins, lipids, and carbohydrates are more stable and more easily detected. The amount of modified macromolecules has been shown to be indicative of the accumulated damage that exists in tissues [33]. Proteins are especially vulnerable to oxidative damage. Oxidative modifications of proteins include nitration of tyrosine, carbonylation of lysine, arginine, proline, threonine, histidine and cystine, and the formation of lipid peroxidation adducts (e.g. 4-hydroxynonenal (HNE)) of lysine, arginine, histidine and cystine [3,30,31]. Oxidatively damaged proteins can be detected by immunoblotting using antibodies specific for these modifications and identified by mass spectrometry (MALDI-TOF) [20]. In addition, it has been shown that such modifications in certain systems can impair the function of these proteins [1,3,4,21,27,29].

In this study, we employ blue-native polyacrylamide gel electrophoresis (BN-PAGE) to resolve intact the respiratory chain Complexes I–V [25] of adult bovine heart submitochondrial particles (SMP), and second dimension denaturing SDS-PAGE to resolve individual subunits of these complexes. Immunoblotting analysis has been used to detect proteins that are modified by HNE, nitration, or carbonylation [17,30]. Proteins shown to be oxidatively damaged were subjected to both MALDI-TOF and LC/MS/MS mass spectrometry to establish their identity. Our studies have identified specific protein subunits of the mitochondrial respiratory chain complexes that are susceptible to and targeted for oxidative damage caused by ROS-mediated protein modification.

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## 2. Materials and methods

Reagents were purchased from Sigma Chemical (St. Louis, MO) and bovine hearts were a kind gift from Sam Kane Meat Packing, Corpus Christi, TX.

Bovine hearts were cooled prior to processing for mitochondrial isolation. The fat and connective tissue were removed from the hearts prior to homogenization of the cardiac muscle. Mitochondrial isolation was carried out by published methods [8,13]. The crude mitochondria were stored in aliquots at −80 °C for further use. For each analysis, fresh aliquots were thawed, centrifuged once and resuspended in the isolation buffer [13]. The mitochondria were then sonicated briefly to generate SMPs and their oxygen consumption activities were measured using an oxygen monitoring system (Strathkelvin Instruments Ltd., Glasgow, UK) with both NADH and succinate as substrates to verify respiratory activity.

BN-PAGE and SDS-PAGE were carried out by published methods [25] with minor modifications. A 5–12% acrylamide gradient was used for the first dimension native gel. Imidazole instead of the Bis–Tris was used as a buffer, and a 10% separating gel and 8% stacking gel were used for the second dimension SDS-PAGE.

Immunoblot analysis was performed as described previously [1]. Individual complex bands were visualized by antibodies against Complex I, 39 kDa subunit; Complex II, 70 kDa subunit; Complex III, FeS subunit; Complex IV, subunit IV, and Complex V, β subunit (Molecular Probes, OR). Protein modifications were identified using a mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology, NY) and anti-HNE Fluorophore antibodies (Calbiochem, CA) [30,31]. Carbonylated proteins were first derivatized with dinitrophenyl hydrazine (DNPH) generating a stable dinitrophenyl hydrazone (DNP) adduct to the carbonyl group [17]. Anti-DNP antibody (Intergen, NY) was then used to detect DNP-derivatized proteins. HRP-conjugated secondary mouse and rabbit antibodies (Amersham Biosciences, UK) were used. The blots were visualized using the SuperSignal chemiluminescent substrate (Pierce, IL), and image recorded using Kodak X-Omat AR films.

Individual protein bands showing ROS-modified proteins were excised from second dimension SDS-PAGE run simultaneously with the gels that were Western blotted and analyzed by the Proteomics Core Facility at UTMB. The proteins were eluted from the gel and digested with trypsin (Promega, WI); the tryptic peptides were then analyzed either by MALDI-TOF or by LC/MS/MS. Mass spectral peak data were submitted to the ProFound (Rockefeller University) online search engine for protein identification using the NCBI database. For LC/MS/MS analysis, digested samples were separated using a Waters CapLC capillary HPLC system consisting of a 75 μm × 10 mm C18 capillary column. The gradient used was 5% acetonitrile/95% water to a maximum acetonitrile content of 80% over a 60-min period. The flow rate was 200 nl/min. The output of the column was inserted directly into the source of a Micromass Q-Tof2 electrospray mass spectrometer. A voltage of 3000 V was applied to the capillary column tip. Samples were analyzed in positive ion mode and MS/MS was performed on all ions that gave a base peak intensity of 10 counts or greater. Data processing for peptide sequencing was done using Micromass ProteinLynx software.

## 3. Results

The oxygen consumption activities of bovine heart SMPs were measured to be 638.8 μmol of $O_2$/min/mg

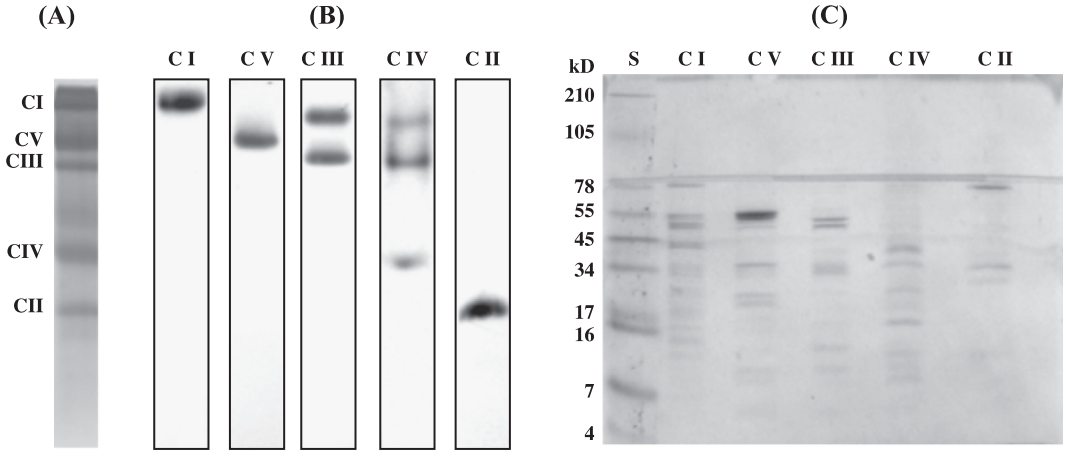


Fig. 1. Separation of bovine heart SMP respiratory chain complex subunits. (A) Bovine Heart SMPs were solubilized and the respiratory chain complexes were separated on a blue-native first dimension gel as described in Materials and methods. Lanes 1, 2 and 3 represent the blue-native first dimension electrophoretic separation of mitochondrial complexes from bovine heart SMPs. (B) Complex specific antibodies were used in immunoblots performed after the blue-native gel to confirm the identity and position of each complex on the blue-native gels. (C) The individual Complexes I–V, resolved in (A), were excised and the subunits of each complex were separated on a second dimension SDS-PAGE. The abbreviations are as following: S = standard, C I = Complex I, C II = Complex II, C III = Complex III, C IV = Complex IV and C V = Complex V.

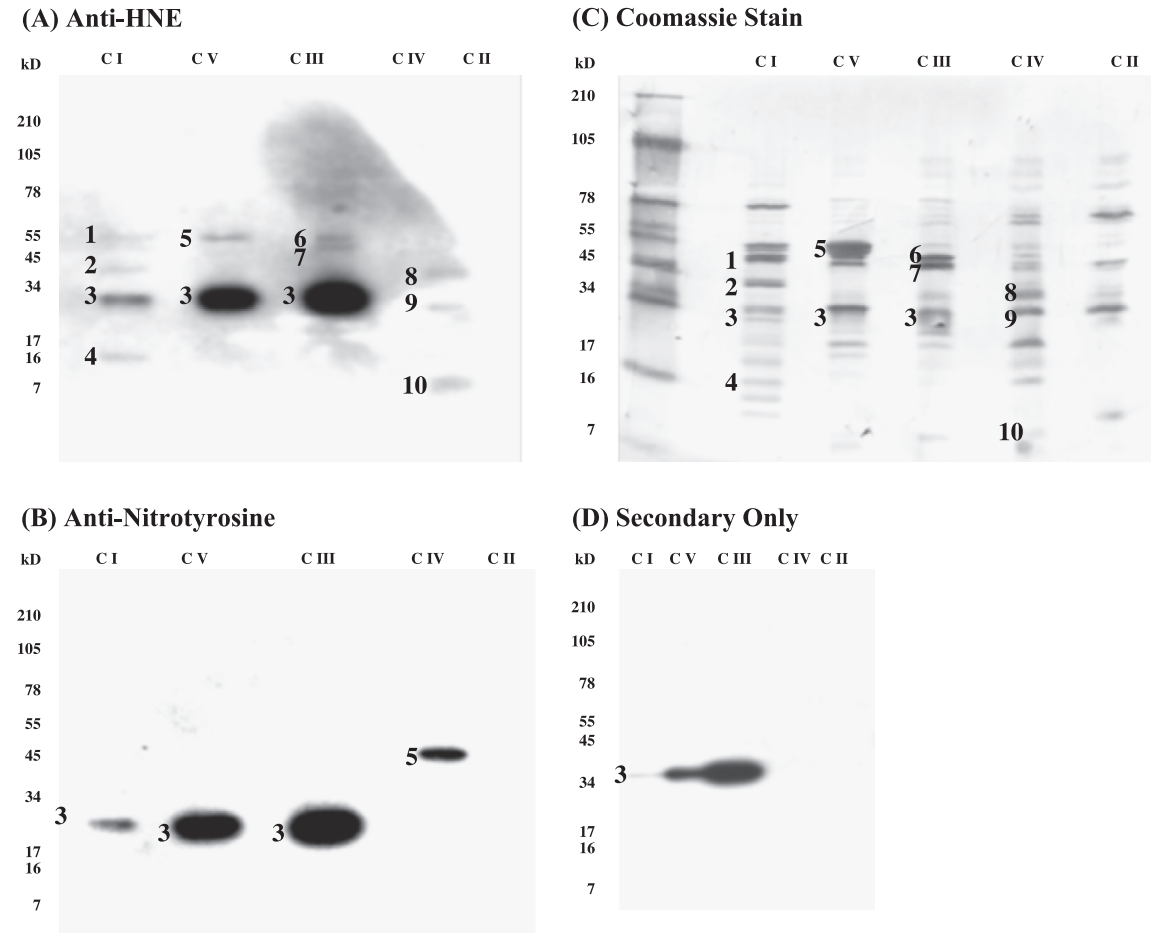


Fig. 2. Identification of oxidatively damaged proteins of bovine heart SMP respiratory chain complexes resolved by second dimension SDS-PAGE. Bovine heart SMP complexes were resolved into their subunits as described in Materials and methods and immunoblots were performed using (A) anti-HNE; (B) anti-nitrotyrosine and (D) secondary only antibodies; (C) Coomassie blue G-250 stain of the second dimension SDS-PAGE used in immunoblots (A) and (B). The molecular weight markers of (A) and (D) correspond to those shown in (C) and (D) and were obtained by overlaying the Coomassie-stained gels over the Western blots. The abbreviations are as follows: C I = Complex I, C II = Complex II, C III = Complex III, C IV = Complex IV and C V = Complex V. Identification of each numbered band is summarized in Table 1.

and 101 μmol of $O_2$/min/mg with NADH and succinate as substrates, respectively. The respiratory rates were completely sensitive to KCN inhibition. These SMPs were solubilized and subjected to BN-PAGE to resolve Complexes I–V [8,13,25]. Fig. 1A shows the first dimension electrophoretic resolution of these SMPs. The complexes were identified by immunoblot using antibodies specific for complex components as seen in Fig. 1B. Based on previous reports, Complex I is made up by 46 subunits and has an approximate molecular mass of 980 kDa [6]; Complex II is made up of 4 protein subunits and has an approximate molecular mass of 130 kDa [23]; Complex III is made up of 11 protein subunits, and in the mitochondria, the functional complex occurs as a dimer with an approximate molecular mass of 500 kDa [26]; Complex IV is made up of 13 protein subunits and has a molecular mass of 200 kDa [15]; and Complex V is made up of 16 polypeptides and their isotypes, and has a molecular mass of 600 kDa [19]. In the BN-PAGE, these complexes separate in the following order, from highest to lowest molecular mass (Fig. 1A):

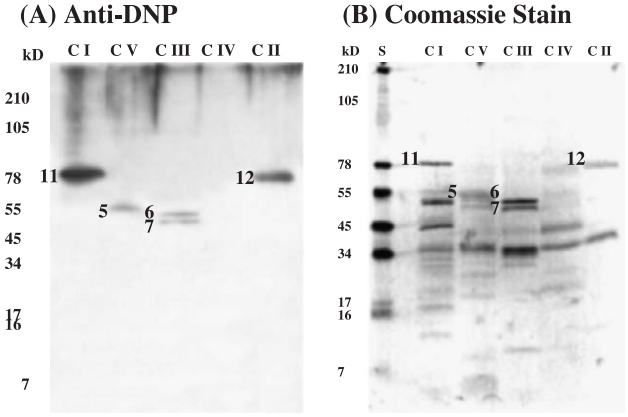


Fig. 3. Identification of carbonylated proteins of bovine heart SMP respiratory chain complexes resolved by second dimension SDS-PAGE. Bovine heart SMP complexes were derivatized using DNPH and resolved into their subunits as described in Materials and methods followed by immunoblot analysis. (A) Anti-DNP antibody, and (B) Coomassie blue G-250 stain of the second dimension SDS-PAGE used in the immunoblot. Identification of each numbered band is summarized in Table 1.

Table 1
HNE, nitrotyrosine-modified and carbonylated subunits of bovine heart SMP respiratory chain complexes

| Band number | MALDI-TOF/ ProFound ID | Z score | Coverage (%) | Mitochondrial localization |
|---|---|---|---|---|
| *HNE-modified* (Fig. 2A) | | | | |
| 1 | NUCM (49.2 kDa) | 2.42 | 41 | Complex I subunit |
| 2 | NUEM (39 kDa) | 2.37 | 40 | Complex I subunit |
| 3[a] | Cyt. $c_1$ (27.2 kDa) | 2.3 | 32 | Complex III subunit |
| 4[a] | B17 (15.5 kDa) | 1.83 | 32 | Complex I subunit |
| 5 | β chain (51.5 kDa) | 2.37 | 52 | Complex V subunit |
| 6 | Core 1 (49.2 kDa) | 2.41 | 43 | Complex III subunit |
| 7 | Core 2 (46.5 kDa) | 2.38 | 29 | Complex III subunit |
| 8 | ANT (T1)(32.9 kDa) | 2.25 | 29 | inner membrane |
| 9 | VDAC 1 (30.7 kDa) | 2.35 | 39 | outer membrane |
| 10 | VIb (10.2 kDa) | 2.31 | 33 | Complex IV subunit |
| *Nitrotyrosine-modified* (Fig. 2B) | | | | |
| 3[a] | Cyt. $c_1$ (27.2 kDa) | 2.3 | 32 | Complex III subunit |
| 5 | β chain (51.5 kDa) | 2.37 | 52 | Complex V subunit |
| *Secondary only* | | | | |
| 3[a] | Cyt. $c_1$ (27.2 kDa) | 2.3 | 32 | Complex III subunit |
| *Carbonylated* (Fig. 3A) | | | | |
| 5 | β chain (51.5 kDa) | 2.37 | 52 | Complex V subunit |
| 6 | Core 1 (49.2 kDa) | 2.41 | 43 | Complex III subunit |
| 7 | Core 2 (46.5 kDa) | 2.38 | 29 | Complex III subunit |
| 11[a] | NUAM (75 kDa) | 2.4 | 30 | Complex I subunit |
| 12[a] | SDHA (70 kDa) | 2.31 | 34 | Complex II subunit |

Cyt. = Cytochrome; ANT = adenine nucleotide translocator; VDAC = voltage-dependent anion channel.
[a] Identified by both MALDI-TOF and LC/MS/MS.

Complex I, Complex V, Complex III, Complex IV, and Complex II. Each complex band was excised and run separately in the order of their separation on the second dimension denaturing gel and the resolved protein bands were visualized by staining with Coomassie blue (Fig. 1C).

Immunoblot analyses of the second dimension gels were carried out to identify oxidatively modified proteins (Figs. 2 and 3). These analyses revealed proteins that were modified by HNE (Fig. 2A) and nitrotyrosine (Fig. 2B) adducts. The proteins modified by carbonylation were detected by DNP-derivatization followed by immunoblot analysis (Fig. 3). These correspondingly modified proteins were cut out from the Coomassie-stained gels (Figs. 2C and 3B) and identified by MALDI-TOF and LC/MS/MS mass spectrometry (Table 1).

Proteins modified by HNE-adduct formation include: Complex I, NUCM subunit (49.2 kDa), NUEM subunit (39 kDa); Complex III, the mitochondrial processing peptidase subunits α (49.2 kDa) and β (46.5 kDa) also known as Complex III Core 1 and Core 2 subunits, respectively; Complex IV, subunit VIb (10.2 kDa), and Complex V, β chain (51.5 kDa). Proteins modified by carbonylation include: Complex I, 75 kDa subunit, Complex II, 70 kDa subunit, Complex III, Core 1 (49.2 kDa) and Core 2 (46.5 kDa) and Complex V β chain (51.5 kDa). Finally, the only protein found to be nitrated is the β chain (51.5 kDa) of Complex V.

The β chain (51.5 kDa) of Complex V (F1 portion) reacted with all three antibodies suggesting multiple sites that are susceptible to oxidative damage. This β chain was also identified in Complex I (by anti-HNE and anti-DNP) and in Complex IV (by anti-nitrotyrosine; Table 1).

Both anti-mouse and anti-rabbit secondary antibodies cross-reacted to cytochrome $c_1$ (27.2 kDa) as seen by the strong signal in secondary antibody blot at an apparent mass of 30 kDa (present in Complex I, V and III in Fig. 2D). However, no other bands were present in this blot compared to blots in Fig. 2A and B, suggesting that all other bands in Fig. 2A and B represent the interactions of oxidatively modified proteins with primary antibodies. Thus, the cytochrome $c_1$ band interacting with secondary antibody repre-

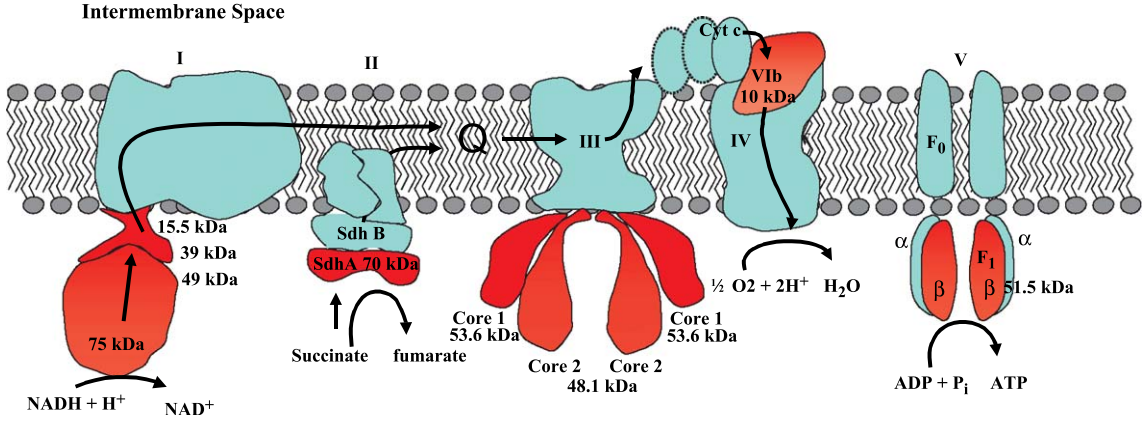


Fig. 4. A diagrammatic presentation of the localization of oxidatively damaged proteins of Complexes I–V of the mitochondrial respiratory chain. The diagram shows the proteins of each complex identified in our studies to be oxidatively damaged by HNE, nitration and/or carbonylation, and that these proteins are localized in the mitochondrial matrix or intermembrane space. The red portions of complexes depict oxidatively damaged subunits and the green portions represent subunits that are not damaged. The pattern of proteins that are modified follows that of the interactions that are carried out by these extramembrane components of the complexes.

sents the abundance of this protein in the complexes and because the protein modification is expected to be lesser than total protein present, this accounts for the significantly higher intensities of cytochrome $c_1$ bands compared to all other oxidatively damaged proteins. Although, it is possible that cytochrome $c_1$ may be modified by oxidation, the recognition by secondary antibodies makes it impossible to determine such modifications. Therefore, further investigation is required to determine whether cytochrome $c_1$ is oxidatively damaged.

In addition to the respiratory chain complex subunits, two proteins, heart-specific T1 isotype of adenine nucleotide translocator (ANT, 32.9 kDa) and voltage-dependent anion channel 1 (VDAC, 30.7 kDa) were also identified among Complex IV proteins modified by HNE formation (Table 1). Both ANT and VDAC are part of a complex that forms the mitochondrial permeability transition pore that has been shown to play a key role in cellular apoptosis.

All proteins we detected as oxidatively modified are known to associate with or are in close proximity to the inner mitochondrial membrane (Fig. 4). These data are consistent with the proposal that ROS generated by dysfunctional/oxidatively stressed respiratory chain complexes may react with the mitochondrial membranes, thereby causing increased lipid peroxidation, and these lipid peroxidation products modify membrane-associated or membrane-proximal subunits of various complexes. In addition, these data also suggest differential targeting of specific subunits of the respiratory chain complexes resulting in differences of nitrotyrosine modification, HNE adduct formation and carbonylation, as summarized in Table 1.

## 4. Discussion

Mitochondria are a major source of ROS in mammalian tissues. However, the difficulties inherent in direct measurement of such short-lived radicals have hampered the identification of ROS formation mechanisms and the targets of oxidative damage. In this study, we used an indirect measurement of ROS damage, i.e. protein modification, to serve as an indicator of damage from mitochondrial ROS. Here, we present for the first time, evidence that specific protein subunits of mitochondrial respiratory chain Complexes I–V are susceptible to oxidative damage in normal unchallenged bovine heart mitochondria (Fig. 4). Modifications due to oxidative damage have been shown to result in either the loss-of-protein function [28] or protein malfunction such as toxic-gain-of-function [22]. For example, it has been shown that the in vitro modification by HNE of subunits I and IV of Complex IV inhibits the enzymatic activity of this complex [7]. In our study, we show that subunit VIb of Complex IV is HNE-modified. This modification may affect the enzymatic activity of Complex IV thereby resulting in mitochondrial dysfunction. Modification of the β chain of F1F0-ATP synthase by all three adducts suggests that this protein is particularly susceptible to oxidative damage. Since the β chain of F1 portion functions to synthesize ATP it is possible that such oxidative damage to this subunit may lead to decreased ATP synthesis. In amyotrophic lateral sclerosis, a gain-of-toxic-function occurs due to point mutations of SOD1 that result in increased enzyme activity and propensity to aggregate [22]. The in vitro oxidation of SOD1 causes an increase in enzyme activity and its propensity to aggregate, thus mimicking the in vivo characteristics of gain-of-toxic-function.

Many diseases characterized by mitochondrial dysfunction result from deficiencies or defects in respiratory chain complex subunits. Mitochondrial myopathy has been attributed to a deficiency in the iron–sulfur cluster containing subunits of Complex I (39 and 49 kDa subunits) [11]. The disproportionate deficiency of 75 kDa iron–sulfur subunit of Complex I plays a major role in mitochondrial encephalomyopathy as well as congenital Complex I deficiency in severe lactic acidosis [14,18]. Down-regulation of the B17 subunit of Complex I in response to oxidative stress, and deficiency of this subunit has been implicated in the defect in Complex I activity in Parkinson's disease [36]. These studies thus suggest that modification due to oxidative damage of specific components of electron transport complexes may play a key role in the gradual decline in tissue function. Furthermore, it has been reported that inherent defects of mitochondrial SDH in humans are associated with various clinical presentations ranging from early-onset of devastating encephalopathy to tumor susceptibility in adulthood or optic atrophy in the elderly [24]. Thus, modification of the 70-kDa subunit of Complex II, the succinate oxidizing subunit, suggests that oxidative damage to this subunit via carbonylation may inhibit the protein's ability to oxidize succinate, thereby contributing to its dysfunction.

We have observed that proteins that are not involved in electron transport have also been modified by oxidative damage. The mitochondrial processing peptidase subunits (Core 1 and Core 2) of Complex III, which are carbonylated and HNE-modified, play a critical, non-respiratory role in mitochondrial function. These proteins function as mitochondrial processing peptidases involved in proper protein folding during the maturation and transport of mitochondrial matrix and inner membrane proteins [9]. Indeed, recent studies have shown that misfolded proteins within the mitochondria activate a mitochondria-specific unfolded protein stress response, indicating the importance of these protein folding–transport processes [38]. In addition, the ROS generation from Complex III is thought to occur at heme $b_H$ of cytochrome *b* subunit or at the quinone reducing $Q_i$ site [37]. Notably, the Core 1 and Core 2 subunits of the mitochondrial processing peptidases are located close to the site of ROS production and their carbonylation and HNE adduct formation suggest that proximity to the site of ROS generation may therefore lead to their oxidation damage. Thus, we propose that subunits proximal to ROS generation sites of respiratory chain complexes may be more suscepti-

ble to oxidative damage and that their modification may lead to mitochondrial dysfunction as seen in oxidative stress.

Two additional proteins, ANT [32,34] and VDAC [2], which are not components of the respiratory chain complexes were also oxidatively modified. Recent studies have shown that in vitro modification of ANT by nitric oxide, peroxynitrite and HNE enhances permeability transition pore opening in proteoliposomes suggesting that this modification may be a regulatory marker for mitochondrial permeability transaction pore opening and cellular apoptosis [32]. However, although the authors reported that none of the endogenous ANT was modified by such adducts, our results clearly show that there is, in fact, an endogenous modification of ANT and VDAC in bovine heart mitochondria. Thus, endogenous modification of these proteins in heart mitochondria may also contribute to the regulation of the permeability transition pore opening and control of apoptosis. The VDAC is localized in the outer mitochondrial membrane that provides a major pathway for the transport of metabolites, e.g. ATP, cholesterol and involved in mitochondrial events that lead up to apoptosis [10]. It is interesting that VDAC proteins are localized only to the peripheral and not to the perinuclear mitochondria [2].

Our data show that the proteins of Complexes I–III, and V, which are oxidatively damaged, are localized in the mitochondrial matrix, while subunit VIb of Complex IV is localized in the intermembrane space. This follows the pathway of electron flow and is, therefore, suggestive of selectivity for extramembrane components of the complexes involved in electron transport (Table 1, Fig. 4). Carbonylation of aconitase, VDAC and core proteins of Complex III have been shown to occur in anoxia-induced oxidative stress in yeast [2]. Interestingly, we show that the same proteins are oxidatively damaged in bovine heart SMPs. These data suggest that the same proteins, across species, of the electron transport complexes may be more susceptible to oxidative damage. We propose that their proximity to the electron transport processes, i.e. their intramitochondrial localization whether yeast or bovine, may contribute to this selectivity.

The results presented here support the hypothesis that mitochondrial respiratory chain complexes generate ROS, which can oxidatively damage key proteins thereby contributing to mitochondrial dysfunction. The oxidative damage from ROS includes proteins of the mitochondrial respiratory chain complex subunits as well as proteins involved in other functions. These modifications occur under normal basal conditions, suggesting a consistent low level of mitochondrial damage in the absence of an oxidative stress challenge.

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