Oxidative Post-translational Modification of Tryptophan Residues in Cardiac Mitochondrial Proteins*

We examined the distribution of N-formylkynurenine, a product of the dioxidation of tryptophan residues in proteins, throughout the human heart mitochondrial proteome. This oxidized amino acid is associated with a distinct subset of proteins, including an overrepresentation of complex I subunits as well as complex V subunits and enzymes involved in redox metabolism. No relationship was observed between the tryptophan modification and methionine oxidation, a known artifact of sample handling. As the mitochondria were isolated from normal human heart tissue and not subject to any artificially induced oxidative stress, we suggest that the susceptible tryptophan residues in this group of proteins are “hot spots” for oxidation in close proximity to a source of reactive oxygen species in respiring mitochondria.

A by-product of oxidative phosphorylation in normally respiring mitochondria is the formation of reactive oxygen species (ROS). If left unchecked, ROS can cause radical-induced damage to proteins, lipids and nucleic acids (1), and elevated oxidative stress has been implicated as a major contributing factor to degenerative diseases and aging (2). Defects in the enzymes associated with scavenging of ROS, such as manganese superoxide dismutase, can have catastrophic consequences for the cell and result in the onset of degenerative pathology (3, 4). Relatively few post-translationally modified products of oxidative damage in mitochondria have been characterized. Those that have been identified come from mitochondria or proteins that have been exposed to simulated oxidative stress in vitro such as peroxynitrite-mediated nitration of a critical tyrosine residue in manganese superoxide dismutase (5, 6) or proteolysis in response to hydrogen peroxide/menadione treatment (7). In unchallenged mitochondria, endogenous S-nitrosylation of mitochondrial caspases regulates their activity and modulates apoptosis (8). In the current investigation, we have examined the occurrence of N-formylkynurenine, a product of double oxidation of tryptophan throughout the mitochondrial proteome of normal human heart tissue and found evidence of selective oxidation in a subset of proteins associated predominantly with redox metabolism.

EXPERIMENTAL PROCEDURES

LC/MSAMS data generated from the human heart mitochondrial proteome project (9) as well as for human and bovine proteins prepared by sucrose density gradient centrifugation or immunoprecipitation using antibodies against complex V (ATP synthase) (10) and complex I (NADH dehydrogenase) (11) were queried against the human or bovine subsets of GenBankTM using the Sonar MS/MS searching algorithm (12) with oxidation of methionine (+16 atomic mass units) and tryptophan (+32 atomic mass units) specified as differential modifications. Corresponding MALDI spectra were manually inspected.

RESULTS AND DISCUSSION

The oxidation of tryptophan to N-formylkynurenine in proteins has been known for over 35 years since Previero and co-workers (13) described it in hen’s egg-white lysozyme in anhydrous formic acid. Kuroda and co-workers (14) subsequently found that inactivation of lysozyme by ozone in aqueous solution occurred only when one critical tryptophan residue was oxidized, thus providing the first evidence that oxidation of a specific tryptophan residue can impair enzyme function. These early reports relied on identification of the tryptophan oxidation products by characteristic electronic absorption spectra. Finley and co-workers (15) exposed α-crystallin from bovine lens tissue to Fenton chemistry in vitro and separated the component tryptic peptides by HPLC. MS/MS spectrometry was used to identify oxidized amino acid sites by +16, +32, and +4 atomic mass unit increases in the molecular mass of peptide fragment ions containing tryptophan residues. Structures corresponding to those mass shifts are shown in Fig. 1.

Other reports have noted +16 and +32 atomic mass unit modifications to peptides containing tryptophan residues from tryptic in-gel protein digests. They have discussed the merits of including these modifications in both peptide mass fingerprinting (16–18) and MS/MS (17) searching algorithms to gain increased protein coverage and therefore increased confidence in an identification. These investigations assumed tryptophan oxidation to be analogous to methionine oxidation, known to be a common artifact of sample handling (19). MacCoss and co-workers (20) performed a shotgun LC/LC/MS/MS study on digests of human cataract lens tissue and searched their large data set of MS/MS spectra for +16 atomic mass unit modifications of tryptophan residues. They verified that a specific tryptophan residue (Trp-60 of αB-crystallin, which was oxidized in the in vitro study (15), was also present in the digests of human lens. Although it could not be demonstrated unequivocally whether oxidation occurred...
FIG. 2. MALDI-TOF mass spectrometry of two peptides from complex I subunit NDUFS4 displaying tryptophan (A) and methionine (B) oxidation. The samples are as follows: i, human heart mitochondria complex I (subject 1) prepared by sucrose density gradient fractionation (SDG) and one-dimensional electrophoresis; ii, human heart mitochondria complex I (subject 2), prepared by immunocapture and one-dimensional electrophoresis; iii, human heart mitochondria complex I (subjects 3, 4, and 5 (pooled)), prepared by SDG and one-dimensional electrophoresis; iv, human heart mitochondria complex I (animal 1) prepared by SDG and one-dimensional electrophoresis; v, bovine heart mitochondria complex I (animal 1) prepared by SDG and one-dimensional electrophoresis; vi, bovine heart mitochondria complex I (animal 2) prepared by SDG and two-dimensional electrophoresis.

Table I

Peptides containing doubly oxidized tryptophan throughout the cardiac mitochondrial proteome

Entries are listed first in order of the most oxidized peptides detected by Sonar MS/MS for a given protein satisfying \( E_{\text{pep}} \) values and criteria listed in Ref. 9 and then in order of decreasing confidence. Lower confidence peptides (\( E_{\text{pep}}/H_{11350}1/\text{H}_{11001}10/\text{H}_{11002}3 \)) are included only if they have been validated by other data in Ref. 9. GI, GenBank™ accession number. Oxidized tryptophan residues are indicated by lowercase w.

| Peptide | \( E_{\text{pep}} \) | GI | Protein description |
|---------|-----------------|---|-------------------|
| VFEISPFEwITR | 1.40E-05 | 6681764 | NDUFA9 |
| FQFIPLSLGwKR | 2.30E-04 | 6681764 | NDUFA9 |
| wLSAEIEDVPKAP | 1.80E-03 | 6681764 | NDUFA9 |
| HAGGVTGWdNLLAVIPGSSTPLPK | 2.10E-04 | 20149568 | NDUFV1 |
| GDARPAEDISlwEISK | 9.40E-04 | 20149568 | NDUFV1 |
| GPDlwLGEIK | 2.40E-03 | 20149568 | NDUFV1 |
| LAALFENPFAwAYKY | 3.20E-05 | 5453559 | ATPase d |
| TIDlwVAEFAEIPQNK | 2.10E-03 | 5453559 | ATPase d |
| YPWlwPHQPIENLK | 7.20E-03 | 5453559 | ATPase d |
| wVVIGDENYGEGSSR | 8.40E-08 | 3600098 | Aconitase precursor |
| VAEKEGwPLDIR | 4.00E-04 | 3600098 | Aconitase precursor |
| LwISNGLADlFTVFAK | 2.90E-08 | 18044943 | Acyl-coenzyme A dehydrogenase, very long chain |
| RPFPEPTTTlwQEDPEDENLYEK | 6.80E-08 | 13938442 | Neuronal protein (NP17.3) |
| ALGVLAQLIwSR | 1.10E-05 | 4758714 | Microsomal glutathione \( S \)-transferase 3 |
| DYlwNTLNSSR | 7.10E-04 | 4758714 | Citrate synthase precursor |
| KLETQAVNlwTAGNSNTR | 1.60E-05 | 5407879 | NDAC-1 |
| wNTDNTLGElwTDGLAR | 5.30E-06 | 5407879 | NDAC-1 |
| VVDGAVGqwLAEFR | 4.70E-05 | 17548911 | Dihydrolipoamide \( S \)-acyltransferase |
| VPEANSwMDTIVR | 6.60E-04 | 17548911 | Dihydrolipoamide \( S \)-acyltransferase |
| SAVTAlwGK | 3.70E-03 | 4504575 | Isocitrate dehydrogenase 2 (NADP\(^+\), mitochondrial) |
| LLVYYPwTQR | 4.30E-03 | 4504575 | Isocitrate dehydrogenase 2 (NADP\(^+\), mitochondrial) |
| NTQYSwLLDGFPKR | 1.00E-06 | 1992344 | Brain myo25 |
| FDLNSpwEAPPYR | 2.10E-05 | 11360206 | NDUFS1 |
| IASGlwLAvwTQNGTR | 2.60E-05 | 5407879 | NDAC-1 |
| GTYHwEDwK | 2.90E-05 | 12001999 | Brain myo25 |
| ASSTSpwEISElwLDQK | 4.00E-05 | 4503607 | Electron transfer flavoprotein alpha polypeptide |
| GRPSTNPWSFPAwTR | 6.40E-05 | 4504575 | Isocitrate dehydrogenase 2 (NADP\(^+\), mitochondrial) |
| GLITwYwEDALSRw | 1.40E-04 | 21411235 | NDUFS1 |
| IPwFQwPIYPYDIR | 1.90E-04 | 6055854 | \( \beta \)-prohibitin |
| GLSDEGWwQVLNVwGK | 2.50E-04 | 229361 | Myoglobin |
| ASwSSLSwDEwel | 3.00E-04 | 5921855 | Cytochrome c oxidase subunit IV, isoform 1 |
| LDDLVwAR | 5.30E-04 | 21750996 | NDUFS7 |
| TlwlwTLPwR | 7.80E-04 | 4505371 | NDUFS8 |
| SYGANFswwNK | 8.70E-04 | 13528960 | NDUFS4 |
| ASHLwYGSIPwGGEPR | 9.90E-04 | 13676336 | Long-chain acyl-coA thioesterase peroxisomal |
| wVEADLQwQPLK | 1.20E-03 | 21903482 | Ubiquinol-cytochrome c reductase complex core protein 2 |
| YEGFFSLwK | 1.30E-03 | 21361114 | Mitochondrial carrier; oxoglutarate carrier |
| LITwQQwLIR | 1.40E-03 | 13272660 | ATP synthase 6 |
| LWEFPLwVQwPADQwK | 1.50E-03 | 4826848 | NDUFS5 |
| IDEAILTlwK | 2.00E-03 | 15991833 | Hexokinase 1 |
| wDOQETLwR | 3.30E-03 | 458862 | Fatty acid-binding protein, heart (hFABP) |
| HwLDSpwPFGFTLDQwPR | 3.40E-03 | 20514262 | 2-Oxoglutarate dehydrogenase E1 component, mitochondrial precursor |
| AwwNGSEGPwKVR | 4.30E-03 | 21754001 | Unnamed protein product |
| ELwPSDDPNwTK | 4.70E-03 | 4757332 | Programmed cell death 8 (apoptosis-inducing factor (AIF)) |
| EQwDTIEELIR | 5.30E-03 | 4503031 | 2,4-Dienoyl CoA reductase 1 precursor |
| GAwSNwLR | 5.30E-03 | 86754 | Carrier adenine nucleotide translocase |
| wYYNANPwFK | 5.30E-03 | 5454152 | Ubiquinol cytochrome c reductase (ubiquinone-binding protein) (VI) |
| ELDwSTEFELPWwG | 5.50E-03 | 8131894 | Mitofin |
| APLAEwBwDwMTMK | 8.10E-03 | 4503053 | Monoamine oxidase B |
| LATwYwYAK | 9.10E-03 | 22096328 | ATP synthase G chain, mitochondrial |
during sample handling or in vivo, the authors noted that all sites of crystallin oxidation that they found had been reported previously in the literature (15). Very recently, Anderson and co-workers (21) described oxidation of a critical conserved tryptophan residue (Trp-352) in luminal loop E of the chloroplast photosystem II protein CP43 providing the first example of selective modification in vivo.

We have been studying modifications to complex I subunits in bovine heart mitochondria in response to the oxidative stress caused by peroxynitrite treatment in vitro and have found evidence of oxidized tryptophan in several subunits, both by MALDI-TOF and by LC/MS/MS. However, we were surprised to discover that the relative intensities of the peaks in the MALDI spectra corresponding to peptides containing N-formylkynurenine were also high in untreated mitochondria from some bovine and human heart preparations, although there was substantial variation. Prior to complex I isolation and electrophoresis, mitochondria were prepared identically from all hearts which were freshly collected, frozen, and thawed immediately prior to analysis. Fig. 2 shows the MALDI spectra of peptides from the human complex I subunit, NDUFS4, and its bovine homologue from five different preparations corresponding to seven different hearts (five human, including one pooled sample of mitochondria from three individual hearts, and two bovine hearts). The relative intensities of m/z 1329.6 and 1361.6 (corresponding to peptides without and with dioxidized tryptophan; Fig. 2A) and 1112.5 and 1128.5 (corresponding to peptides without and with oxidized methionine; Fig. 2B) were used as a rough measure of protein oxidation. It was found that there is no correlation between the extent of tryptophan and methionine oxidation, thereby suggesting that they occur via different mechanisms. The dioxidation of tryptophan is clearly discernable in Fig. 2A, i and ii, in which complex I was purified by different methods, sucrose density gradient centrifugation or immunoprecipitation, respectively, but corresponds to mitochondria from the same human heart. This finding suggests that the method of preparation is not a factor in determining the extent of oxidation but rather that it is a characteristic of the donor from which it was obtained (in this case, a 41-year-old male Caucasian who died of brain cancer). The other human donor, displaying far less extensive oxidation of tryptophan as seen in Fig. 2A, iii, was a 62-year-old female Caucasian who died of intracranial bleeding. In contrast, NDUFS4 from a pool of mitochondria from three human hearts displayed an extensively oxidized tryptophan-containing peptide (Fig. 2A, iv) (9). Again the degree of oxidation in the pooled sample was not commensurate with the degree of oxidation for the methionine-containing fragment (Fig. 2B, iv).

Having just completed an extensive survey of the mitochondrial proteome including the acquisition of over 800,000 MS/MS spectra identifying more than 600 distinct gene products (9), we were prompted to search for the distribution of the oxidative modification by reanalyzing the data with N-formylkynurenine selected as a differential modification of tryptophan (+32 atomic mass units) using the Sonar MS/MS algorithm (12). Table I lists all N-formylkynurenine-containing peptides found with peptide expect scores (E_{pep}) values of $1 \times 10^{-3}$ (99% confidence). Of this list of 51 peptide sequences from 39 proteins, nine subunits of complex I had N-formylkynurenine-containing tryptic peptides including a newly discovered subunit neuronal protein (NP17.3) (11). We used this subset of proteins to compare tryptophan oxidation versus methionine oxidation as a function of the ability to observe a peptide in any given LC/MS/MS experiment. In Fig. 3, we have plotted the number of distinct peptides containing tryptophan (A) and methionine (B) for a given complex I subunit which had a Sonar MS/MS E_{pep} score of $1 \times 10^{-3}$, and on that plot we have indicated whether the corresponding oxidized residue was observed. Methionine oxidation appears to be related directly to the number of observable peptides, as would be expected if oxidation were a random sample-handling artifact. In contrast, tryptophan oxidation appears much more specific to selected subunits, with the greatest modification being noted for NDUFV1 (51-kDa flavoprotein 1) and NDUFA9 (a 39-kDa reductase/isomerase subunit). In addition, five subunits of the iron-protein component are oxidized, polypeptides that would be expected to be in proximity to centers of ROS generation.

Interestingly, the next most oxidized of the components of the oxidative phosphorylation machinery is complex V, site of proton pumping and phosphorylation of ADP to ATP but not necessarily directly associated with redox chemistry. Three subunits with oxidized tryptophan residues were observed in complex V, the most oxidized being subunit d. Interestingly, a homologous peptide from subunit d was also reported to contain N-formylkynurenine by Bienvenut and co-workers (17) in bovine heart, suggesting that there may be a specificity for tryptophan oxidation in certain sequences across species as observed in Fig. 2A for NDUF54. We also note that the three oxidized subunits come from the membrane-bound F_{0} component of the complex and not from the subunits of the F_{1} component, e.g. ATP synthase subunits α and β that yield far more tryptic peptides (22). In plant mitochondria, these latter subunits are proteolytically degraded following hydrogen peroxide-induced oxidative stress (7). Only two oxidized peptides were observed for complex III subunits (ubiquinol-cytochrome c reductase), one from complex IV (cytochrome c oxidase) and none for complex II (succinate dehydrogenase). A recent report on the crystal structure of the bacterial analogue of mitochondrial complex II has revealed that the redox
centers are arranged in a manner that prevents generation of ROS at the flavin adenine dinucleotide (23). The absence of oxidized tryptophan in complex II, therefore, supports our hypothesis that the degree of tryptophan oxidation reflects the exposure of proteins to ROS-generating sites.

The remainder of Table I is dominated by redox proteins and certain metalloproteins (including globin contaminants) that may be exposed to ROS or are susceptible to Fenton chemistry. The outer mitochondrial membrane protein, VDAC 1 (voltage-dependent anion channel isoform 1), was also reported to be susceptible to tryptophan oxidation by Thiede and co-workers (18). Based upon the m/z of the mono-oxidized tryptic peptide reported, the sequence is identical to the VDAC 1 peptide reported by us in Table I (KLETAVNLAWTAGNSNTR). This result would suggest that the susceptible sequence is independent of tissue type, as human Jurkat T cells were examined in that study. Adenine nucleotide translocase also exhibits susceptibility to tryptophan oxidation in the sequence list in Table I and, together with VDAC, forms part of the permeability transition pore (24) that is modulated by oxidative stress (25). The presence of prohibitin is interesting in view of its proposed role as a part of a complex that acts as a molecular chaperone for mitochondrial respiratory complex assembly (26). Nijtmans et al. (26) named the complex PHB and proposed renaming the protein hPHB2 to better reflect its function, i.e., proteins that hold badly formed subunits. Given that our results suggest that PHB may be in the proximity of a site of ROS generation, it is tempting to speculate that, in addition to targeting improperly processed or mutated respiratory complex subunits for proteolytic degradation, the PHB complex may also target mature oxidatively damaged and dysfunctional subunits to mitochondrial proteases. The reason for the presence of other protein classes in Table I, including the two kinases, the apoptosis-inducing factor, and the other proteins of unknown function, remains to be determined.

In summary, we have assembled a subset of proteins from cardiac mitochondria that appear to be susceptible to double oxidation of their tryptophan residues. Tryptophan oxidation does not appear to be correlated with methionine oxidation, a known artifact of sample handling. We therefore suggest that this subset of proteins represent hot spots for oxidative modification in respiring mitochondria.

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