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# POST-TRANSLATIONAL MODIFICATIONS OF RAT LIVER MITOCHONDIAL OUTER MEMBRANE PROTEINS IDENTIFIED BY MASS SPECTROMETRY

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## SUMMARY

The identification of post-translational modifications is difficult especially for hydrophobic membrane proteins. Here we present the identification of several types of protein modifications on membrane proteins isolated from mitochondrial outer membranes. We show, *in vivo*, the mature rat liver mitochondrial carnitine palmitoyltransferase-I enzyme is N-terminally acetylated, phosphorylated on two threonine residues, and nitrated on two tyrosine residues. We show long chain acyl-CoA synthetase 1 is acetylated at both the N-terminal end and at a lysine residue and tyrosine residues are found to be phosphorylated and nitrated. For the three voltage-dependent anion channel isoforms present in the mitochondria, the N-terminal regions of the protein were determined and sites of phosphorylation were identified. These novel findings raise questions about regulatory aspects of carnitine palmitoyltransferase-I, long chain acyl-CoA synthetase and voltage dependent anion channel and further studies should advance our understanding about regulation of mitochondrial fatty acid oxidation in general and these three proteins in specific.

### Keywords

Carnitine palmitoyltransferase-I; Long chain acyl-CoA synthetase; Voltage dependent anion channel; post-translational modifications; mitochondria

## INTRODUCTION

Numerous proteins are post-translationally modified and most of these modifications are needed for proper function in the cell. There are over 200 covalent modifications some of which include phosphorylation, nitrosylation, acetylation, methylation, and hydroxylation. Of these

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modifications, phosphorylation is one of the most frequent with estimates of about one third [1] of proteins being phosphorylated. Furthermore, it has been suggested that the majority of phosphorylated human proteins may be phosphorylated at multiple sites [2]. Protein phosphorylation is seen as the primary means of altering the activity of a protein rapidly and, for this reason, seen as a key event in many signal transduction pathways. While less common than phosphorylation, tyrosine nitration also is an important modification in cellular function. Nitration of tyrosine residues in a protein can induce a diverse physiological and pathological response. Protein acetylation is another frequent protein modification. Acetylation can occur co-translationally at the N-terminus and post-translationally at lysine residues. Although N-terminal acetylation is widespread in eukaryotes the biological relevance of this modification is only known for a few substrates [3,4,5]. In contrast, lysine acetylation is reversible with a wide range of functional consequences [6,7].

While important to cellular function, post-translational modifications are often difficult to detect using analytical techniques such as gel electrophoresis and mass spectrometry. These difficulties arise from a number of reasons including the lower relative abundance of modified protein when compared to the unmodified protein. Modifications such as phosphorylation may be unstable during sample preparation and under mass spectrometric conditions. For these reasons, high sequence coverage and a sensitive mass spectrometric technique are required for the detection of post-translational modifications.

Recently, many proteomic studies have focused on the study of sub-cellular systems such as lysosomes [8], Golgi complex [9,10,11], endoplasmic reticulum [12], peroxisomes [13], and mitochondria [14,15,16,17,18]. Mitochondria are of particular interest due to their well-known importance in energy production and their proposed role in apoptosis [19,20,21]. The majority of mitochondrial proteomic studies identifies or focuses on soluble proteins from the matrix of the mitochondria and only a few studies detail integral membrane proteins of the inner membrane. To our knowledge, there are no comprehensive studies detailing the proteins of mitochondrial outer membrane. Many of these integral membrane proteins are involved in the transport of metabolites across these membranes. Among these polytopic membrane proteins, long chain acyl-CoA synthetase (ACS), voltage dependent anion channel (VDAC), and carnitine palmitoyltransferase-I (CPT-I) localized in the mitochondrial outer membrane are of special interest as they play an obligatory role in the mitochondrial uptake of long-chain fatty acids, the major substrate for energy production in many tissues [22]. In addition to the role of these enzymes in fatty acid oxidation, they are involved in energy exchange and metabolite trafficking (VDAC) [23], apoptosis (VDAC, CPT-I) [24,25,26], and serve as a docking site for cytosolic proteins (VDAC) [27,28].

The goal of the present work was to determine whether the CPT-I, VDAC, and ACS proteins are modified and use this information in subsequent functional studies. Recently, we described a protocol that allowed high sequence coverage (82–99%) of these three polytopic rat liver mitochondrial outer membrane proteins [29]. Using this method combined with bioinformatics, we document the co-translational (N-terminal acetylation) and post-translational (phosphorylation, nitration, acetylation) modifications on all three integral membrane proteins.

## Experimental Procedure

Experimental procedures are described in detail previously [29] and are therefore discussed here briefly.

**Animals—**Male Sprague-Dawley rats (200–400g) were obtained from Charles River Laboratories (Wilmington, MA) and had free access to food and water. All procedures were approved by the VA Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for care and use of animals in research.

**Chemicals—**The matrix, α-cyano-4-hydroxycinnamic acid and calibration standards were purchased from Sigma (St. Louis, MO). Dithiothreitol and iodoacetamide was purchased from Bio-Rad Laboratories (Hercules, CA). Chemically modified porcine trypsin was purchased from Promega (Madison, WI). Proteinase K was purchased from Roche (Indianapolis, IN). All other chemicals were obtained in the commercially available purest form.

**Isolation of rat liver mitochondria and rat liver mitochondrial outer membranes—**Percoll-purified rat liver mitochondria served as the starting material for isolation of the outer membranes. Mitochondria were isolated in 220 mM mannitol, 70 mM sucrose, 2 mM EDTA, and 5 mM MOPS at a pH 7.4 as referenced [30]. This buffer was supplemented with protein phosphatase inhibitor cocktail that included 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 4 mM β-glycerophosphate. High purity rat liver mitochondrial outer membranes and contact sites were isolated by the swell/shrink technique and discontinuous sucrose gradient centrifugation as detailed previously [31] and resuspended in 20 mM MOPS at pH 7.4 supplemented with the protein phosphatase inhibitor cocktail described above.

**SDS-PAGE, Isolation of Proteins, and Protease Digestions—**Proteins were isolated from Percoll-purified rat liver mitochondrial outer membranes by semi-preparative SDS-PAGE as described earlier [32,33,29]. Briefly, 1.0 mg of outer membranes was subjected to semi-preparative SDS-PAGE (100mm × 72mm × 1.5mm, 10% separating, 4% concentrating gel) and the separated proteins visualized by a brief staining with Coomassie Brilliant Blue R250. The bands around 32, 78, and 88 kDa were excised from the gel. The proteins in the excised gel piece were then electroeluted for further analysis. All trypsin and proteinase K digestions were carried out as previously described [29].

**Mass Spectrometry—**A saturated matrix solution was made by dissolving α-cyano-4-hydroxycinnamic acid in a 1:1 solution of acetonitrile and water. Samples were prepared by mixing 1μL of analyte with 1μL of matrix on the stainless steel sample plate. Reflectron MALDI mass spectra were recorded on a Bruker (Billerica, MA) BiFlex III MALDI-TOF instrument equipped with nitrogen laser (337 nm, 3 ns pulse). All spectra were obtained in positive ionization mode using an accelerating voltage of 20 kV. Angiotensin II, ACTH clip (amino acids 18–39), and insulin were used as calibrants for these experiments.

Electrospray spectra were acquired on a ThermoElectron (San Jose, CA) LTQ linear quadrupole ion trap mass spectrometer. The instrument used either an Upchurch (Oak Harbor, WA) micro injector valve sample delivery system for sample introduction or the ThermoElectron Surveyor Autosampler. Experimental results for the LTQ data were acquired using a PepMap C18 column from LC Packings (Sunnyvale, CA). The flow rate was approximately 200 nL/min. The samples were diluted to a concentration of approximately 100 fmolμL and 2μL of the sample were injected. The mobile phases used were 0.1% formic acid in HPLC grade water and 0.1% formic acid in HPLC grade acetonitrile. Sample analyses were performed using data-dependent/dynamic exclusion with a repeat count of one in order to maximize coverage. The LTQ method employed 8 full MS/MS scans following one full scan mass spectrum (400–2000 Da). All data files were searched against the NR database using Bioworks 3.1 (ThermoElectron, San Jose CA) as described previously [29]. Phosphorylation (serine, threonine, and tyrosine), nitration (tyrosine), oxidation (methionine), and acetylation (N-terminus of protein, lysine) were selected as possible differential modifications.

## RESULTS AND DISCUSSION

Recently, we developed a targeted proteomic approach for characterization of integral membrane proteins which provides high sequence coverage [29]. Using this mass

spectrometric approach, ACS, VDAC, and CPT-I isolated from liver mitochondrial outer membranes of fed rats were structurally characterized. The results of this study are summarized in Table 1.

## Acetylation

**N-terminal Acetylation—**The open-reading frame of rat liver mitochondrial CPT-I predicts a 773 amino acid residue polypeptide [34]. Although it has been demonstrated that the amino acid residues 4–238 from the cDNA deduced sequence are present in the mature liver isoform [35] the exact N-terminal sequence is not known. Using ESI-MS/MS, we have identified a peptide from the N-terminus, Figure 1. The sequence is written above the spectrum and indicates the fragmentation of the peptide throughout the spectrum by demonstrating the spacing of the b ions. In the spectrum, peaks are labeled according to Biemann nomenclature and the m/z values of select peaks are indicated in the table within the figure. The peptide sequence in Figure 1 represents amino acids 2–22 with the cDNA deduced alanine at the 2 position being acetylated. All of the mass spectral peaks identified in the spectrum exhibit the shift of 42 mass units expected with N-terminal acetylation. This proves the methionine predicted by the cDNA is absent in the mature protein and the N-terminal alanine is acetylated. Our data explain earlier experiments that failed to identify the N-terminal amino acid of the rat liver CPT-I isoform by Edman degradation [36 and unpublished observations from this laboratory].

In addition to CPT-I, the ACS-1 protein was found to be N-terminally acetylated. However, the cDNA deduced N-terminal methionine was retained and this methionine was acetylated. The open-reading frame of rat liver mitochondrial ACS-1 predicts a 699 amino acid residue polypeptide [37]. In agreement with data published by Arnold et al. [38], we have identified the N-terminal tryptic peptide of ACS-1, $^{1}M_{ac}EVHELFR^{8}$ (Table 1 and Figure 1 in Supplement).

Included in our extensive coverage of the VDAC proteins, we have identified the N-terminal portions of the three VDAC isoforms. For VDAC-1, the cDNA encodes for a 283 amino acid protein with methionine as the N-terminus. We have determined the N-terminal amino acid residue is alanine and this alanine is acetylated, $^{2}A_{ac}VPPTYADLGK^{12}$ (Table 1 and Figure 2 in Supplement). For VDAC-2, the cDNA encodes for a polypeptide with a deduced size is 295 amino acids [39] with a putative signal peptide cleavage site present at position 25 [40]. We have verified that the purported signal peptide is present in the mature protein and the N-terminal methionine predicted by the cDNA is present in the mature protein. For the VDAC 3 isoform, the cDNA deduced protein consists of 283 amino acid residues with the predicted methionine present in the mature protein.

The biological significance of the N-terminal acetylation is unknown. Unlike ε-lysine modification, N-terminal acetylation, catalyzed by N-terminal acetyltransferases is irreversible and occurs co-translationally [for reviews see 41 and 42]. It has been suggested that co-translational N-acetylation modifies protein-protein interaction [43], affects accumulation of the mature protein(s) in target organelles [44], and confers metabolic stability on the protein by providing general protection from peptidases and the ubiquitin-mediated pathway of protein degradation [45]. It is also of interest to note that the C-terminal portions of CPT-I, ACS-1, and all three VDAC isoforms were characterized and no modifications of the C-terminal end were identified [29]. As expected from the lack of mRNA expression of the VDAC3 splice variant in the liver, this VDAC3 isoform was not detected in our rat liver mitochondrial outer membrane preparation [46].

**Lysine acetylation—**Acetylation of specific lysine residues is a reversible and highly regulated posttranslational protein modification. Although the importance of reversible lysine

acetylation of histone proteins is well established, the importance of protein modification by reversible acetylation in intermediary metabolism is just emerging. It has been shown recently, that both isoforms of acetyl-CoA synthetase (cytosolic - AceCS1, mitochondrial AceCS2) are reversible acetylated resulting in loss-of-function (acetylation) or gain-in-function (deacetylation) [47,48,49]. It has been proposed that acetylation modulates the activity of all AMP-forming family of enzymes, including long-chain acyl-CoA synthetases [47]. We have identified the peptide $^{628}$NKDINK$_{ac}$AILEDMVK$^{641}$ from ACS-1, Figure 2. In the spectrum, peaks are labeled according to Biemann nomenclature and the m/z values of select peaks are indicated in the table within the figure. The non-acetylated version of the peptide was also detected in this experiment.

## Nitration

As shown in Figure 3 in addition to N-terminal acetylation, rat liver mitochondrial CPT-I also is nitrated. In a previous study using immunoblotting, tyrosine nitration was detected particularly under stress by Fukumoto *et al.* [50]. Using the described approach, we have identified two sites of nitration on the CPT-I protein. First, we identified the unmodified peptide, $^{585}$FCLTYEASMTR$^{595}$, and the modified peptide, $^{585}$FCLTY$_{nit}$EASM$_{ox}$TR$^{595}$, Figure 3. In Figure 3A, the MS/MS spectrum of the ions at 720 Da representing the modified peptide is shown and in Figure 3B the MS/MS spectrum of the ions at 690 Da representing the unmodified peptide is shown. Again, the sequence of each peptide is written above the spectrum. In this figure, the sequencing of the peptide throughout the spectrum is demonstrated using the spacing of the b ions. Masses for select y ions for each spectrum are shown in the table within the spectrum. The mass shifts due to the nitration of tyrosine and oxidation of methionine are clearly visible in the y7-y9 ions. A mass difference of m/z 208 between the b4 and b5 and y6 and y7 ions is representative of a nitrated tyrosine. The methionine is found to be oxidized in Figure 3A. This oxidation is most likely an artifact from the treatment of the sample during electrophoresis and does not have any functional implications since methionine oxidation was a commonly identified modification. The detection of these two peptides documents the nitration of CPT-I liver isoform at tyrosine 589. Additionally, a second peptide $^{272}$AGNTIHAILLY$_{nit}$R$^{283}$ nitrated on tyrosine 282 was identified from the MS/MS spectrum of the ions at 931 Da, Table 1 and Figure 3 in the Supplement. The sequence of the peptide in the spectrum indicates the fragmentation pattern of the peptide demonstrated with the b ion series. The y2 ion is detected with a m/z value indicative of an arginine and a nitrated tyrosine. Also, the mass shift between the b10 and b11 ions is m/z 208, the mass of a nitrated tyrosine. This data shows, unequivocally, the nitration of mature CPT-I *in vivo*.

Additionally, we found ACS1 to be nitrated on peptide $^{9}$Y$_{nit}$FRMPELIDIR$^{19}$, Table 1 and Figure 4 in Supplement. We have also identified the peptide $^{82}$LLLY$_{phos}$Y$_{nit}$YDDVR$^{91}$, Table 1 and Figure 5 in Supplement. ACS nitration was reported by Elfering et al. by immunoblotting [51]. However, the isoform was not determined. Although the biological significance of protein tyrosine nitration is not known, it occurs in cells and tissues during oxidative stress and inflammation, probably through generation of peroxynitrite. Protein tyrosine nitration has been reported in mitochondria from diabetic mice [52], in liver of endotoxemic rats [53,50], and brain of Alzheimer's patients [54]. While protein tyrosine nitration has been considered as the consequence of a pathological disease process and oxidative stress, evidence that nitration meets criteria for physiologically relevant signal are accumulating [for reviews see 55 and 51]. In the present study we documented nitration of tyrosines 282 and 589 of CPT-I and 9 and 86 in ACS1. The enzymes were isolated from rat liver mitochondrial outer membranes of non-stressed animals show that tyrosine nitration occurs under conditions with no apparent stress or illness.

## Phosphorylation

Previously we have shown phosphorylation of CPT-I by protein kinase CKII *in vitro* and identified the sites of phosphorylation as serine 741 and serine 747 using peptide mass fingerprinting and ESI-MS/MS [33]. These two sites of phosphorylation were identified on CPT-I isolated from rat liver mitochondrial outer membranes treated with casein kinase II.

Here we show that rat liver mitochondrial CPT-I is phosphorylated *in vivo*, Table 1. When CPT-I isolated from outer membranes prepared in the presence of protein phosphatase inhibitors was subjected to digestion with trypsin followed by proteinase K, a peptide phosphorylated on threonine 588 was identified, Figure 4, as well as its non-phosphorylated form was identified (shown previously in Figure 3). As shown in Figure 4, the phosphorylated peptide ($^{585}$FCLT$_{phos}$Y$_{nit}$EASM$_{ox}$TR$^{595}$) was nitrated on tyrosine 589 and methionine 593 was oxidized. Again, this methionine oxidation is likely an artifact from sample treatment. The spacing of the b ions is used to indicate the sequence of the peptide in the spectrum and the m/z values of select peaks are shown in the table within the spectrum. The m/z difference in the b3 and b4 ions is indicative of a phosphorylated threonine. There are several peaks in the spectrum resulting from the neutral loss of water or $H_3PO_4$. These neutral losses suggest the presence of a phosphate group on the peptide. Threonine 588 corresponds to glycogen synthetase kinase 3 (GSK3) consensus sequence. Phosphorylation of threonine 588 by GSK3 would require serine 592 four residues downstream to be phosphorylated. Thus, phosphorylation of threonine 588 either is catalyzed by a different protein kinase, or tyrosine 589 nitration allows phosphorylation at this site by GSK3.

In addition to phosphorylation of CPT-I isolated from outer membranes, we have identified that CPT-I isolated from rat liver mitochondrial contact sites is phosphorylated at a different site. The CPT-I was isolated from contact sites and digested like the outer membrane CPT-I. When analyzing CPT-I from contact sites, we identified the peptide $^{599}$EGRTET$_{phos}$VR$^{606}$ from the MS/MS spectrum of the ions at 504 Da, Table 1 and Figure 6 in Supplement. The most abundant ions are formed by the loss of a water molecule from the precursor ion indicating the presence of a phosphate group on the peptide. The sequence of the phosphopeptide is shown in the spectrum with spacing indicative of the b ion spacing. The mass shift seen between the b5 and b6 peaks is m/z 181, the mass of a phosphothreonine. There also is a neutral loss of a phosphate seen from the b6 ion. This phosphopeptide is exclusively detected from CPT-I isolated from contact sites. When analyzing CPT-I isolated from outer membranes, only the non-phosphorylated peptide was detected. Threonine at residue 604 represents a high probability phosphorylation site corresponding to a protein kinase A/protein kinase C/$Ca^{2+}$/calmodulin-dependent protein kinase (PKA/PKC/CaMII) consensus site. Differences in *in vivo* phosphorylation of CPT-I observed in different membrane preparations could arise from differences in the structure of the C-terminal segment when the enzyme is present in the outer membrane versus contact sites. On the other hand, the difference in phosphorylated residues between *in vivo* and published *in vitro* experiment reflect the addition of exogenous protein kinase CKII *in vitro*. Based on the predicted membrane topology of rat liver mitochondrial CPT-I [56], all identified phosphorylation sites are localized in the C-terminal segment and thus *in vivo* should be readily accessible to protein kinases present in the cytosol and *in vitro* to added protein kinases.

Phosphorylation was identified on the ACS-1 protein, Table 1 and Figure 5 in Supplement. ACS-1 was phosphorylated on Tyr-85, an insulin receptor (INSR) consensus site. There are no reports of the phosphorylation of ACS-1 in the literature in either *in vivo* or *in vitro* studies. ACS isoforms are distinctly regulated across tissues and within a given tissue suggesting post-translational modification(s) [57].

When the proteins electroeluted from the 30 kDa band were digested and analyzed using ESI-MS/MS, several phosphorylated proteins were identified, Table 1. All three VDAC isoforms were identified as discussed previously [29] and phosphorylation was detected on all three proteins. In VDAC-1, two serine residues were identified as phosphorylated, Ser-136 and Ser-12 (Table 1 and Figure 7 and 8 in Supplement) to a PKC and CaMII/GSK3 consensus site. In VDAC-2, Tyr-237 (INSR site) was identified as phosphorylated (Figure 9 in Supplement). In VDAC-3, Ser-241 (CKI, PKA site, Figure 10 in Supplement) and Thr-33 (PKC site, Figure 11 in Supplement) were identified as phosphorylated, Table 1. Phosphorylation of VDAC has been suggested earlier, but the localization of the site has not been identified in any study. Using 2D electrophoresis and MALDI-MS Yoo et al. [58] reported the presence of three VDAC1 forms in post-mortem brain of Alzheimer's and Down syndrome patients which differed in their isoelectric points. Changes in the isoelectric point of a protein are often due to post-translational modifications including, but not limited to, phosphorylation. Using a functional proteomics approach involving MALDI-TOF mass spectrometry, Yan *et al.* identified VDAC as phosphorylated, but neither the isoform or the site of phosphorylation was specified [59]. VDAC-1 and -2 were found to contain phosphorylated tyrosines by immunoblotting in guinea pig brain synaptosomes under hypoxic conditions [60]. The location of phosphotyrosine was not identified. Additionally, phosphorylation of VDAC has been reported *in vitro*. Using the catalytic subunit of cAMP-dependent protein kinase and VDAC purified from rat liver mitochondria Bera et al. [61] demonstrated that VDAC can be phosphorylated by PKA. In cell culture, Pastorin et al demonstrated the phosphorylation of VDAC-1 by glycogen synthase kinase 3β (GSK3) by immunoblotting [62]. VDAC1 also can be phosphorylated as shown by incubating GST-VDAC1 with recombinant PKCε *in vitro* [63].

In contrast to the above referenced studies on VDAC phosphorylation where the phosphorylated residue or residues have not been identified, in the present study we have identified the site and type of phosphorylated amino acid. Based on the proposed membrane topology models of bovine and human VDAC1 [64,65] and mouse VDAC2 [66] and the three dimensional model of all three VDAC proteins [67], we have addressed the membrane orientation of the phosphorylated amino acid residues in all three VDAC isoforms. In VDAC1 the two phosphorylated serine residues reside on the opposite side of the outer membrane. Ser-12 is localized in the α-helix on the the cytosolic side and Ser-136 in the third loop facing the intermembrane space. Tyr-237 in VDAC2 is localized in the large (third) cytosolic loop and Ser-241 and Thr-33 in VDAC3 are localized in the sixth loop on the intermembrane side and first beta strand close to the cytosol, respectively. Thus, in all three VDAC isoforms the phosphorylated amino acid residues are accessible to modifying protein kinases and phosphatases localized in the cytosol and intermembrane space and could explain the different roles VDAC isoforms play in metabolism [22–28].

Posttranslational modification by reversible phosphorylation is the most frequent protein modification with estimates of more than one third of all proteins being phosphorylated [1,2]. Approximately 90% of all phosphorylated proteins are believed to be phosphorylated on multiple sites. To our knowledge this is the first study documenting the *in vivo* phosphorylation of the liver isoform of CPT-I, ACS1, and all three VDAC isoforms by ESI-MS/MS. The finding that CPT-I is phosphorylated in liver of fed, unstressed animals suggests that under physiological conditions the enzyme is regulated by phosphorylation. Furthermore, the differences in phosphorylation sites between CPT-I isolated from outer membranes (Thr-588) and contact sites (Thr-604) suggest regulation is dependent on membrane localization. These differences in phosphorylation sites observed on CPT-I in outer membranes and contact sites on the one hand and between *in vivo* and *in vitro* phosphorylation on the other could arise from differences in the structure of the C-terminal segment when present in the outer membrane versus contact sites and from the absence of protein kinase CKII *in vivo*. Based on the predicted

membrane topology of rat liver mitochondrial CPT-I [68], all identified phosphorylation sites are localized in the C-terminal segment and thus *in vivo* should be readily accessible to protein kinases present in the cytosol and *in vitro* to added protein kinases. Previously we reported that *in vitro* phosphorylation of CPT-I alters both activity and regulation by malonyl-CoA [33]. Addressing the phosphorylation based regulation of CPT-I will require the quantitation of phosphorylation under different physiological/pathophysiological conditions.

The differences in phosphorylation consensus sites between the three VDAC isoforms and the presence of multiple phosphorylation sites may reflect the functional differences the isoforms fulfill *in vivo*. Phosphorylation of VDAC (isoform not specified) by GSK3 *in vitro* promotes apoptosis by preventing hexokinase II binding to mitochondria [62]. In a recent study it was shown that alterations in the pore size of VDAC in rat brain mitochondria after interaction with Bax and tBid are controlled via phosphorylation of VDAC. This increase in pore size allows the release of cytochrome c and other apoptogenic molecules into the cytosol leading to cell death [69]. It has been shown that *in vitro* phosphorylation of VDAC by protein kinase A reduces the single-channel current and opening probability at negative clamping potentials [70] and that protein kinase Cε inhibits the permeability transition pore in cardiac mitochondria probably via phosphorylation of VDAC [63].

### Conclusion

In summary, we document several post-translational modifications of three mitochondrial integral outer membrane proteins. We have shown for CPT-I: a) the mature rat liver mitochondrial CPT-I is composed of 772 amino acids with the N-terminal alanine being acetylated; b) *in vivo* the enzyme is phosphorylated on threonines 588 and 604 with the phosphorylation at position 604 detected only in CPT-I isolated from contact sites and c) under basal conditions the enzyme is nitrated on tyrosines 282 and 589. We have shown for ACS-1: a) the mature protein is composed of 699 amino acids with the N-terminal methionine being acetylated, b) lysine 633 is acetylated, c) the protein is phosphorylated at tyrosine 85, and d) tyrosines 7 and 86 are nitrated. For VDAC-1, the protein exhibits phosphorylation at serine 12 and 136 and N-terminal acetylation of the alanine residue. For the VDAC-2, a phosphorylated tyrosine was identified at position 237. For VDAC-3, there are two sites of phosphorylation at Ser-241 and Thr-33. These novel findings raise questions about regulatory aspects of CPT-I, ACS, and VDAC and further studies should advance our understanding about regulation of mitochondrial fatty acid oxidation in general and these three proteins in specific.

Identification of novel sites of post-translational modification raises questions about regulatory aspects of these three mitochondrial outer membrane proteins. Further studies should advance our understanding about regulation of mitochondrial fatty acid oxidation.

## Supplementary Figures

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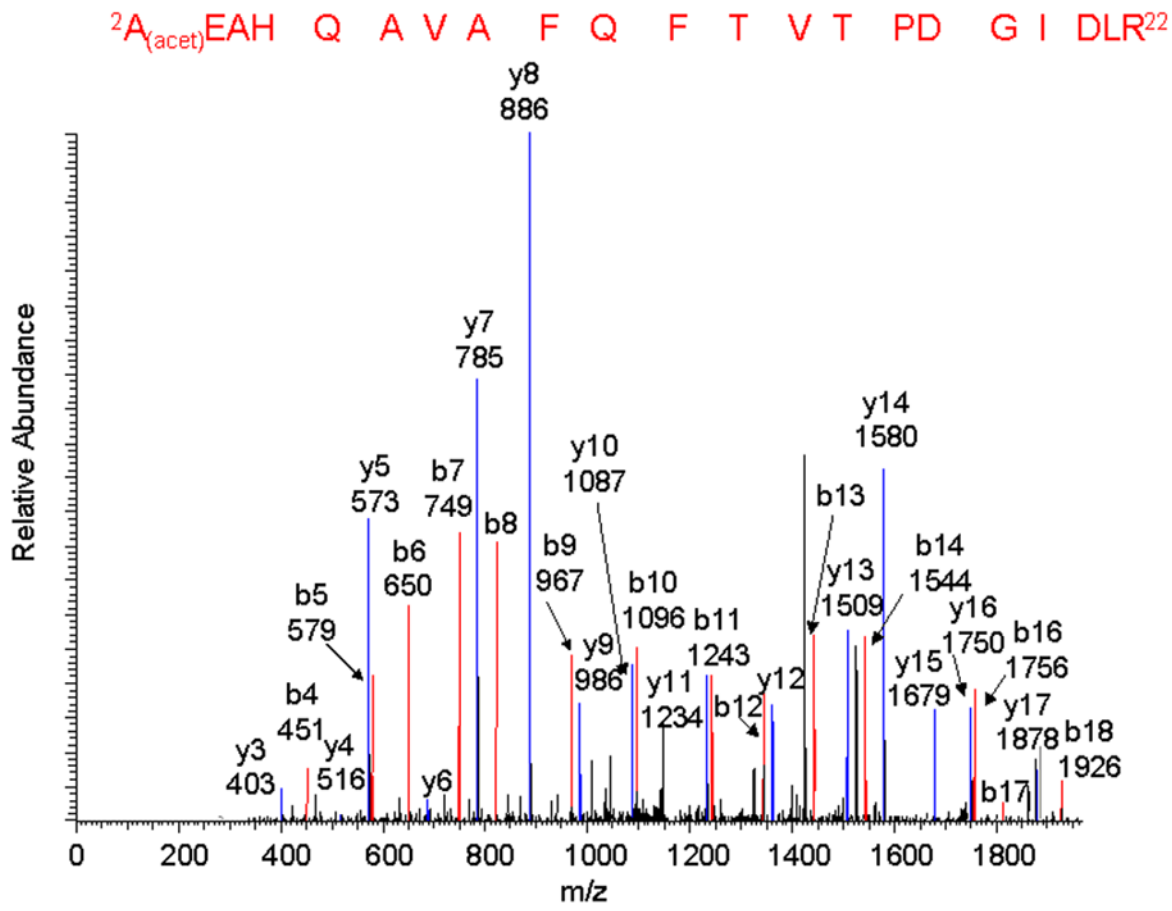


**Figure 1.**

N-terminal acetylation of the CPT-I protein: MS/MS spectrum of the ions at 1165 Da. The sequence of the peptide is shown in the spectrum and the spacing of the sequence indicates b ion fragmentation pattern. B ions (in red) and y ions (in blue) are labeled according to Biemann nomenclature.

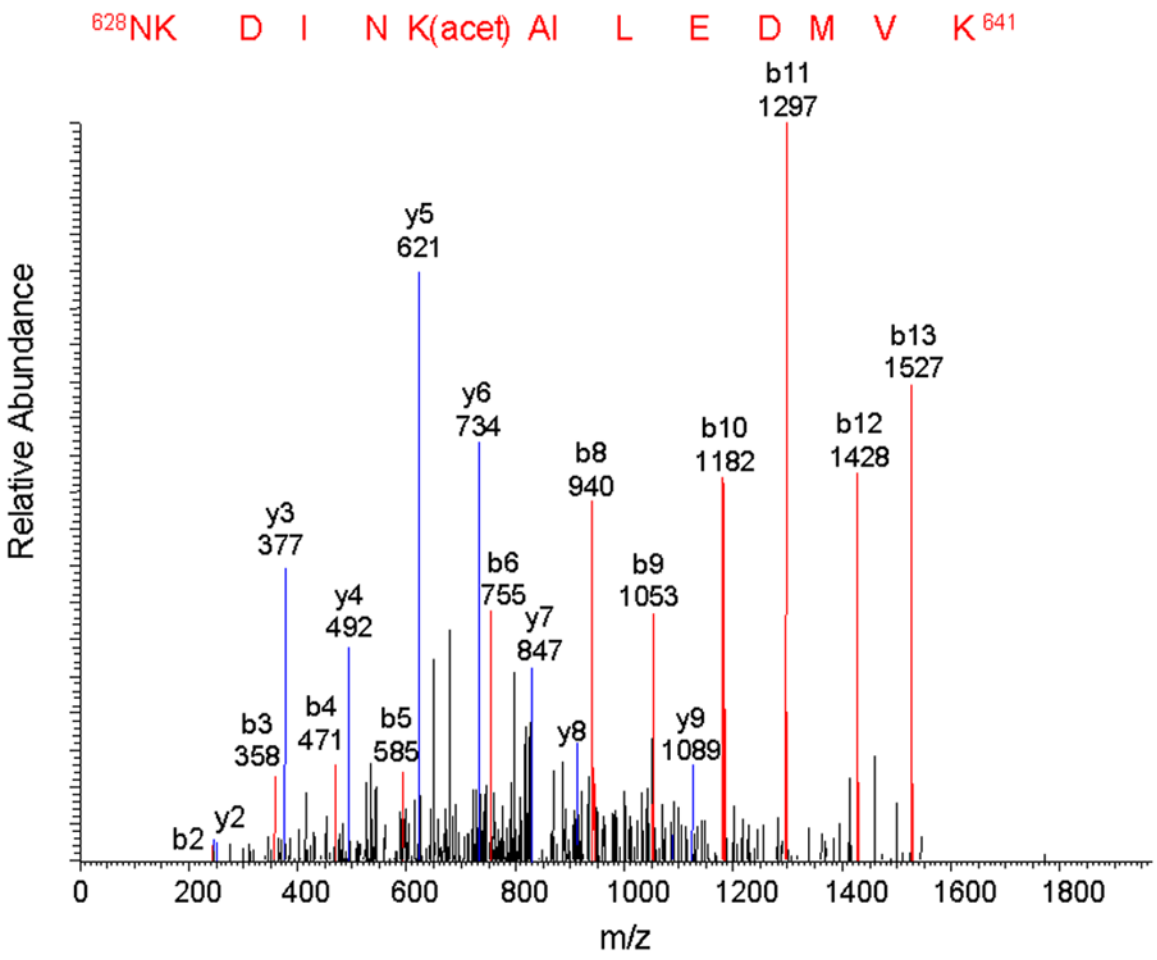


**Figure 2.**

Lysine acetylation of the LCAS 1 protein: MS/MS spectrum of the ions at 837 Da. The sequence of the peptide is shown in the spectrum and indicates fragmentation pattern of the b ions. B ions (in red) and y ions (in blue) are labeled according to Biemann nomenclature.

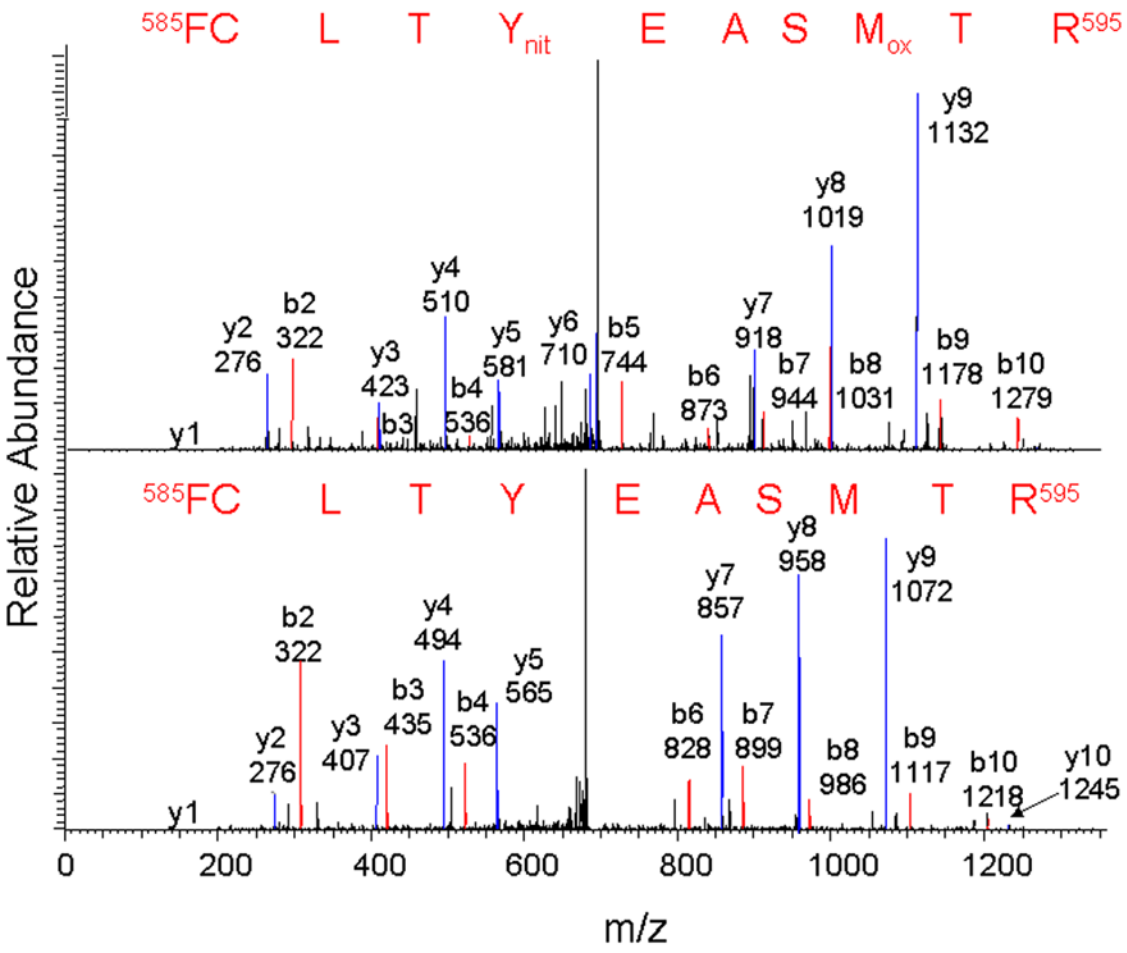


**Figure 3.**

Tyrosine nitration of the CPT-I protein: MS/MS spectra of the ions at 720 Da (top) and 690 Da (bottom). The sequences of these peptides are shown in the spectra with two post-translational modifications (top) and no modifications (bottom). The spacing of the sequences indicates the b ion fragmentation pattern. B ions (in red) and y ions (in blue) are labeled according to Biemann nomenclature.

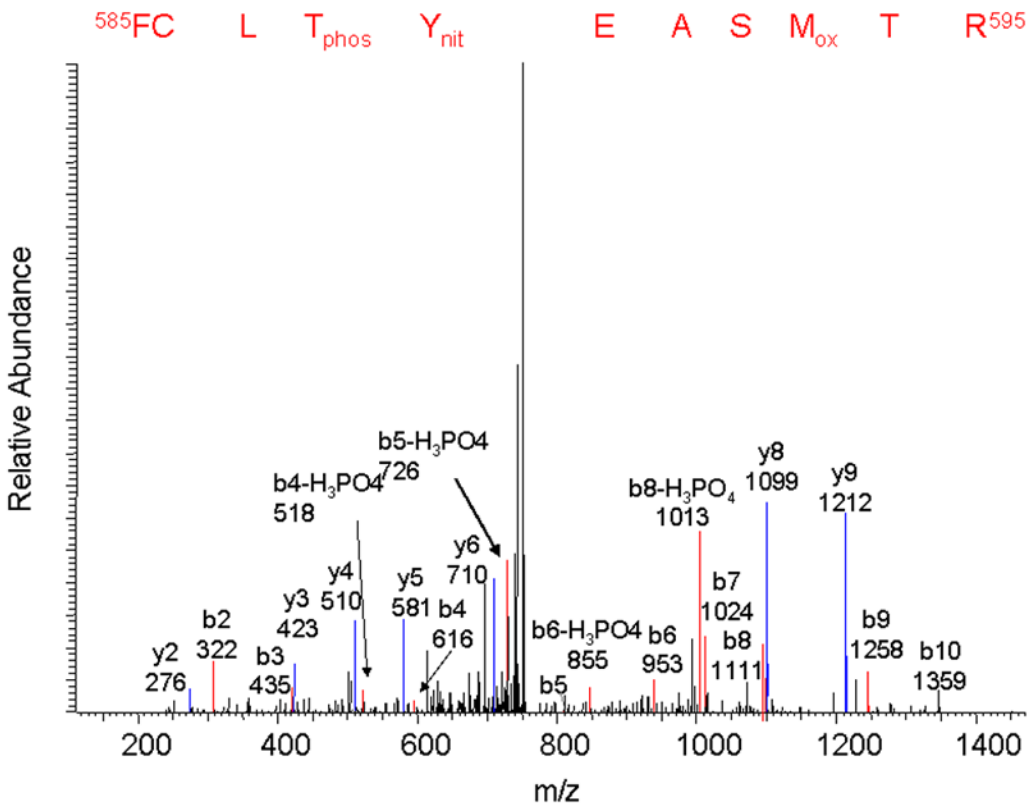


**Figure 4.**

Phosphorylation of the CPT-I protein: MS/MS spectrum of the ions at 760 Da. This peptide is the phosphorylated peptide shown in Figure 3. The sequence of the peptide is shown in the figure with the spacing of the sequence indicating fragmentation of the b ions. B ions (in red) and y ions (in blue) are labeled according to Biemann nomenclature.

**Table 1**

Post-translational Modification of CPT-I, ACS-1, and VDAC

| Protein | Acetylation | Phosphorylation | Nitration |
|---|---|---|---|
| CPT-I | Ala-2[a]<br>(Met-1 not present) | Thr-588 (PKC)<br>Thr-604 (PKC)[b]<br>Ser-741[c](CKII)<br>Ser-749[c](CaM-II, cdc2, GSK3) | Tyr-282[b]<br>Tyr-589 |
| ACS-1 | Met-1[a,b]<br>Lys-633 | Tyr-85 (INSR)[b] | Tyr-9[b]<br>Tyr-86[b] |
| VDAC-1 | Ala-2[a,b]<br>(Met-1 not present) | Ser-12 (CaM-II, GSK3)[b]<br>Ser-136 (PKC)[b] | |
| VDAC-2 | | Tyr-237 (INSR)[b] | |
| VDAC-3 | | Ser-241 (CKI, PKA)[b]<br>Thr-33 (PKC)[b] | |

Kinase consensus sites were predicted using the NetPhosK 1.0 Server. Kinases are shown in parentheses after the phosphorylated amino acid.

[a] N-terminal acetylation

[b] Data shown in Supplement

[c] Identified previously from *in vitro* experiments using CKII and ATP (detailed in ref. 33).