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Post-translational modifications of rat liver mitochondrial outer membrane proteins identified by mass spectrometry

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Abstract

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*, that 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 that 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

1. 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 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 [3–5]. 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

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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 [9–11], endoplasmic reticulum [12], peroxisomes [13], and mitochondria [14–18]. Mitochondria are of particular interest due to their well-known importance in energy production and their proposed role in apoptosis [19–21]. 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) [24–26], 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.

2. Experimental procedures

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

2.1. Animals

Male Sprague–Dawley rats (200–400 g) 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.

2.2. 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.

2.3. 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.

2.4. 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 (100 mm × 72 mm × 1.5 mm, 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].

2.5. 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 1 μL of analyte with 1 μL 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 fmol/μL and