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PPAR α Activation Induces N^ε-Lys-Acetylation of Rat Liver Peroxisomal Multifunctional Enzyme Type 1

Miguel A. Contreras,

Department of Pediatrics, The Darby Children's Research Institute, Medical University of South Carolina, Charleston, SC 29425, USA

Oscar Alzate,

Systems Proteomics Core Laboratory, Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC 27599, USA

Avtar K. Singh, and

Department of Pediatrics, The Darby Children's Research Institute, Medical University of South Carolina, Charleston, SC 29425, USA

Department of Pathology, Ralph H. Johnson VA Medical Center, Charleston, SC 29401, USA

Inderjit Singh

Department of Pediatrics, The Darby Children's Research Institute, Medical University of South Carolina, Charleston, SC 29425, USA

Miguel A. Contreras: miguel.contreras@nih.gov; Oscar Alzate: oscar.alzate@ttuhsc.edu; Inderjit Singh: singhi@musc.edu

Abstract

Peroxisomes are ubiquitous subcellular organelles that participate in metabolic and disease processes, with few of its proteins undergoing posttranslational modifications. As the role of lysine-acetylation has expanded into the cellular intermediary metabolism, we used a combination of differential centrifugation, organelle isolation by linear density gradient centrifugation, western blot analysis, and peptide fingerprinting and amino acid sequencing by mass spectrometry to investigate protein acetylation in control and ciprofibrate-treated rat liver peroxisomes. Organelle protein samples isolated by density gradient centrifugation from PPAR α -agonist treated rat liver screened with an anti-N^ε-acetyl lysine antibody revealed a single protein band of 75 kDa. Immunoprecipitation with this antibody resulted in the precipitation of a protein from the protein pool of ciprofibrate-induced peroxisomes, but not from the protein pool of non-induced peroxisomes. Peptide mass fingerprinting analysis identified the protein as the peroxisomal multifunctional enzyme type 1. In addition, mass spectrometry-based amino acid sequencing resulted in the identification of unique peptides containing 4 acetylated-Lys residues (K¹⁵⁵, K¹⁷³, K¹⁹⁰, and K⁵⁸³). This is the first report that demonstrates posttranslational acetylation of a peroxisomal enzyme in PPAR α -dependent proliferation of peroxisomes in rat liver.

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Correspondence to: Inderjit Singh, singhi@musc.edu.

Present Address: M. A. Contreras, Office of Research Infrastructure Programs, Division of Comparative Medicine, National Institutes of Health, 6701 Democracy Boulevard, Room 945, Bethesda, MD 20892, USA

Present Address: O. Alzate, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, 5001 El Paso Dr., El Paso, TX 79905, USA

Electronic supplementary material

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Conflict of interest

The authors declare that they do not have conflicts of interest.

Keywords

Peroxisome proliferator-activated receptor alpha (PPAR α); Lysine-acetylation; Acetylation; Peroxisomes; Rat peroxisomal multifunctional enzyme type 1 (rpMFE-1); Peroxisome proliferation

Introduction

Peroxisomes are ubiquitous subcellular organelles which play a role in many important cellular metabolic pathways, related genetic peroxisomal disorders and other pathologic processes. One of the most studied functions of peroxisomes is the β -oxidation of very long chain fatty acids (VLCFA; more than C₂₂ in length) and several aliphatic substrates [1]. In addition, other functions include detoxification of hydrogen peroxide [2] and participation in the biosynthesis of ether phospholipids (plasmalogens) [3], bile acids [4] and n-3 polyunsaturated fatty acids [5].

The relevance of this organelle to human health is evident from the several known inherited diseases due to defects in peroxisomal biogenesis factors, as is the case in Zellweger syndrome [6], or in single protein malfunction as in X-linked adrenoleukodystrophy [7], and for its involvement in the nervous system development/homeostasis [8–11]; its susceptibility to inflammatory processes in neural and peripheral tissues and organs [12, 13], and its receptor-mediated proliferation in rodent livers leading to tumor formation [14, 15].

In rodents, many structurally diverse chemicals designated as peroxisome proliferators are able to induce their proliferation [15, 16] by activating the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR α) [17]. Further, PPAR α binds to peroxisome proliferator response elements present in the promoter regions of genes encoding inducible enzymes/proteins involved specially in the β -oxidation of fatty acids in peroxisomes [18]. This β -oxidation system consists of four steps: (1) oxidation catalyzed by acyl-Coenzyme A (CoA) oxidase (ACOX), (2) and (3) hydration and dehydrogenation of fatty acid catalyzed by a multifunctional enzyme (hydratase and dehydrogenase activities), and (4) thiolysis, catalyzed by 3-ketoacyl-CoA thiolase, which results in the release of a molecule of acetyl-CoA and a molecule of acyl-CoA which is two carbon atoms shorter than the precursor molecule [1, 19]. These four steps can be catalyzed by at least two sets of proteins. The most studied set, known as the inducible pathway, depends on PPAR α for its gene expression [19], and is composed of straight chain fatty acyl-CoA oxidase (ACOX-1), enoyl-CoA hydratase/(3S)-hydroxyacyl-CoA dehydrogenase bifunctional enzyme or multifunctional enzyme type 1 (MFE-1; also known as L-bifunctional protein or LBP) and 3-ketoacyl-CoA thiolase B [1, 19]. The other pathway, referred to as the non-inducible pathway, is composed of the respective isoenzymes [20–22].

Originally, peroxisomal proteins were reported to be synthesized on free polyribosomes, followed by their transport to preexisting peroxisomes, without undergoing posttranslational modifications (PTMs) [23]. However, recently this concept has changed, and the role played by the endoplasmic reticulum in the biogenesis of peroxisomes is now well established [24]. In addition, over the last 40 years, more than 70 proteins have been identified as peroxisomal proteins [1, 7]; of which only a few membrane-related proteins have been described to be susceptible to PTMs. Indeed, peroxins (proteins involved in peroxisome biogenesis) Pex19p, Pex5p and the ABC transporters D1 (ABCD1) and D3 (ABCD3) are posttranslationally modified by farnesylation [25], ubiquitination [26] and phosphorylation [27], respectively.

Proteomic analysis has demonstrated that protein *N*^ε-lysine acetylation is a broad cellular mechanism used to regulate the proteins function of the intermediary metabolism, in addition to histones and transcription factors [28–31].

Peroxisomes are dynamic organelles that play diverse metabolic roles in response to cellular demands under normal and pathological conditions; some of which can alter their size and number, and hence functionality [12]. To gain insight into whether peroxisomal proteins may be subjected to *N*^ε-lysine acetylation in response to cellular demands, we investigated this particular PTM in the proteins of the peroxisome present in PPARα agonist-treated rat liver. In this manuscript, using immuno-detection and proteomic analysis, we report for the first time that the rat peroxisomal multifunctional enzyme type 1 (rpMFE-1) undergoes PTM on several *N*^ε-Lys residues under PPARα-mediated peroxisome proliferation. These events may play a role in the proliferation of this organelle.

Materials and Methods

Reagents

All reagents were analytical grade of the highest purity commercially available. Accudenz A.G. was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY). Ciprofibrate (CIP) was a kind gift by Dr. A. Soria, Sterling-Winthrop Research Institute (Rensselaer, NY) [32]. Nitrocellulose membranes and non-fat milk powder were from BioRad Laboratories (Hercules, CA).

Animal Treatment

Animal work was performed under a protocol approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Six SD male rats (180–200 g) were purchased from Jackson Laboratories (Bar Harbor, ME) and kept at the University animal facility. Rodents were fed ad libitum either a standard diet (3 control animals) or a standard diet supplemented with 0.025 % (w/w) CIP (3 treated animals) for 2 weeks (Ciprofibrate is a PPARα agonist that induces the transcription of a number of genes that facilitate lipid metabolism, especially of those for peroxisomal proteins) [16, 32].

Isolation of Peroxisomes from Rat Liver

Animals were euthanized by an intraperitoneal injection (60 mg/kg body weight) of pentobarbital (Nembutal Sodium) after overnight fasting. The livers were harvested and a peroxisome-enriched fraction (λ fraction) was obtained by differential centrifugation. This fraction was used further for the isolation of peroxisomes by centrifugation in a linear density gradient of Accudenz (0–50 %) [12]. The gradient fractions were assayed for the subcellular organelle marker enzyme catalase as described elsewhere [12, 16].

Immunoprecipitation of Acetyl Lysine-Modified Proteins

Peroxisomal proteins (150 µg each from untreated or treated gradient fractions) were incubated at 4°C with 5 µg of anti-*N*^ε-acetyl-lysine antibodies (ImmuneChem Pharmaceuticals, Burnaby, British Columbia, Canada [28]) in a total volume of 250 µL for 24 h. Following addition of protein A/G-agarose (50 µL; Santa Cruz Biotechnology, Santa Cruz, CA) the solution was incubated for 2 h at 4 °C, centrifuged and the agarose complexes extensively washed with Tris-buffered saline containing 0.05 % (v/v) Tween-20. Pellets were stored at –20°C until further analysis by SDS-polyacrylamide (PAA) gel electrophoresis (SDS-PAGE). Control samples without peroxisomes or antibodies were run in parallel. Precision Plus protein standards (prestained all blue, BioRad) were loaded in parallel to samples.

Western Blot Analysis

Equal amounts of peroxisomal proteins (50 µg, precipitated with 10 % acetone (v/v), from untreated and treated gradient fractions) and integral membrane proteins obtained from purified peroxisomes [12] were resolved by SDS-PAGE (pre-cast 4–20 % PAA gradient gels, BioRad), transferred to nitrocellulose membranes, and analyzed by western blot as described previously [13]. Antibodies used were: anti-*N*^ε-acetyl-lysine (ImmuneChem Pharmaceuticals [28]); anti-peroxisomal 3-ketoacyl-CoA thiolase (custom made against residues 388–402 of the rat protein sequence); anti-PMP-70 (custom made against C-terminal residues 644–659 of the human peroxisomal membrane protein (PMP or ABCD3) sequence); anti-very long chain acyl-CoA synthetase (custom made against C-terminal residues 576–585 of the mouse protein sequence); anticytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA); anti-rpMFE-1 (a generous gift by Professor Takashi Hashimoto, Shinshu University, Japan [12]). The blots were developed by treatment with Lumi-Phos WB reagent (Pierce Biotechnology, Rockford, IL) and exposure to CL-Xposure films (Pierce). SDS-gels were stained with a solution of Coomassie blue 0.025 % in methanol:acetic acid:water (40:10:50) by volume and destained with methanol:acetic acid:water in the proportion indicated above. Prestained protein standards (Precision Plus, Bio-Rad) were resolved in parallel to samples as indicators of proteins transference to the nitrocellulose membranes. Images were obtained using an Alpha Innotech FluorChem 8000 Imager (San Leandro, CA).

Gel Plug Processing and Trypsin Digest

A gel piece containing the protein of interest was excised from the SDS-gel and submitted for analysis at the Systems Proteomics Core Laboratory, University of North Carolina, Chapel-Hill, NC. The gel piece was washed with 50 mM ammonium bicarbonate for 10 min. Next, it was de-stained using 50 mM ammonium bicarbonate in 50 % acetonitrile (ACN) for 10 min, repeated until the gel was clear. The gel was dehydrated with 100 µL ACN for 10 min, and then air-dried. The gel piece was covered with sequencing-grade modified trypsin (Promega, Madison, WI), and incubated in ice for 20 min. Then, the excess trypsin was removed, followed by incubation at 37 °C overnight. To extract the peptides, the gel piece was centrifuged at 10,000g for 2 min, and the digest buffer was removed from the gel. Then 50 µL of ACN/0.5 % trifluoroacetic acid (TFA) was added and the tube was shaken in a thermomixer for 10 min at 25 °C. The solutions were combined and transferred to Axygen low-retention microcentrifuge tubes. Peptides were further extracted with two washes of 50 % ACN/0.5 % TFA for 10 min each. The supernatants were collected and pooled, and then dried down to ~5 µL. Prior to MS analysis the samples were reconstituted with 5 µL of 50 % methanol/0.1 % TFA [33].

Protein Identification by Mass Spectrometry

Peptides were analyzed by tandem mass spectrometry (MS/MS) using a 4800 Proteomics Analyzer MALDI-TOF/TOF (matrix-assisted laser desorption ionization-time of flight/time of flight; Applied Biosystems, Inc., Foster City, CA). MS and MS/MS peak spectra were acquired and the 15 most intense peaks with a signal-to-noise ratio greater than 20 were automatically selected for MS/MS analysis. The peptide mass fingerprinting and sequence tag data from the TOF/TOF were evaluated with AB's GPS Explorer. The MS and MS/MS spectra were used by the Mascot search engine to identify proteins from non-redundant databases including NCBI nr as described previously [34, 35].

Peptides Sequence Analysis

Trypsin-digested samples were analyzed via liquid chromatography–electrospray ionization–tandem mass spectrometry (LC/ESI–MS/MS) on a linear ion trap mass

spectrometer (LTQ, Thermo-Finnigan, San Jose, CA) coupled to a Dionex/LC Packings nano LC system (Dionex, Sunnyvale, CA). A 75- μ m i.d. C18 reversed phase LC column (Micro-tech Scientific, Vista, CA) was utilized with a 60 min gradient from 2 % ACN, 0.2 % formic acid to 70 % ACN, 0.2 % formic acid. Data Dependent Analysis was used on the LTQ to perform MS/MS on all ions above an ion count of 1,000. Dynamic Exclusion was set to exclude ions from MS/MS selection for 3 min after being selected 2 times in a 30-s window. The MS/MS data were searched against the NCBI mouse or rat database with the Thermo Finnigan Bioworks 3.3 software package. To be considered a valid identification, the following criteria must be met: a Protein Probability of 1.0 E-3 or better, an X_{corr} vs. charge state >1.5, 2.0, 2.5 for +1, +2, and +3 ions, with at least 2 unique peptides matching the protein, and a good match for at least 4 consecutive “y” or “b” ion series from the MS/MS spectra.

In-Silico Analysis

In-Silico prediction of N^{ϵ} -acetyl lysine residues in a protein amino acid sequence was performed using the PAIL algorithm on the web server at <http://bdmpail.biocuckoo.org/prediction.php> (set at high stringency conditions) [36].

Mapping the Modified Residues to the Protein Tertiary Structure

Amino acid residues mapping to the protein tertiary structure of the full length of rat peroxisomal multifunctional enzyme type 1 (rpMFE-1) (PDB ID: 2 $\times$ 58) [37] was performed with PyMOL (the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC; <http://pymol.org/>).

Results

N^{ϵ} -acetyl Lysine Posttranslational Modification in Induced Peroxisomes

There is compelling evidence indicating that regulation of protein function by acetylation outside the nucleus is increasingly important for the regulation of the intermediary metabolism [30, 31]. To determine whether or not specific protein acetylation is important for peroxisome regulation, we isolated proteins from subcellular organelles from PPAR α -agonist treated rat livers using density gradient centrifugation. Isolated proteins were screened for modifications of lysine residues, especially acetylation at N^{ϵ} -lysine, using specific antibody against N^{ϵ} -acetyl-lysine residues (N^{ϵ} -AcK) [28]. Subcellular organelles present in a peroxisomal enriched fraction (λ fraction) [12] were purified by centrifugation in a linear density gradient. Determination of the protein profile showed that the proteins were distributed into two peaks in the gradients (Fig. 1a). The first protein peak co-localized with catalase, the enzymatic marker for peroxisomes (fractions 4–6; Fig. 1b); whereas the second peak of proteins co-localized with the mitochondrial protein cytochrome c (fractions 10–11; Fig. 1c). The second peak also co-localized with very long chain acyl-CoA synthetase, an enzyme mainly localized in the endoplasmic reticulum (ER), determined by immunoreaction with a specific antibody (fractions 12–15; Fig. 1d). These data indicate that the individual subcellular organelles were very well resolved. Screening of the gradient for proteins carrying N^{ϵ} -AcK residues by western blot analysis resulted in two areas of reactivity: one located in the gradient's higher density, co-localized with the peroxisome marker and containing a 75-kDa band (fractions 4–6; Fig. 1e); and another area, in the central region of the gradient, containing two main bands that co-localized with the mitochondrial fractions (fractions 10–11; Fig. 1e). Since the presence of acetylated proteins in mitochondria has been previously reported [28], we focused our efforts in the characterization of the signal present in the peroxisomal fractions.

First, the specificity of the anti- N^{ϵ} -AcK antibody [28] was confirmed by a competitive blocking of the antibody-dependent signal in the peroxisomal and mitochondrial fractions, using a custom-made peptide carrying a single N^{ϵ} -AcK residue (Supplemental Fig. 1); and second, the selectivity of the posttranslational acetylation process was demonstrated by the observation of signal in peroxisomes isolated from PPAR α -agonist treated animals (Fig. 2a, CIP) but not in peroxisomes from untreated animals (Fig. 2a, UT) when screened with the same anti- N^{ϵ} -AcK antibody (Fig. 2). 3-Ketoacyl-CoA thiolase B, a wellknown PPAR α dependent inducible peroxisomal enzyme, was used as standard of peroxisomal proteins induction (Fig. 2b). This analysis further indicates that the acetylation process is only associated with the induction of peroxisomal proteins during peroxisome proliferation.

Time-Dependent N^{ϵ} -acetyl Lys Modification of the Induced Peroxisomal Protein

To understand the significance of protein acetylation with regard to the protein induction/peroxisome proliferation events, we studied the time-dependent expression of the N^{ϵ} -AcK signal by western blot analysis, using equal amounts of purified peroxisomes isolated from untreated and peroxisomes isolated at different time periods since starting the treatment of the animals (1, 2, 4, 6, 9, 11 and 14 days; Fig. 3a). These data were compared with protein levels (induction) of the PPAR α -dependent peroxisomal enzyme acyl-CoA oxidase-1 (ACOX-1) 50 kDa subunit (Fig. 3b). There is a clear correlation between the appearance of the N^{ϵ} -AcK modified protein(s) and the induction of the ACOX-1 enzyme as early as 4–6 days from starting the dietary treatment (Fig. 3a, b). These observations strongly indicate a relationship between acetylation of the target protein and the induction of PPAR α -dependent proteins, suggesting that the acetylation process may play a role in the peroxisome proliferation process.

Distribution of N^{ϵ} -acetylated Proteins in Induced Peroxisomes

To gain further insight into distribution and identification of the modified protein(s), a pool of induced peroxisomal fractions (fractions 4–6) was screened by western blot for N^{ϵ} -lysine acetylation. A single band of apparent molecular weight of 75 kDa was reactive with the anti- N^{ϵ} -AcK antibody (Fig. 4a, CIP). Sub-fractionation of peroxisomes into integral membrane (M) and soluble (S) proteins using alkaline carbonate treatment [12] and further screening with anti- N^{ϵ} -AcK antibody demonstrated that the modified protein was associated with the peroxisomal fraction containing soluble proteins (S1–S2) but not peroxisomal membrane proteins (M1–M2; Fig. 4a). An antibody against the peroxisomal transporter ABCD3 (membrane protein of 70 kDa, PMP-70) recognized its antigen as a 70 kDa protein in total peroxisomes (Fig. 4b, CIP) as well as in the membrane fraction (M1–M2) but not in the soluble fraction (S1–S2; Fig. 4b). These observations document that the N^{ϵ} -acetyl Lys modified protein(s) in the soluble fraction of peroxisomes is a component of the peroxisomal matrix.

Immunoprecipitation of N^{ϵ} -acetylated Proteins and Identification by Mass Spectrometry

To identify the N^{ϵ} -Lys modified protein(s), the pool of peroxisomal proteins isolated from the liver of CIP-treated (CIP) animals was immunoprecipitated (IP) with antibodies against N^{ϵ} -AcK and the precipitated complex was resolved by SDS-PAGE (Fig. 5). Sample controls without antibodies (Fig. 5, lane 3) or peroxisomal proteins (Fig. 5, lane 4) were also included in the experiment. The Coomassie stained gel showed the presence of a band of 75 kDa in IP sample from the CIP-treated peroxisomal pool (Fig. 5, lane 2; arrowhead). A piece from that gel band was excised and analyzed by MS. The protein present in the gel was identified as the rat peroxisomal multifunctional enzyme type 1 (rpMFE-1; containing the enoyl-CoA hydratase/(3S)-hydroxyacyl-CoA dehydrogenase activities; UniProt Accession Number: P07896) (Table 1; Supplemental Fig. 2). The 39 unique peptides detected and used

to identify rpMFE-1 covered 54 % of the protein's amino acid sequence. To further support this finding, we used polyclonal antibodies raised against rpMFE-1 to screen the IPs of UT or CIP-treated peroxisome samples by western blot analysis (Fig. 6; lanes 2–3). The western blot analysis indicated that the rpMFE-1 antibody reacted with the IP band of 75 kDa present in the CIP samples (Fig. 6, lane 3; arrowhead) as well as with a peroxisomal fraction isolated from livers of CIP-treated rats, run as a positive control (Fig. 6, lane 1), but not with the UT sample (Fig. 6, lane 2) nor a negative control (Fig. 6, lane 4). These results support the identification of the N^{ϵ} -AcK-modified protein as rpMFE-1.

Identification of the N^{ϵ} -acetylated Lysine Residues in rpMFE-1

The MALDI-TOF/TOF analysis also detected two possible peptides bearing potential N^{ϵ} -acetylation at lysine (K) residues present in rpMFE-1 peptide sequences. Peptide 1 ($^{141}\text{VGVPVALDLITSGKYLSADEALR}^{164}$) contained a possible acetylation moiety at position K¹⁵⁵ and peptide 2 ($^{187}\text{IIDKPIEPR}^{195}$) contained a possible acetylation moiety at position K¹⁹⁰ (Table 1; Supplemental Fig. 2). Further analysis was performed with LC/ESI-MS/MS to obtain the peptides' amino acid sequences and identity of residues that are modified. There were 5 modified peptides, 4 of which belonged to the 140–195 region of the rpMFE-1 primary sequence (Table 1). Those 4 peptides were:

- (1) $^{165}\text{LGILDAVVKSDPVEEAIK}^{182}$, containing the highest ion score, modified at the K¹⁷³ residue and it was identified having two different charges;
- (2) $^{141}\text{VGVPVALDLITSGKYLSADEALR}^{164}$, having the next best ion score, modified at the K¹⁵⁵ residue was also present with two different charges (this amino acid sequence matched the sequence of peptide 1 identified by MALDI analysis Table 1 and Supplemental Fig. 2); (3) $^{183}\text{FAQKIIDKPIEPR}^{195}$, and (4) $^{187}\text{IIDKPIEPR}^{195}$. Sequences 3 and 4 overlapped the sequence of peptide 2 identified by MALDI-MS ($^{187}\text{IIDKPIEPR}^{195}$) and hence, all containing the acetylated-K¹⁹⁰ residue. Finally, peptide 5, $^{577}\text{GWYQYDKPLGR}^{587}$, found outside the stretch of residues 140–195, was modified at its K⁵⁸³ residue.

To further support these findings, an *in silico* analysis using the PAIL algorithm (<http://bdmpail.biocuckoo.org/prediction.php>) was performed to predict potential N^{ϵ} -acetyl-Lys sites in the rpMFE-1 amino acid sequence. The analysis, using settings at high stringency, predicted 22 potential lysine residues susceptible to N^{ϵ} -acetylation (Table 2). Of those 22 potential sites, the predicted acetylation of K¹⁵⁵ residue (DLITSGKYLSADE; Table 2) clearly matched the N^{ϵ} -acetyl-Lys found in peptide 1 as well as in two others peptides identified by LC/ESI-MS/MS (Table 1).

Mapping of Residues Lys¹⁵⁵ and Lys⁵⁸³ to the Tertiary Structure of rpMFE-1

Taking advantage of the availability of the crystal structure of the rat peroxisomal multifunctional enzyme type 1 (pMFE-1) [37, 38], we visualized the location of Lys¹⁵⁵ in the hydratase domain and Lys⁵⁸³ in the dehydrogenase domain, using PyMOL software. The *in silico* mapping of the Lys¹⁵⁵ residue indicated that this residue is located in domain A between α -helix 5 (H5) and β -strand 5 (B5), with its positively charged side chain pointing into a “tunnel” structure (Fig. 7a, b; yellow residue), that links the active sites present in the N-terminal part (hydratase/isomerase) with the active site of the C-terminal part (dehydrogenase) [37]. On the other hand, Lys⁵⁸³ residue was localized between helix DH5 and helix DH6, an area that adopts the fold of a three-stranded antiparallel β -meander, in domain D, located at the C-terminus of the dehydrogenase enzyme (Fig. 7a, b; white Lys residue) [38].

Discussion

A growing body of experimental evidence indicates that peroxisomal cellular homeostasis constitutes a dynamic system that originates from the endoplasmic reticulum and plays diverse metabolic roles in response to cellular demands under normal and pathological conditions [1, 7]. Since their discovery, more than 70 proteins have been identified to be confined to peroxisomes [1–3, 7]. Using proteomic methodologies, new proteins/enzymes have been found to localize in peroxisomes, thus expanding our knowledge of the functional pathways of this organelle [39, 40]. Indeed, some examples are Lon protease [41], glutathione-*S*-transferase [42], nudix hydrolase [43], among others [44]. In addition, some studies have demonstrated that individual proteins may be present as different isoforms in peroxisomes [42, 45], suggesting that PTMs may be affecting protein structure/functions, and hence metabolic pathways in peroxisomes.

Using an antibody against *N*^ε-acetyl-Lys (*N*^ε-AcK) [28], in combination with MS-based analysis we identified, for the first time, a chemically modified *N*^ε-Lys acetylated protein localized in peroxisomes isolated from liver of CIP-treated animals. Immuno-blotting of density gradient protein fractions and immunoaffinity-purified peroxisomal proteins supported these results. This protein was identified as the rat peroxisomal multifunctional enzyme type 1 (rpMFE-1). Furthermore, in silico analysis of Lys-acetylation in rpMFE-1 using its amino acid sequence predicted 22 possible Lys sites under high-stringency conditions. One of those predicted sites matched the Lys¹⁵⁵ residue found acetylated by peptide fingerprinting (MALDI-TOF/TOF) and peptide sequence analysis (LC/ESI-MS/MS). Therefore, rpMFE-1 is reported here as a new protein in the short list of peroxisomal proteins that undergo PTMs. This list includes the membrane-related proteins peroxins Pex19p and Pex5p, and the membrane half transporters ABCD1 and ABCD3 [25–27]. Since *N*^ε-Lys acetylation of pMFE-1 was only observed in proliferating peroxisomes induced by PPARα-agonist, MFE-1 is the first enzyme in peroxisomes and first matrix protein that has been documented to be acetylated in PPARα-agonist induced/proliferating peroxisomes.

rpMFE-1 has three catalytic activities dependent on two active sites. The Δ^2 -enoyl-CoA hydratase-1 and the Δ^3 , Δ^2 -enoyl-CoA isomerase (H/I) activities occur at the active site present at its N-terminal and the (3S)-hydroxyacyl-CoA dehydrogenase (HAD) activity occurs at its active site present at the enzyme C-terminal [21, 37, 38]. The precise functional role of pMFE-1 is not clear, as it shares substrate and catalytic specificity with the non-inducible isoenzyme pMFE-2 (peroxisomal multifunctional enzyme type 2) [1, 46] and with other non-peroxisomal monofunctional enzymes, i.e. isomerases, hydratases (also known as crotonases), and dehydrogenases. Indeed, based on rpMFE-1 sequence analysis, its N- and C-terminal parts present homology to two different superfamilies: the crotonase [47], and the HAD superfamily [48], respectively. In addition to the two functional units, this protein presents five structural and functional domains (domains A–E; Fig. 7a) [48]. The N-terminal region contains the domains A and B; and the C-terminal region domains B–E, with domain B being a linker helix, which functionally belongs to the N-terminal part but structurally to the C-terminal part [38].

Three of the four identified Lys acetylated residues occur in domain A of the H/I fragment (N-terminal part), and of those three modified residues (K¹⁵⁵, K¹⁷³, K¹⁹⁰), the Lys¹⁵⁵ residue was identified by two different mass spectrometry analyses and predicted by in silico screening of rpMFE-1 primary sequence as indicated above. The Lys¹⁵⁵ residue is part of the eight lysine and five arginine residues whose positively charged side chains point towards the tunnel structure (T in Fig. 7a) that separates the two active sites (H/I and HAD) of the enzyme [37]. This particular location of the Lys¹⁵⁵ residue may suggest a possible physiological role of the PTM on regulation of the enzyme functions.

The last modification, identified on the Lys⁵⁸³ residue, corresponds to a lysine residue forming part of the ⁵⁸³KPLGR region in domain D of the enzyme's C-terminal dehydrogenase part [38]. Under experimental conditions, where only the C-terminal dehydrogenase part of the rpMFE-1 is expressed under culture conditions (i.e. lacking the N-terminal H/I fragment), domain D plays a key role in protein dimerization, an event which does not occur under physiological conditions [38]. Therefore, the acetylation of Lys⁵⁸³ may play a role in the regulation of conformational changes in the quaternary structure of the rpMFE-1.

In addition, because the acetylation of Lys residues in rpMFE-1 is only seen under PPAR α -agonist treated conditions, we hypothesize that these modifications may be important for peroxisome proliferation. The concept that a peroxisomal matrix protein may participate in the regulation of the peroxisome number is not new, as it has been suggested from yeast studies [49, 50].

The fact that the acetylation of rpMFE-1 occurs only in PPAR α induced rat liver peroxisomes, parallel to the increased expression of the inducible peroxisomal enzyme ACOX-1, and not in peroxisomes from liver of control rats, suggests that this PTM is highly selective and is related to the peroxisome proliferation process. Supporting this role is the observation that pMFE-1-deficient mice, when challenged with a peroxisome proliferator drug, increase the PPAR α -dependent levels of mRNA and proteins in the liver, but display an appreciable blunting of the peroxisome proliferative response as compared to similarly treated wild type mice [51]. This effect may be attributed to the absence of pMFE-1 protein in the peroxisomes of the deficient mice liver, because all the other inducible proteins studied are expressed to the same extent in wild type and pMFE-1 deficient mice when treated with the same peroxisome proliferator drug [51]. Therefore, only under conditions where PPAR α is activated, the presence of MFE-1 inside peroxisomes seems to be required for proper organelle proliferation; otherwise pMFE-1-deficient mice have normal "numerical density" of peroxisomes when on a normal diet [51]. These observations strongly suggest that pMFE-1 is required for the PPAR α -dependent proliferation process of peroxisomes in rat liver. Moreover, additional support for such a role comes from studies in yeast. The peroxisomal division process in yeast involves a specific signal transmitted from inside the mature organelle [50]. This signal is carried out by the redistribution of an enzyme from the matrix to the organelle membrane. On the membrane, the interaction of the enzyme with Pex16p, a membrane associated protein that negatively regulates peroxisome division, allows mature peroxisomes to divide [50]. What triggers the redistribution of the enzyme is unknown, but we can suspect the involvement of PTM of the enzyme.

In summary, this is the first report which identifies that the peroxisomal matrix enzyme MFE-1 undergoes *N*^ε-acetyl-modification at four different lysine residues which belong to domains A and D of the protein. This process occurs only during PPAR α -agonist induced peroxisome proliferation in rat liver, and suggests that acetylation may play a functional role in the organelle proliferative process by regulating the structure/function of rpMFE-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABC	ATP-binding cassette
ACN	Acetonitrile
ACOX	Acyl-CoA oxidase
CIP	Ciprofibrate
CoA	Coenzyme A
HAD	Hydroxyacyl-CoA dehydrogenase
LC/ESI-MS/MS	Liquid chromatography–electrospray ionization–tandem mass spectrometry
MALDI-TOF/TOF	Matrix-assisted laser desorption ionization-time of flight/time of flight
MFE-2	Multifunctional enzyme type 2
MS	Mass spectrometry
N^ε-AcK	N ^ε -acetyl-lysine
PAA	Polyacrylamide
Pex	Peroxin
PI	Protein immunoprecipitation
PPARα	Peroxisome proliferator-activated receptor alpha
PTM	Posttranslational modifications
rpMFE-1	Rat peroxisomal multifunctional enzyme type 1
SCPx	Sterol carrier protein x
TFA	Trifluoroacetic acid
VLCFA	Very long chain fatty acids

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Fig. 1.

Fractionation of subcellular organelles and *N*^ε-lysine acetylated proteins signal profile in density gradient centrifugation from PPAR α -agonist treated rat liver. Rat liver subcellular organelles were isolated from a peroxisome enriched fraction by Accudenz density gradient centrifugation and were analyzed for protein and organelle markers as described in the "Materials and Methods". The distribution of the organelles in the gradient was determined by the protein distribution (a) and organelles marker proteins: enzymatic activity of catalase for peroxisomes (b), western blots analysis of cytochrome c (12 kDa) for mitochondria (c) and very long chain acyl-CoA synthetase (70 kDa) for endoplasmic reticulum (d). Western

blot analysis of protein N^{ϵ} -lysine acetylation was also determined (**e**). The *line on top of fractions 4–6* (**a**) indicates the fractions with the highest specific activity of catalase (**b**), which were pooled together and used as the peroxisome fraction. The *arrowhead* indicates a protein band of approximately 75 kDa recognized by the antibody against N^{ϵ} -acetylated lysine (**e**). The data represent the means $\pm$ SD of the values for $n = 3$ gradients obtained from ciprofibrate-treated animals. The *bar (top of a)* represents the density of the gradient's fractions (0–50 %). *Dotted lines* across figures **a–e** indicate the regions of the gradient where peroxisomal and mitochondrial peaks are localized. Western blots are representative of at least two individual analyses (equal amounts of protein from each gradient fraction were loaded on the SDS-PAGE)

Fig. 2.

The N^{ϵ} -lysine acetylated protein is present only in peroxi-somal fractions isolated from PPAR α -agonist treated rat liver. Modified (N^{ϵ} -lysine acetylated) proteins present in 50 μ g of peroxisome purified fractions obtained from untreated (UT) and PPAR α -agonist (ciprofibrate; CIP) treated livers were screened with a specific antibody against N^{ϵ} -acetyl-lysine using western blot (a) and compared with the western blot signal obtained using an antibody against the PPAR α -agonist inducible peroxisomal enzyme 3-ketoacyl-CoA thiolase B (41 kDa) (b). The *arrowhead* indicates a protein band of approximately 75 kDa (a). Prestained protein standards were also loaded in the gel. The western blot signal was

developed using chemo-luminescence (Lumi-Phos WB) and exposure to CL-Xposure films, as indicated in the method section

Fig. 3. *N*^ε-lysine acetylation in peroxisome fractions parallels the induction (proliferation) of peroxisomes in the liver of PPAR α -agonist treated animals. Equal amounts (50 μ g) of peroxisomal pool fractions proteins isolated from livers of untreated (UT) and ciprofibrate-treated (CIP) animals at different treatment intervals (1, 2, 4, 6, 9, 11, 14 days) were resolved by SDS-electrophoresis, transferred to nitrocellulose and analyzed by western blot using anti-*N*^ε-acetyl-lysine antibody (**a**) and antibodies against the PPAR α -agonist inducible peroxisomal enzyme Acyl-CoA oxidase-I (ACOX-1; 50 kDa subunit) (**b**), as described in the "Materials and Methods". Western blots are representative of at least two individual analyses

Fig. 4.

N^ε-lysine acetylation is associated with a soluble protein fraction of peroxisomes.

Peroxisomes isolated from liver of treated animals (*CIP*) were separated into membrane (*M1–M2*) and soluble (*S1–S2*) protein fractions using carbonate treatment as indicated in the “Materials and Methods”. The samples were resolved by electro-phoresis and analyzed by western blot using antibodies against *N^ε*-acetyl-lysine (**a**) or against the peroxisomal transporter ABCD3 (peroxisomal membrane protein of 70 kDa) (**b**). The blots were developed using chemo-luminescence and exposed to X-Ray film as indicated in the “Materials and Methods”. The arrowhead indicates a protein band of approximately 75 kDa

Fig. 5.

The antibody against N^ε-acetyl lysine immunoprecipitated a protein of 75 kDa. Proteins of peroxisome enriched fractions isolated from ciprofibrate treated (CIP) rat liver were subjected to immuno-precipitation using antibodies against-N^ε-acetyl lysine, and resolved by gel electrophoresis. The immune-complex was sedimented using protein A/G-agarose. After extensive washing, the immunoprecipitated protein samples were resolved by SDS-electrophoresis (4–20 % precast gel) and visualized by staining the gel with Coomassie blue as indicated in the “Materials and Methods”. Prestained protein standard markers (Precision Plus all Blue) were loaded in parallel to samples (*lane 1*), the immune complex (*lane 2*),

immunoprecipitation sample without antibodies (*lane 3*) or without peroxisome proteins (*lane 4*) onto the precast gel. After running, the gel was stained with Coomassie blue, destained and the image acquired as indicated in the “Materials and Methods”. *The arrowhead* indicates an immuno-precipitated protein band of 75 kDa. The *asterisk* indicates the protein band corresponding to IgG heavy chain. The acquired gel image was rendered using Adobe Photoshop

Fig. 6. Identification of the immunoprecipitated N^ε-acetylated perox-isomal proteins by western blot. An antibody raised against rat peroxisomal multifunctional protein-1 (rpMFE-1) was used to confirm the identity of the acetylated protein in peroxisomes. Proteins of the peroxisome enriched fractions isolated from untreated (UT, *lane 2*) or ciprofibrate treated (CIP, *lane 3*) rat liver were subjected to immunoprecipitation using antibodies against-N^ε-acetyl lysine (*lanes 2–3*), as indicated in the “Materials and Methods”. A sample without peroxisomal proteins was included in the immunoprecipitation experiment (*lane 4*). The immunocomplex was sedimented using protein A/G-agarose and washed extensively. The

immunoprecipitated samples (*lanes 2–4*) and a peroxisome enriched sample from ciprofibrate-treated rat liver (25 μ g; *lane 1*) were resolved by SDS-electrophoresis (4–20 % precast gel), transferred to nitrocellulose membranes and immunoscreened with antibodies raised against rpMFE-1. The antibody signal was detected by chemo-luminescence and exposure to X-Ray films, as indicated in the “Materials and Methods”. The *arrowhead* indicates the protein band of 75 kDa recognized by the anti-MFE-1 antibody. The *asterisk* indicates the protein band corresponding to IgG heavy chain

Fig. 7.

Folding of pMFE-1. Ribbon representation of the three dimensional folding of pMFE-1 shows the protein different structural domains (A–E) (a). Domain A is located at the N-terminal of the protein and contains the hydratase/isomerase active sites, domain B is the linker helix, domain C is the NAD-binding domain of the dehydrogenase part and also contains important catalytic residues of the HAD active site. Domains D and E are tightly associated with each other, with domain E not seen because it is covered by domain D [37]. T represents the tunnel area. The ribbon representation (a) and the three dimensional space-filling rendering (b) show a positively charged side chain near the tunnel, corresponding to Lys¹⁵⁵ residue (yellow) located in domain A and Lys⁵⁸³ residue (white) in domain D

Proteins identified by multiple fragmentation spectra (peptide mass fingerprinting) and their modified (acetylated) peptides resolved and sequenced by liquid chromatography mass spectrometry

Table 1

Method	Accession number	Unique peptides detected	Sequence coverage (%)	Calculated mass	Observed mass	Ion score	Modified sequences identified ^c
MALDI-TOF/TOF	NP_598290 ^a pMFE-1 ^b (<i>Rattus norvegicus</i>)	39	54			1,590	
		PEPTIDE 1		2,528.3970	2,528.3877	44	141VVGVVPVALDLITSGK Y LSADEALR ¹⁶⁴
		PEPTIDE 2		1,122.6517	1,122.6511	28	187IIDK P IEPR ¹⁹⁵
Method	Accession number	Unique modified peptides detected	Calculated mass	Observed mass	Ion Score	Charge	Modified Sequences identified ^c
LC/ESI-MS/MS	NP_598290 ^a pMFE-1 ^b (<i>Rattus norvegicus</i>)	5	1,937.0564	1,937.0721	94.5	2	165LGILDAVVVKSDPVEEAIK ¹⁸²
			2,527.3696	2,527.3897	68.6	2	141VVGVVPVALDLITSGK Y LSADEALR ¹⁶⁴
			2,527.3706	2,527.3897	56.6	3	141VVGVVPVALDLITSGK Y LSADEALR ¹⁶⁴
			1,423.6782	1,423.6884	56.1	2	577GWYQYDK P PLGR ⁵⁸⁷
			1,595.8932	1,595.9035	53.6	3	183FAQKIIDK P IEPR ¹⁹⁵
			1,937.0575	1,937.0721	47.6	3	165LGILDAVVVKSDPVEEAIK ¹⁸²
			1,121.6360	1,121.6444	33.8	2	187IIDK P IEPR ¹⁹⁵

^aNCBI Ref Seq
^bPeroxisomal multifunctional enzyme type 1
^cBold and underlined character represents a N^ε-acetylated lysine residue (see also Supplemental Fig. 2)

Table 2

In-silico prediction of lysine N^{ϵ} -acetylated residues in the primary sequence of the rat peroxisomal multifunctional enzyme type 1 (rpMFE-1)

Peptide	Position	Score	Threshold
EIQRYQ K PVLAAI	87	1.19	0.5
<u>DLITSGKYLSADE</u>	155	1.11	0.5
AIKFAQ K IIDKPI	186	0.65	0.5
SIQASV K HPYEVG	241	1.09	0.5
GIKEEE K LFMYLRL	253	0.88	0.5
RASGQA K ALQYAF	265	1.05	0.5
Y AFFAE K SANKWS	275	0.83	0.5
PSGASW K TASAQP	289	2.43	0.5
NGQASA K PKLRFS	359	2.21	0.5
QASAK K LRFS	361	2.64	0.5
RFSSST K ELSTVD	369	1.12	0.5
DMNLK K KVFAELS	390	0.59	0.5
ELSALC K PGAFLC	400	1.21	0.5
VMSLS K KIGKIGV	464	1.34	0.5
LSKKIG K IGVVVG	467	0.65	0.5
GLDVGW K IRKGQG	531	0.71	0.5
VGWKIR K GQGLTG	534	1.05	0.5
PGTPVR K RGNSRY	551	1.69	0.5
FGQKTG K GWYQYD	576	1.75	0.5
LPTVLE K LQKYR	677	0.61	0.5
QGSPL K EWQSLA	709	0.83	0.5
AGPHGS K L	721	13.33	0.5

Potential lysine residues (**K**) N^{ϵ} -acetylated in the amino acid sequence of the rpMFE-1 were predicted using the PAIL algorithm (high stringency settings) on the web server at <http://bdmpail.biocuckoo.org/prediction.php>.

Underlined peptide sequence correspond to the sequence of an acet-ylated peptide identified by Mass Spectrometry (see text)