Phosphorylation of Rat Liver Mitochondrial Carnitine Palmitoyltransferase-I

EFFECT ON THE KINETIC PROPERTIES OF THE ENZYME*

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Janos Kerner‡§, Anne M. Distler¶§, Paul Minkler†, William Parland**, Scott M. Peterman‡¶, and Charles L. Hoppel**‡§

From the Department of Nutrition, Biochemistry, and Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106, Medical Research Service, Louis Stokes Department of Veterans Affairs Medical Center, Cleveland, Ohio 44106, and Thermo Electron, Somerset, New Jersey 08873

Hepatic carnitine palmitoyltransferase-I (CPT-I) isolated from mitochondrial outer membranes obtained in the presence of protein phosphatase inhibitors is readily recognized by phosphoamino acid antibodies. Mass spectrometric analysis of CPT-I, tryptic digests revealed the presence of three phosphopeptides including one with a protein kinase CKII (CKII) consensus site. Incubation of dephosphorylated outer membranes with protein kinases and [γ-32P]ATP resulted in radiolabeling of CPT-I only by CKII. Using mass spectrometry, only one region of phosphorylation was detected in CPT-I isolated from CKII-treated mitochondria. The sequence of the peptide and position of phosphorylated amino acids have been determined unequivocally as FpSSPETDpSHRFGK (residues 740–752). Furthermore, incubation of dephosphorylated outer membranes with CKII and unlabeled ATP led to increased catalytic activity and rendered malonyl-CoA inhibition of CPT-I from competitive to uncompetitive. These observations identify a new mechanism for regulation of hepatic CPT-I by phosphorylation.

β-Oxidation of long-chain fatty acids represents a main energy-yielding process for many organs, especially for muscle and liver. The liver plays a central role in both physiological and pathological conditions by switching from a carbohydrate to fatty acid-based metabolism. Carnitine palmitoyltransferase-I (CPT-I), localized in the outer mitochondrial membrane, catalyzes the formation of long-chain acylcarnitines from their respective CoA esters, committing them to β-oxidation in the mitochondrial matrix. By virtue of its inhibition by malonyl-CoA, CPT-I represents the key regulatory site controlling the flux through β-oxidation. Consistent with its central role in mitochondrial fatty acid oxidation, the enzyme exists in at least two isoforms, the liver (CPT-I_L) and muscle types (CPT-I_M) (1). Recently a third form, the brain-specific isofrom (CPT-I_B), has been cloned and expressed (2). Although the protein sequences of mouse and human CPT-I show high similarity to those of CPT-I_L and CPT-I_M, only malonyl-CoA binding but no catalytic activity is observed with the protein expressed in yeast. The liver and muscle isoforms differ in their regulatory properties. The muscle isoform is thought to be regulated solely by changes in malonyl-CoA concentration. However, Sugden et al. (3) have shown that hyperthyroidism facilitates myocardial fatty acid oxidation by a mechanism that involves increased activity and decreased malonyl-CoA sensitivity of CPT-I and that the two parameters can apparently be regulated independently from each other. The liver isoform, in addition to being regulated via changes in malonyl-CoA concentrations, also is regulated by changes in the sensitivity of the enzymes to malonyl-CoA inhibition (1, 4, 5). Thus, CPT-I_L is unique in responding to different physiological states, e.g. starvation, insulin deficiency, and hypoxia and hyperthyroidism by changing its sensitivity to inhibitor severalfold (6–9). These changes amplify the effects of fluctuations in the cytosolic concentration of malonyl-CoA occurring under these conditions.

Although changes in malonyl-CoA sensitivity are considered the major means for the regulation of the liver isofrom of CPT-I and thus for hepatic mitochondrial fatty acid oxidation, the mechanism for the decrease in malonyl-CoA sensitivity has not been uncovered. It has been suggested that changes in the lipid composition of the membrane microenvironment in which CPT-I resides are important for alteration in malonyl-CoA sensitivity (10–12). Alternatively, covalent modification of CPT-I protein itself could also be responsible for these changes in the malonyl-CoA sensitivity of the enzymes. Recent molecular biology approaches suggest that modulation of interdomain interactions may be involved in the changes in malonyl-CoA sensitivity displayed by the liver isoform under different physiological conditions (13–19). These changes in the interactions may arise from covalent modification (phosphorylation) of the protein. Phosphorylation of one or more amino acid residues in CPT-I would affect the charge distribution and consequently alter the intramolecular interactions between the cytosolic N- and C-terminal domains resulting in altered malonyl-CoA sensitivity and catalytic activity. Thus, Pegorier et al. (20) reported that glucagon or dibutyryl-cAMP treatment of fetal rabbit hepatocytes decreases the malonyl-CoA sensitivity of CPT-I in isolated mitochondria 5- and 10-fold, respectively, with no effect on the catalytic activity. These data sug-
gest that the changes observed in malonyl-CoA sensitivity of CPT-I are brought about by a cAMP-dependent signaling event. In addition to malonyl-CoA sensitivity, phosphorylation-based regulation of CPT-I, catalytic activity has also been suggested. In isolated rat hepatocytes glucagon or dibutyryl-cAMP increased CPT-I activity without affecting malonyl-CoA sensitivity, an effect that was mimicked by the protein phosphatase inhibitor, okadaic acid (21). Subsequent studies from the same authors indicated that phosphorylation of cytoskeletal components rather than phosphorylation of CPT-I is responsible for the increased catalytic activity (22, 23). Thus, the mechanism(s) by which malonyl-CoA sensitivity and/or catalytic activity are altered are not known.

Based on these observations and on our preliminary findings with protein phosphatase inhibitors on malonyl-CoA sensitivity of hepatic CPT-I (24, 25), we initiated studies using a proteomic approach to elucidate the potential role of phosphorylation/dephosphorylation on the regulation of rat liver mitochondrial CPT-I activity and malonyl-CoA sensitivity.

**EXPERIMENTAL PROCEDURES**

**Animals**—Male Sprague-Dawley rats (200–400 g) were obtained from Zivic-Miller (Allison Park, PA) and had free access to food and water. New Zealand White male rabbits for production of polyclonal antisera were purchased from Covance Research (Denver, PA). All procedures were approved by the Veterans Administration Institutional Care and Use Committee and performed in accordance with National Institutes of Health guidelines for care and use of animals in research.

**Chemicals**—Polyclonal antisera against oligopeptides specific for CPT-I, were prepared as described earlier (26), and the specific antibodies were isolated by affinity chromatography using peptides coupled to Sulfo-Link resin (Pierce). Recombinant catalytic subunit of protein kinase A, catalytic subunit of protein kinase C, the recombinant α-subunit of protein kinase C (calmodulin kinase, CaMKII), and human casein kinase II (or protein kinase CKII (CKII)) were purchased from Calbiochem, and constitutively active AMP-activated protein kinase was a generous gift from Dr. G. Lopashuck (University of Alberta, Edmonton, Canada). Protease inhibitor mixture and protein phosphatase inhibitor mixtures I and II were from Sigma. L-[1-methyl-14C]Carnitine was synthesized as described by Ingalls et al. (27). All other chemicals were of the highest commercially available purity. The matrix, e-erythro-4-hydroxycinnamic acid, and calibration standards were purchased from Sigma.

**Isolation of Rat Liver Mitochondria and Rat Liver Mitochondrial Outer Membranes**—Percoll-purified rat liver mitochondria were isolated as described in Ref. 28 in 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4, supplemented or not with protein phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 4 mM β-glycerophosphate). Mitochondria were used to assay CPT-I activity and malonyl-CoA sensitivity as described below or served as the starting material for isolation of mitochondrial outer membranes.

High purity rat liver mitochondrial outer membranes were isolated by the swell/shrink technique and discontinuous sucrose gradient centrifugation as detailed elsewhere (29) in the presence and absence of the above protein phosphatase inhibitors and resuspended in 20 mM MOPS, pH 7.4, supplemented or not with protein phosphatase inhibitors. Where indicated, crude outer membranes isolated in the absence of protein phosphatase inhibitors were treated by incubating the membrane fraction (6 mg of protein) for 30 min at room temperature with a mitochondrial matrix fraction (2.0 ml, 15 mg/ml) complemented with protease inhibitors at 1/100 dilution (Sigma) before discontinuous sucrose gradient centrifugation. This step was included to dephosphorylate the outer membranes. For mass spectrometric analysis for CPT-I phosphorylation by CKII, the above experiment was scaled up using 1.0 mg of dephosphorylated rat liver outer membranes and 6.0 μg of recombinant CKII. Following isolation of the membranes, the membranes were recovered by centrifugation (30 min, 230,000 × g), solubilized in 30 μl of Laemmli sample buffer supplemented with 8 M urea, and 15-μl aliquots were subjected to SDS-PAGE separation. The separated polypeptides were transferred to polyvinylidene difluoride membranes for autoradiography, and the same membranes were then probed with affinity-purified antibodies specific for CPT-I, peptide and protein kinase CKII antibodies by Western blotting.

In experiments where the effect of phosphorylation on CPT-I activity and malonyl-CoA sensitivity was assessed, dephosphorylated rat liver mitochondrial outer membranes (180 μg) were incubated with 1.8 μg of recombinant CKII in the presence of 5% glycerol and 2.5 mM ATP for 60 min at 37 °C in a final volume of 150 μl supplemented with protein phosphatase inhibitor mixtures I and II and protease inhibitor mixture at a 1/100-fold dilution. Controls were incubated as above either with 2.5 mM ATP alone or without CKII and ATP.

For mass spectrometric analysis for CPT-I phosphorylation by CKII, the above experiment was scaled up using 1.0 mg of dephosphorylated rat liver outer membranes and 6.0 μg of recombinant CKII. Following isolation of the membranes, the membranes were recovered by centrifugation (30 min, 230,000 × g) and the CPT-I was isolated by semi-preparative SDS-PAGE and electroelution.

**Determination of CPT-I Activity**—CPT-I activity was measured using the modified radiochemical forward assay and is expressed as the activity inhibited by 200 μM malonyl-CoA (28). Malonyl-CoA sensitivity of CPT-I was assessed by determining the IC50 values at 50 μM malonyl-CoA or by determining the K values at 25 and 50 μM palmitoyl-CoA concentrations. Malonyl-CoA concentrations are given in the appropriate figure legend (Figs. 1 and 8). Using isolated rat liver mitochondria or rat liver homogenate the assay was carried out in the absence and presence of the protein phosphatase inhibitors sodium fluoride (10 mM), sodium orthovanadate (1.0 mM), sodium pyrophosphate (1.0 mM), and β-glycerophosphate (4 mM). Rat liver homogenate was prepared from powdered rat liver in 220 mM sucrose, 70 mM mannitol, 5 mM MOPS supplemented or not with sodium fluoride (40.0 mM), sodium orthovanadate (1.0 mM), sodium pyrophosphate (1.0 mM), and β-glycerophosphate (40 mM), pH 7.4 (31), and the CPT-I activity was determined as described above for isolated mitochondria.

**Isolation of CPT-I**—CPT-I was isolated from purified rat liver mitochondrial outer membranes by semi-preparative SDS-PAGE as described for contact site proteins (29). Briefly, 1.0 mg of phosphorylated (prepared in the presence of protein phosphatase inhibitors), dephosphorylated (prepared in the absence of protein phosphatase inhibitors and exposed to matrix proteases as described above), and protein kinase CKII phosphorylated rat liver outer membranes were subjected to semi-preparative SDS-PAGE; the separated polypeptides were visualized by brief staining with Coomassie Brilliant Blue R250, and the ~88 kDa band corresponding to CPT-I was cut out and electroeluted (see Fig. 2). For tryptic digestion the electroeluted CPT-I was precipitated with ethanol (80%), and the precipitated protein was washed twice with 50% ethanol to remove excess protein salt and subjected to reduction with DTT and carboxymethylation with iodoacetamide using standard protocols. Before tryptic treatment, excess reagents were removed by ethanol (80%) precipitation of CPT-I. Tryptic digestion of CPT-I isolated from 1.0 mg of mitochondrial outer membranes was carried out at 37 °C for 24 h in 50 mM ammonium bicarbonate, 1 mM DTT, and 1 mM hydroxylamine (1 each) in matrix protease (2.5 μg) and 40 μg of purified recombinant CKII. The digest that was not dephosphorylated was transferred to polyvinylidene difluoride membranes for autoradiography, and the same membranes were then probed with affinity-purified antibodies specific for CPT-I, and the same membranes were then probed with affinity-purified antibodies specific for CPT-I, peptide and protein kinase CKII antibodies by Western blotting.

**SDS-PAGE and Immunoblot Analyses**—CPT-I, isolated by semi-preparative SDS-PAGE was subjected to analytical SDS-PAGE, and the CPT-I polypeptide was transferred to polyvinylidene difluoride membrane and probed with affinity-purified CPT-I peptide antibodies or commercial mouse monoclonal antibodies raised against phospho-specific sequences. The polypeptide from CPT-I (1.0 mg) was visualized immunologically as described earlier (26), and phosphorylation was visualized by enhanced chemiluminescence detection. In the latter case, bovine serum albumin was used instead of fat-free dry milk, and 50 μM NaF was included in all buffers containing bovine serum albumin.
Mass Spectrometry—The 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 a stainless steel sample plate. Reflectron MALDI mass spectra were recorded on a Bruker (Billerica, MA) BiFlex III MALDI-TOF instrument equipped with a nitrogen laser (337 nm, 3-na pulse). All spectra were obtained in positive ionization mode using an accelerating voltage of 20 kV. Angiotensin II, ACTH dip (amino acids 18–39), and insulin were used as calibrants for these experiments.

Electrospray spectra were acquired on ThermoFinnigan LCQ Deca quadrupole ion trap or LTQ linear quadrupole ion trap mass spectrometers. Both systems used an Upchurch microinjector valve sample delivery system for sample introduction. Experimental results for the LTQ data were acquired using a PepMap C18 column from LC Packings (Sunnyvale, CA). The flow rate was ~200 nM/min. The samples were diluted to a concentration of 100 fmol/μl, and 2 μl 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 to maximize coverage. The LTQ method utilized eight data-dependent full ESI tandem mass spectrometry (MS/MS) scans following one full scan mass spectrum from 400 to 2000 Da. Analyses performed on the LCQ Deca were acquired using a C18 packed picofrit column purchased from New Objectives, Inc. (Woburn, MA). The mobile phases used were 0.1% formic acid in HPLC-grade water and 0.1% formic acid in HPLC-grade acetonitrile. The LCQ method allowed for three full MS/MS scans following one full scan mass spectrum. All data files were searched against a rat subset taken from the NR data base. The rat subset was indexed with carbamoylated cysteines. The results of the searches were filtered by removing all peptide matches containing an XCorr score lower than 1.0 regardless of charge state.

RESULTS AND DISCUSSION

As shown in Fig. 1A, when homogenates from quick-frozen rat liver were prepared in the presence of a mixture of broad range protein phosphatase inhibitors (PPIs) as described by Vavvas et al. (31) and were assayed in the presence of these PPIs, there was a significant decrease in CPT-I inhibition by 10 μM malonyl-CoA with no change in the apparent reaction velocity, reflecting a decreased sensitivity to inhibition by malonyl-CoA.

Similar observations were made with mitochondria from rat livers isolated in the presence and absence of PPIs. As shown in Fig. 1B, when liver mitochondria are isolated in the presence of PPIs from fed rats, CPT-I in these mitochondria display decreased malonyl-CoA sensitivity (IC_{50} > 16 μM) when compared with mitochondria isolated in the absence of PPIs (IC_{50} < 4 μM) with no change in the catalytic activity of the enzyme. Although these data are consistent with regulation of CPT-I by phosphorylation/dephosphorylation, an indirect effect of PPIs.

With immunological evidence of CPT-I phosphorylation, the protein was evaluated for post-translational modifications using a mass spectrometric approach. Although the hydrophobicity of membrane proteins complicates mass spectrometric analyses, success has been reported with a variety of techniques. Intact membrane proteins have been studied using both MALDI (33, 34) and ESI (35, 36) techniques to desorb and ionize the analyte. To determine the sequence of the protein and identify any post-translational modifications, a “bottom-up” approach to proteomics is often taken. For these experiments, isolated CPT-I was ethanol precipitated, the cysteines were reduced and carboxymethylated, and the protein was subjected to trypsin digestion. Tryptic digests of membrane proteins yield hydrophobic peptides, especially those peptides from the membrane spanning region of the protein. Hydrophobic peptides have been studied successfully using both MALDI (37–39) and ESI (40, 41). Recent publications have discussed the complementary nature of the two ionization methods to increase proteome coverage (42). In our analyses, the resulting digests were examined by both MALDI-TOF mass spectrometry (MS) and ESI-quadrupole ion trap-MS using a nanospray source. The MALDI-TOF data provided a quick screening method for protein coverage, and the MS/MS experiments allowed for sequence determination of the tryptic peptides.

The tryptic digest of the CPT-I protein was first analyzed using MALDI-TOF MS, Fig. 3. The mass fingerprint analysis of this spectrum yielded 74% coverage of the liver isoform of the protein. Although the mass fingerprint indicates the presence of the CPT-I protein, sequence information was needed as well. The sample was then analyzed using nanospray ESI interfaced to a Finnigan-LTQ linear ion trap mass spectrometer. The resulting total ion current chromatogram is shown in Fig. 4A. In this analysis, 62% coverage by mass was obtained for the protein with 473 of 773 amino acids being sequenced. To demonstrate the quality of the data used to sequence the tryptic peptides, MS/MS spectra for three different peptides are shown in Fig. 4, B–D. Fig. 4, B–D, includes the amino acid sequence of each peptide, the m/z value of the precursor ion, and the m/z value of the protonated peptide detected in the MALDI-TOF MS experiment. These peptides are labeled (b), (c), and (d) in the total ion current chromatogram, Fig. 4A. In Fig. 4B, the MS/MS spectrum of the peptide eluting at 19.86 min is shown. Fifteen of the 18 predicted ions were detected, allowing for coverage of all 10 amino acids of this peptide. In Fig. 4C, the MS/MS spectrum for the peak denoted (d) eluting at 21.3 min is shown. Despite the large molecular mass of this peptide (2511 Da), 50% of all the predicted fragment ions were detected in the singly, doubly, or triply charged state, allowing for 20/22 amino acids to be sequenced. Last, Fig. 4D shows the MS/MS spectrum of the peptide eluting at 21.6 min. For this peptide, 23/34 ions were detected in both the singly and doubly charged states allowing for 15 of the 18 amino acids to be sequenced.

Previously, the mass spectrometric analysis of rat liver CPT-I had not been performed. With our approach we were able to obtain 61% sequence coverage of this protein using ESI-MS/MS data acquired on the linear quadrupole ion trap instrument and 74% coverage using a mass fingerprint analysis of the MALDI-TOF data. It is also important to note the peptides sequenced in Fig. 4 are exclusive to the liver isoform of rat CPT-I. For each of the peptides shown, 3/10 (30%), 7/22 (32%), and 8/18 (44%) amino acids are unique to the liver isoform. In these mass spectrometry experiments, no data indicated the presence of the muscle isoform of CPT-I.

In addition to the mass fingerprint of CPT-I, the MALDI-TOF MS data also identified three potential sites of phosphorylation. These potential sites are listed in Table I. In the mass
spectrum, there is a peak at \( m/z \) 3021 Da that matches the molecular weight of the singly phosphorylated peptide, \(^{149}\)IW-MAMVKVLSGRPFKMLYSFQTSLPR\(^{173}\). A second potential phosphopeptide was detected at \( m/z \) 761 Da, corresponding to the monophosphorylated peptide \(^{97}\)MSSQTR\(^{102}\). Last, the ions detected at \( m/z \) 1322 Da could represent the doubly phosphorylated peptide \(^{740}\)FSSPETDSHR\(^{752}\). In addition, there were several other peaks in the mass spectrum that could represent peptides from these three regions differing only in the number of missed cleavage sites (Table I). For example, the peak at 1322 Da was assigned to a peptide that contains two phosphate groups and no missed cleavage sites. Also found in the MALDI-TOF mass spectrum was a peak at 1654 Da that could represent the diphosphopeptide \(^{740}\)FSSPETDSHRFGK\(^{752}\) with one missed cleavage site and the peak at 2061 Da representing the diphosphopeptide \(^{740}\)FSSPETDSHRFGKHLR\(^{755}\) with two
missed cleavage sites. The non-phosphorylated peptides were detected at 1495 Da containing one missed cleavage site and at 1162 Da with no missed cleavage sites. The diphosphopeptide in the C-terminal region (residues 740–749) of the protein was of particular interest as it could be phosphorylated in vitro by protein kinase CKII (see below).

Fig. 3. Positive ion MALDI-TOF mass spectrum of the tryptic digest of the CPT-I protein isolated from rat liver mitochondrial outer membranes prepared in the presence of PPIs. 74% coverage was obtained using a mass fingerprint analysis.

Fig. 4. A, total ion current chromatogram of the tryptic digest of the CPT-I protein isolated from rat liver mitochondrial outer membranes prepared in the presence of PPIs. B, MS/MS spectrum of the ion at \( m/z \) 577.6. C, MS/MS spectrum of the ion at \( m/z \) 837.8. D, MS/MS spectrum of the ion at \( m/z \) 1063.4. B–D, the sequence of the peptide is shown along with mass of the protonated ion detected in the MALDI experiment and the \( m/z \) value of the precursor ion. In the MS/MS spectra, peaks representing the b ions are in red, y ions are in blue, doubly charged b ions are in purple, and doubly charged y ions are in green.
Additional in vitro experiments using isolated rat liver mitochondrial outer membranes (isolated in the absence of PPIs and dephosphorylated by incubation with a soluble mitochondrial matrix fraction prepared from frozen/thawed rat liver mitochondria), commercially available protein kinases, and \[^{32}P\]ATP were carried out to identify the protein kinase(s) involved in phosphorylation in CPT-I. The protein kinases, protein kinase A, protein kinase C, CaMII, and CKII, were chosen based on the consensus sequences present in the identified phosphopeptides. In addition, AMPK was selected because it plays a key role in regulation of fuel selection for energy production.

Using freshly isolated rat liver mitochondrial outer membranes, we observed that rat liver mitochondria prepared in the absence of PPIs did not contain phosphoserine. Because these mitochondria had been frozen before analysis, we reasoned that the disruption of the mitochondrial membrane system by freezing releases matrix containing phosphoprotein phosphatases, as has been shown by Signorile (30). The release of these phosphoprotein phosphatases causes the dephosphorylation of CPT-I. Thus, we exposed crude rat liver mitochondrial outer membranes to mitochondrial matrix fraction (prepared as a high speed supernatant from frozen mitoplasts) and then completed the isolation using a discontinuous sucrose gradient. CPT-I isolated from these outer membranes did not contain identifiable phosphopeptides by MALDI-TOF mass fingerprinting.

![Fig. 5. Phosphorylation of rat liver mitochondrial outer membranes. A, \[^{32}P\] incorporation into rat liver mitochondrial outer membrane proteins in the absence of exogenous protein kinase (lane 1) and in the presence of protein kinase CKII (lane 2), CaMII (lane 3), protein kinase C (lane 4), AMPK (lane 5) and protein kinase A (lane 6) and \[^{32}P\]ATP. B, for comparison the same membranes were probed with affinity-purified CPT-I antibodies. Lane MW contains prestained molecular weight markers.](image URI)

### Table I

| Fragment | Sequence | Measured mass (Da) | Expected mass (Da) | Deviation (Da) | Number of Phosphates | Localization of fragment | Consensus site |
|----------|----------|--------------------|--------------------|---------------|---------------------|-------------------------|--------------|
| TLDYTGRMSSQTK | 90-102 | 1585.5 | 1585.6 | -0.1 | 2 | Intermembrane | CaMII, PKA |
| MSSQTK | 97-102 | 761.0 | 761.2 | -0.2 | 1 | Intermembrane | CaMII, PKA |
| STKLVAMVKVLGR | 146-160 | 1866.9 | 1866.8 | +0.1 | 2 | Cytosol, (C-term) | PKC, CKI |
| IWMVKVLKGRPMYSLFQTSR | 149-173 | 3019.7 | 3019.5 | +0.2 | 1 | Cytosol, (C-term) | PKC, CKI |
| FSSEPDSHR | 740-749 | 1322.3 | 1322.4 | -0.1 | 2 | Cytosol, (C-term) | CKI/CKII, PKC |
| FSSEPDSHRFGK | 740-749 | 1654.5 | 1654.6 | -0.1 | 2 | Cytosol, (C-term) | CKI/CKII, PKC |
| FSSEPDSHRFGKHLR | 740-755 | 2060.9 | 2060.9 | +0.2 | 2 | Cytosol, (C-term) | CKI/CKII, PKC |

![Fig. 5. Phosphorylation of rat liver mitochondrial outer membranes. A, \[^{32}P\] incorporation into rat liver mitochondrial outer membrane proteins in the absence of exogenous protein kinase (lane 1) and in the presence of protein kinase CKII (lane 2), CaMII (lane 3), protein kinase C (lane 4), AMPK (lane 5) and protein kinase A (lane 6) and \[^{32}P\]ATP. B, for comparison the same membranes were probed with affinity-purified CPT-I antibodies. Lane MW contains prestained molecular weight markers.](image URI)
FIG. 6. Mass spectrometric analyses of the tryptic digest of CPT-I. The protein was isolated from dephosphorylated rat liver mitochondrial outer membranes after treatment with protein kinase CKII and unlabelled ATP. A, positive ion MALDI-TOF mass spectrum. 74% coverage was obtained using a mass fingerprint analysis. B, enhanced view of the peak at 1654 Da in the MALDI mass spectrum shown in A. The ions at 1654 Da match the mass of the protonated peptide with two phosphorylated amino acids. Note that this peptide contains one missed cleavage site. C, enhanced view of the peak at 1162 Da in the MALDI mass spectrum of the tryptic CPT-I digest. The ions at 1162 Da match the mass of the protonated peptide. This peptide contains no missed cleavage sites. D, MS/MS spectrum of the CPT-I peptide at 1654 Da shown in B. This peptide, amino acids 740–752, contains two phosphorylation sites and one missed cleavage site. The phosphorylated amino acids are shown in the sequence. E, MS/MS spectrum of the peptide at 1162 Da shown in C. This peptide, amino acids 740–749, contains no phosphorylation and no missed cleavage sites. In D and E, the peaks are labeled using Biemann nomenclature, and doubly charged peaks are denoted with the charge in parentheses.
In the presence of protein kinase A, AMPK, protein kinase C, and CaMII, there is no further incorporation of $^{32}$P into proteins of freshly isolated and dephosphorylated outer membranes. In contrast, in the presence of protein kinase CKII on top of the background labeling, a heavy labeling of an 88-kDa protein with another less intense new $^{32}$P band at 45-kDa band is observed (Fig. 5A). The band at 88 kDa is identified as CPT-IL by immunoblotting with affinity-purified peptide antibodies specific for the liver isoform and is shown for these reactions in Fig. 5B.

The data show that in freshly isolated and dephosphorylated outer membrane, only protein kinase CKII leads to significant incorporation of $^{32}$P into CPT-I. To determine the exact position of the phosphorylated amino acids, a mass spectrometric approach was used. CPT-I was harvested from the dephosphorylated outer membrane. Again, 74% coverage of the protein was obtained using mass fingerprinting analysis. There was no evidence of phosphorylated amino acids in this preparation. These same freshly isolated, dephosphorylated outer membranes were also incubated with protein kinase CKII and unlabelled ATP. CPT-I was harvested as outlined in Fig. 2, and the electroeluted protein was reduced, carboxymethylated, and digested with trypsin. The resulting tryptic digest was examined using MALDI-TOF MS, Fig. 6A. The mass fingerprinting again provided 74% coverage of the protein. Examination of the mass spectrum revealed only one region that could contain phosphorylated amino acids, the C-terminal region of the protein from amino acid 740 to 749 (Table I). The $m/z$ values of the peptides detected were identical to those described above for the native phosphorylated CPT-I shown in Fig. 3. This region is again represented by the diphosphopeptides with differing numbers of missed cleavage sites. Because of the presence of the phosphate groups, the trypsin may be unable to cleave at each of the predicted sites resulting in the formation of phosphopeptides.

### Table 1: Coverage of CPT-I Protein

| Peptide | Coverage (%) |
|---------|--------------|
| CPT-I   | 74%          |

The coverage map for the CPT-I protein from two different preparations. The top sequence is the CPT-I protein isolated in the presence of protein phosphatase inhibitors (labeled +). The bottom sequence is the CPT-I protein isolated from the dephosphorylated CPT-I protein treated with protein kinase CKII and ATP (labeled CK). The regions of the proteins covered by both MALDI mass fingerprinting and ESI-MS/MS are shown in green. The regions covered only by MALDI mass fingerprinting are shown in red. These regions covered only by ESI-MS/MS are displayed in blue. The boxed regions are the transmembrane domains. Using ESI-MS/MS, 61.1% of the amino acids were covered for the CPT+ sample and 51.7% for the CPT-CK sample. Using MALDI-MS/MS, 74.2% of the CPT+ sample is covered and 73.8% of the CPT CK sample is covered. By combining the MALDI-TOF MS and ESI-MS/MS data, 76.7% of the CPT+ protein is covered, and 77.0% of the CPT-CK protein is covered.
with internal arginine or lysine residues. In Fig. 6B, a region of the MALDI-TOF mass spectrum is expanded to show the peak representing the diphosphopeptide containing a missed cleavage site. For comparison, the region of the MALDI mass spectrum with the peak representing the dephosphorylated peptide is shown in Fig. 6C.

Although the MALDI-TOF mass spectrum suggested the phosphorylation of CPT-I, sequence information would provide both confirmation of the phosphorylation and location of the phosphorylated amino acids. Using a quadrupole ion trap equipped with a nanospray source, MS/MS spectra were acquired for ions that could have formed from those peptides detected in the MALDI experiment, shown in Fig. 6B and C. The MS/MS spectrum for the doubly charged ion detected at m/z 828 is shown in Fig. 6D, and the MS/MS spectrum for the ion at m/z 582 is shown in Fig. 6E. Based on the fragment ions detected in the MS/MS spectrum shown in Fig. 6E, the peptide was determined to be FFSSPETDSH (residues 740–749). The peaks in the spectrum are labeled with their corresponding assignments according to Biemann nomenclature for peptides. For clarity, not all peaks are labeled.

When examining the MS/MS spectrum for m/z 828, there are fragment ions detected that are characteristic of a doubly phosphorylated peptide. The fragment ion at m/z 729 represents the diphosphorylated peptide, and the fragment ion at m/z 779 represents the monophosphorylated peptide. Many fragment ions were detected in the MS/MS spectrum allowing for high coverage of the amino acids of the peptide and identification of the phosphorylated amino acids. In the spectrum, the y6 ion with phosphate at m/z 811 and without a phosphate group at m/z 713 are both detected. The y5 ion also is detected at m/z 644. To further confirm the position of a phosphate group, a fragment ion is detected at m/z 461 that represents the internal peptide fragment ion, pSHR (Ser-747). To locate the other phosphorylated amino acid, the high m/z region of the MS/MS spectrum was examined. The fragment ions at m/z 1409 and 1311 are the y12 ions with the loss of one phosphate and two phosphates, respectively. The y11 ion is also detected at m/z 1340 and the y11 ion with the loss of one phosphate is detected at m/z 1242. The mass difference between the fragment ions at 1409 and 1242 is indicative of a phosphoserine residue and indicates phosphorylation at Ser-741. By analysis of the fragment ions detected in the MS/MS spectrum of m/z 828, the peptide is determined to be FpSSPETpSHRFGK (residues 740–752).

To detect the post-translational modifications using mass spectrometry, it is crucial to cover as much of the protein as possible. To demonstrate the sequence coverage of the CPT-I protein in these experiments, coverage maps are shown in Fig. 7 for the CPT-I protein isolated in the presence of PPIs and the dephosphorylated CPT-I protein treated with ATP and protein kinase CKII. The coverage maps in Fig. 7 demonstrate the coverage achieved from the peptide mass fingerprint of the MALDI-TOF MS data and from the ESI-MS/MS data.
Next, we tested whether the phosphorylation of CPT-I by protein kinase CKII alters its kinetic properties. Purified rat liver mitochondrial outer membranes that had been dephosphorylated (see above) were incubated with CKII plus ATP, only ATP, or in the absence of either the kinase or ATP. The data obtained support the hypothesis that covalent modification of CPT-I by phosphorylation occurs and renders malonyl-CoA inhibition of CPT-I from competitive to uncompetitive (Fig. 8A). In addition, the velocity increased about 20–25% under these same conditions (Fig. 8B).

In conclusion, we have demonstrated for the first time that the liver isoform of CPT-I is phosphorylated. Furthermore, phosphorylation at the CKII site in the C-terminal end of CPT-I in vitro leads to decreased malonyl-CoA sensitivity and increased catalytic activity. Thus, phosphorylation of CPT-I provides a new mechanism for control of fatty acid oxidation.

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Janos Kerner, Anne M. Distler, Paul Minkler, William Parland, Scott M. Peterman and Charles L. Hoppel

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