

Subcellular Localization and Regulation of Coenzyme A Synthase*

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Alexander Zhyvoloup†§, Ivan Nemazany†¶, Ganna Panasyuk†¶*, Taras Valovka§, Tim Fenton§, Heike Rebholz§, Mong-Lien Wang§, Richard Foxon§, Valeriy Lyzogubov†‡, Vasylij Usenko†‡, Ramziya Kyyamova†, Olena Gorbenko†, Genadiy Matsuka†, Valeriy Filonenko†, and Ivan T. Gout†§ §§¶¶

From the †Department of Structure and Function of Nucleic Acid, The Institute of Molecular Biology and Genetics, Kyiv 03143, Ukraine, §Ludwig Institute for Cancer Research, London W1W 7BS, United Kingdom, ¶National University "Kyiv-Mohyla Academy," Kyiv 04070, Ukraine, ‡Morphological Laboratory BIONTEC, Dnipropetrovsk 94000, Ukraine, and §§Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, London WC1E 6BT, United Kingdom

CoA synthase mediates the last two steps in the sequence of enzymatic reactions, leading to CoA biosynthesis. We have recently identified cDNA for CoA synthase and demonstrated that it encodes a bifunctional enzyme possessing 4'-phosphopantetheine adenyltransferase and dephospho-CoA kinase activities. Molecular cloning of CoA synthase provided us with necessary tools to study subcellular localization and the regulation of this bifunctional enzyme. Transient expression studies and confocal microscopy allowed us to demonstrate that full-length CoA synthase is associated with the mitochondria, whereas the removal of the N-terminal region relocates the enzyme to the cytosol. In addition, we showed that the N-terminal sequence of CoA synthase (amino acids 1–29) exhibits a hydrophobic profile and targets green fluorescent protein exclusively to mitochondria. Further analysis, involving subcellular fractionation and limited proteolysis, indicated that CoA synthase is localized on the mitochondrial outer membrane. Moreover, we demonstrate for the first time that phosphatidylcholine and phosphatidylethanolamine, which are the main components of the mitochondrial outer membrane, are potent activators of both enzymatic activities of CoA synthase *in vitro*. Taken together, these data provide the evidence that the final stages of CoA biosynthesis take place on mitochondria and the activity of CoA synthase is regulated by phospholipids.

Coenzyme A (CoA) is an essential cofactor in numerous biosynthetic and degradative pathways of cellular metabolism, including the tricarboxylic acid cycle, the synthesis and β -oxidation of fatty acids, and the degradation of amino acids. The biosynthesis of CoA is highly conserved from prokaryotes to eukaryotes and involves five enzymatic steps, which use pan-

tothenate (vitamin B₅), ATP, and cysteine (1, 2). The pathway is initiated by pantothenate kinase, which converts pantothenic acid into 4'-phosphopantothenic acid. The 4'-phosphopantothenoylcysteine synthase and phosphopantothenoylcysteine decarboxylase catalyze the formation of 4'-phospho-pantothenoylcysteine and 4'-phosphopantetheine (4'PP),¹ respectively. The last two steps of CoA biosynthesis are mediated by CoA synthase, which is a bifunctional enzyme, possessing 4'PP adenyltransferase and dephospho-CoA kinase activities. Therefore, CoA synthase mediates coupling of phosphopantetheine with ATP to form dephospho-CoA and the subsequent phosphorylation of the 3'-hydroxyl group to generate CoA.

Biosynthesis of CoA is regulated by various extracellular stimuli, including nutrients, hormones of metabolic homeostasis, and cellular metabolites. Numerous studies have demonstrated that tissue level of CoA is reduced by insulin, glucose, fatty acids, and pyruvate, whereas glucagon and glucocorticoids stimulate CoA biosynthesis (3–6). Changes in the rate of CoA biosynthesis occur with fasting, re-feeding, and several pathological conditions, such as diabetes, Reye syndrome, cancer, vitamin B₁₂ deficiency, and cardiac hypertrophy (7–11). Moreover, the ratio between CoA and its thioester derivatives is important for maintaining cellular homeostasis. It has been demonstrated that starvation and diabetes increase the Acyl-CoA:CoA ratio, which leads to mobilization of fatty acids (2, 12). In addition, the ratio between acyl-CoA and CoA affects the activity of a number of cellular enzymes, including pyruvate dehydrogenase, pyruvate carboxylase, carnitine acetyltransferase, and citrate synthase (13–15).

The analysis of CoA metabolic intermediates in bacteria and mammalian cells demonstrated that both pantothenate and 4'PP could accumulate in significant amounts, indicating the existence of two rate-limiting steps in CoA biosynthesis (16, 17). Studies from several laboratories provided evidence that pantothenate kinase is the main regulatory enzyme in CoA biosynthesis (3, 17). The activity of pantothenate kinase is strongly inhibited in a feedback regulatory mode by CoA and all of its acyl esters (6, 18, 19). So far, very little is known about the regulation of 4'PP adenyltransferase activity of CoA synthase, which seems to be another control point in maintaining

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¶¶ To whom reprint requests should be addressed: The Ludwig Institute for Cancer Research, 91 Riding House St., London W1W 7BS, UK. Tel.: 0044-207-8784088; Fax: 0044-207-8784040; E-mail: ivan@ludwig.ucl.ac.uk.

¹ The abbreviations used are: 4'PP, 4'-phosphopantetheine; EGF, epidermal growth factor; SOD, superoxide dismutase; CoAsy, coenzyme A synthase; VDAC, voltage-dependent anion channel; PPAT, phosphopantetheine adenyltransferase; dPCoAK, dephospho-coenzyme A kinase; EGFP, enhanced green fluorescent protein; MOM, mitochondrial outer membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

CoA homeostasis. No physiological regulators of CoA synthase have been reported so far. However, the regulation of this step may control the re-utilization of 4'PP arising from the turnover of the acyl carrier protein or the degradation of CoA by a phosphodiesterase.

The compartmentalization of the CoA biosynthetic pathway is very important but is not well understood. In baker's yeast, a multienzymatic CoA-synthesizing complex has been isolated and shown to contain all enzymatic activities involved in CoA biosynthesis (20, 21). However, such a complex has not been observed in other organisms. In mammalian cells, the first three enzymes in the pathway of CoA biosynthesis are cytosolic proteins (17, 22, 23). The data on the localization of CoA synthase are somewhat controversial. The 4'PP adenyllyltransferase and dephospho-CoA kinase activities of CoA synthase have been reported in the cytosol (3), the mitochondrial outer membrane (25) and in the mitochondrial matrix (26). Molecular cloning of CoA synthase cDNA has facilitated the task of analyzing subcellular localization of the enzyme and its regulation in response to various extracellular stimuli and metabolic changes (27–29).

In this study, we demonstrate by confocal microscopy and subcellular fractionation that CoA synthase is localized on mitochondria. Furthermore, CoA synthase was mapped more precisely to the mitochondrial outer membrane. Mitochondrial localization of CoA synthase was shown to be mediated by the N-terminal sequence, which targets green fluorescent protein solely to mitochondria. We also provide evidence for the first time that both activities of CoA synthase are activated by phosphatidylcholine and phosphatidylethanolamine. This study demonstrates that the last two steps of CoA biosynthesis occur on the outer mitochondrial membrane and that phospholipids can modulate the activity of CoA synthase.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media and reagents were purchased from Invitrogen; insulin-like growth factor, epidermal growth factor (EGF), phorbol 12-myristate 13-acetate, dephospho-CoA, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol were from Sigma; mitochondrial marker MitoTracker Orange CMT-MRos was from Molecular Probes. All chemicals used in this study were of analytical reagent grade.

Cell Cultures and Antibodies—MCF7 and human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum. The anti-VDAC1, anti-Mn-SOD and anti-Myc monoclonal antibodies (9E-10) were purchased from Santa Cruz Biotechnology. Fluorescein isothiocyanate-conjugated goat anti-mouse antibodies were from Jackson ImmunoResearch Laboratories. Generation of rabbit polyclonal anti-CoAsy antibodies was described previously (27).

Plasmid Construction, Expression and Purification of Recombinant Proteins—Full-length coding sequence of CoAsy was PCR-amplified by Vent polymerase and cloned into pFastBacHTb vector in frame with the His-tag epitope at the N terminus (Invitrogen). Generation of recombinant baculovirus was carried out using Bac-to-Bac baculovirus expression system (Invitrogen). Amplification of recombinant baculovirus, infection of Sf9 cells, and affinity purification of His-CoAsy were performed according to manufacturer's recommendations. To generate mammalian expression constructs, PCR-amplified sequences of full-length CoAsy (amino acids 1–563), Δ TMCAsy (amino acids 29–563), Δ NCoAsy (amino acids 187–563), and dPCoAK (amino acids 349–563) were cloned into pcDNA3.1 vector (Invitrogen) in frame with the C-terminal Myc-tag epitope. An 87-bp cDNA fragment coding for the first 29 amino acids of CoAsy was amplified by PCR and cloned in frame with the 5'-end of the green fluorescent protein (EGFP) cDNA into pEGFP-N1 vector (Clontech). All constructs were verified by DNA sequencing using an ABI 373A automated sequencer.

Immunofluorescence Analysis—NIH3T3 cells, grown on 13-mm coverslips, were transfected with 0.5 μ g of pcDNA 3.1-CoAsy, pcDNA 3.1- Δ TMCAsy, pcDNA 3.1-dPCoAK, or pcDNA 3.1- Δ NCoAsy constructs using PolyFect under recommended conditions (QIAGEN).

Transfected cells were cultured for 24 h and then treated with 100 nM MitoTracker Orange CMTMRos (Molecular Probes) for 15 min before fixation with 4% paraformaldehyde and permeabilization with 0.2% Triton X-100. Primary anti-Myc antibodies (1:1000 dilution) were incubated with fixed cells for 1 h followed by incubation with secondary fluorescein isothiocyanate-anti-mouse antibodies. Transfection of human embryonic kidney 293 cells with pEGFP-N1 and pEGFP-N1-CoAsyN plasmids was carried out using LipofectAMINE according to manufacturer's recommendations (Invitrogen). Fluorescently labeled cells were viewed with a Zeiss LSM510 confocal microscope, and the images were analyzed using the LSM510 image browser software.

Isolation of Mitochondria—Crude heavy and light mitochondrial fractions were obtained by differential centrifugation as recommended in OptiPrep Application Sheets (Axis-Shield PoC AS; www.axis-shield-poc.com/density/dtech.htm). Briefly, MCF-7 cells were collected from two 150-mm plates and resuspended in 2 ml of buffer A (0.25 M sucrose, 1 mM EDTA, and 10 mM HEPES-NaOH, pH 7.4). Cell suspension was homogenized with 15–20 passages through a 25-gauge needle. The homogenate was then centrifuged at $1,000 \times g$ for 10 min. The supernatant was subsequently centrifuged at $3,000 \times g$ for 10 min. The pellet was retained and the supernatant further centrifuged at $17,000 \times g$ for 10 min. The last two pellets were termed heavy and light mitochondrial fractions and used for isopycnic fractionation in an OptiPrep density gradient.

Fractionation of Organelles with Preformed Density Gradient—Fractionation of the light and heavy mitochondrial pellets was performed in a continuous 10–30% OptiPrep (iodixanol) gradient according to manufacturer's recommendations (Axis-Shield PoC AS). Briefly, the gradient was formed by diffusion of 10/20/25/30% steps for 60 min. Heavy and light mitochondrial fractions were re-suspended in 5% OptiPrep, layered on the top of the gradient, and centrifuged in a swinging-bucket rotor at $80,000 \times g$ for 2 h. After unloading the gradient, obtained fractions were frozen in an ethanol/dry ice bath and stored at -80°C .

Limited Proteolysis, Alkaline and Urea Extractions—To determine the sensitivity of CoAsy to protease treatment, a light mitochondrial fraction (100 μ g of proteins) was incubated with 1 μ g of proteinase K for 30 min at room temperature in buffer containing 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, and 2 mM CaCl_2 . The reaction was stopped by adding 2 mM phenylmethylsulfonyl fluoride and boiling in gel loading buffer. The samples were separated by SDS-PAGE and immunoblotted for detection of CoAsy, VDAC1, and Mn-SOD.

The same quantity of a light mitochondrial fraction was extracted with 3 M urea and 0.1 M Na_2CO_3 , pH 11.0, or under neutral conditions as described previously (30). The pellets and soluble fractions obtained in all types of extraction were separated by SDS-PAGE and probed with anti-CoAsy, anti-VDAC1, and anti-Mn-SOD antibodies.

Enzymatic Assays—PPAT and dPCoAK activities were measured as described previously (27). Enzymatic assays were performed in the presence or absence of freshly prepared phospholipid vesicles. All phospholipids were dissolved in chloroform and stored at -80°C in small aliquots. Appropriate amounts of phospholipid solutions were mixed and solvent was vaporized in a SpeedVac at room temperature for 1 h. Lipid vesicles were freshly prepared in $1\times$ assay buffer by vigorous vortexing of mixed phospholipids followed by sonication for 1 min at room temperature. The quality of prepared vesicles was analyzed by phase-contrast microscopy.

RESULTS

The N-terminal Region of CoAsy Possesses a Conserved Hydrophobic Stretch—We and others have recently cloned cDNAs encoding mammalian CoAsy (27–29). Sequence analysis and biochemical studies revealed that CoAsy is a multidomain protein, possessing two enzymatic domains: 4'PP adenyllyltransferase in the middle and dephospho-CoA kinase at the C terminus. No significant homology to known protein domains or motifs was reported for the N-terminal region of CoAsy in previous studies. However, further inspection of CoAsy revealed the presence of a hydrophobic segment, extending from residues 7 to 29 (Fig. 1). It is followed by a long peptide segment that is typically hydrophilic. This finding suggested that the N-terminal sequence of CoAsy might be involved in anchoring the enzyme to a membranous component of the cell.

CoAsy Is Localized on Mitochondria—To analyze the subcellular localization of CoAsy, we employed immunofluorescent-confocal microscopy and subcellular fractionation approaches.

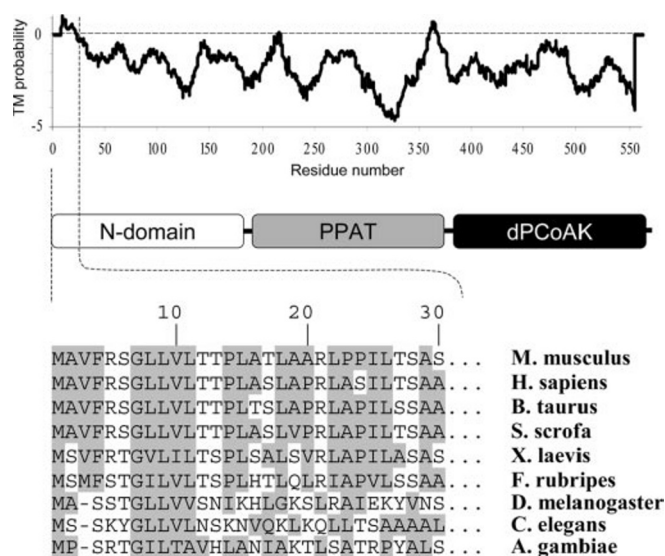


FIG. 1. CoAsy possesses a conserved hydrophobic stretch at the N terminus. The hydrophobicity profile of CoAsy was generated by TMpred program (Refs. 31–33; www.ch.embnet.org/software/TMPRED_form.html). A modular domain structure of CoAsy is represented in the middle. The alignment of the first 30 amino acids of CoA synthases from various organisms is shown below. The hydrophobic residues are shaded.

When NIH3T3 cells were transfected with Myc-tagged CoAsy, the fluorescent signal was localized within discrete structures in the cytoplasm, resembling mitochondria (Fig. 2A). To confirm this, Myc-tagged, CoAsy-transfected cells were treated with MitoTracker 15 min before fixation. As shown in Fig. 2B, the green fluorescent signal corresponding to Myc-tagged CoAsy completely overlaps with the orange fluorescence of MitoTracker. The specificity of the antibody signal was tested by probing NIH3T3 cells transfected with empty vector using both anti-Myc and the fluorescein isothiocyanate-labeled secondary antibodies. NIH3T3 cells expressing the Myc-tagged CoAsy constructs were also probed with secondary antibody alone. In both cases, the fluorescent signal was at background level (data not shown). Mitochondrial localization of Myc-CoAsy was also confirmed in transiently transfected human embryonic kidney 293 cells (data not shown).

A series of CoAsy deletion mutants were constructed to identify the region involved in targeting the enzyme to mitochondria (Fig. 2A). Initially, we generated and tested two truncated mutants: Myc-ΔNCoAsy and Myc-dPCoAK. Transient expression of these constructs in NIH3T3 cells clearly indicated that these deletions yielded proteins that lose mitochondrial localization (Fig. 2B). Based on these data and the prediction analysis, we have created a mutant that lacks 29 amino acids from the N terminus but contains the conserved hydrophobic stretch. When expressed in NIH3T3 cells, this mutant also lacked mitochondrial localization (Fig. 2B). Taken together, these data indicate that CoAsy is localized on mitochondria and that this association is mediated by the N-terminal sequence.

The Extreme N-terminal Sequences Target CoAsy to Mitochondria—Green fluorescent protein has proven to be a powerful and convenient fluorescent tag for monitoring the expression and subcellular localization of genes of interest, protein domains, or specific motifs. We therefore cloned the N-terminal sequence of CoAsy coding the first 29 amino acids into the pEGFP-N1 mammalian expression vector as described under “Experimental Procedures.” Immunofluorescent microscopic analysis showed that cells transfected with control plasmid pEGFP-N1 exhibited a strong green fluorescent signal throughout the cells (Fig. 3). In contrast, cells with pEGFP-

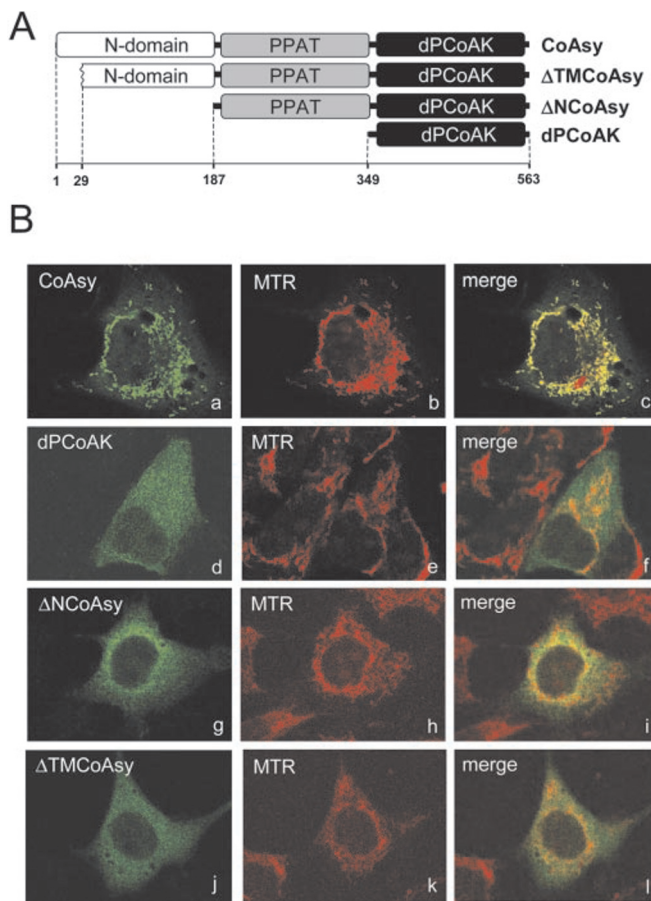


FIG. 2. Mitochondrial localization of CoAsy is mediated by the N-terminal region. A, modular representation of different CoAsy constructs used in this study. The length of deleted mutants is marked below in amino acids. B, NIH3T3 cells were transiently transfected with Myc-tagged CoAsy (a–c), dPCoAK (d–f), ΔNCoAsy (g–i), or ΔTMCoAsy (j–l) plasmids. Cells were incubated with MitoTracker Orange CMTM-Ros (MTR) before being fixed, permeabilized, and processed for immunofluorescence confocal microscopy using the Myc-tag antibody and fluorescein isothiocyanate-anti-mouse secondary antibodies. Yellow in the overlay (merge) images appears where the green fluorescence of Myc-CoAsy colocalizes with the red fluorescence of MitoTracker Orange CMTM-Ros.

CoAsyN construct showed the green fluorescent product CoAsyN-EGFP to be localized to specific sites in the cytosol, resembling mitochondria (Fig. 3). To confirm that these sites correspond to mitochondria, transfected cells were treated with MitoTracker 15 min before fixation. Converging the two images, gave a yellow fluorescent pattern, suggesting the colocalization of both fluorescent signals on the same cellular organelles, namely mitochondria. Based on this finding, we conclude that the extreme N-terminal sequences of CoAsy, corresponding to amino acids 1–29, are responsible for mitochondrial targeting of CoAsy.

CoAsy Cofractionates with Purified Mitochondria—We have also employed the technique of subcellular fractionation to study the localization of CoAsy in MCF-7 cells, which express significant levels of endogenous enzyme (27). The light and heavy mitochondrial pellets were obtained from exponentially growing MCF-7 cells and further fractionated in 10–30% OptiPrep gradient. Unloaded fractions were resolved by SDS-PAGE and immunoblotted with antibodies directed against CoAsy and VDAC1. The results shown in Fig. 4A indicate the presence of CoAsy in the light mitochondrial fraction and that its fractionation pattern is similar to that of VDAC1, which is a specific marker of the mitochondrial outer membrane (34).

FIG. 3. The N-terminal 29 amino acids of CoAsy localize green fluorescent protein exclusively on mitochondria. Human embryonic kidney 293 cells were transiently transfected with pEGFP-N1 vector (*a–c*) or with pEGFP-N1-CoAsyN (*d–f*). Cells were incubated with MitoTracker Orange CMTMRos (*MTR*) before being fixed, permeabilized, and exposed for immunofluorescence-confocal microscopy. The intracellular distribution of green fluorescent protein is given in green (*a, d*), mitochondria are stained in red (*b, e*), the yellow (*c, f*) appears when fluorescence of EGFP and MitoTracker are colocalized.

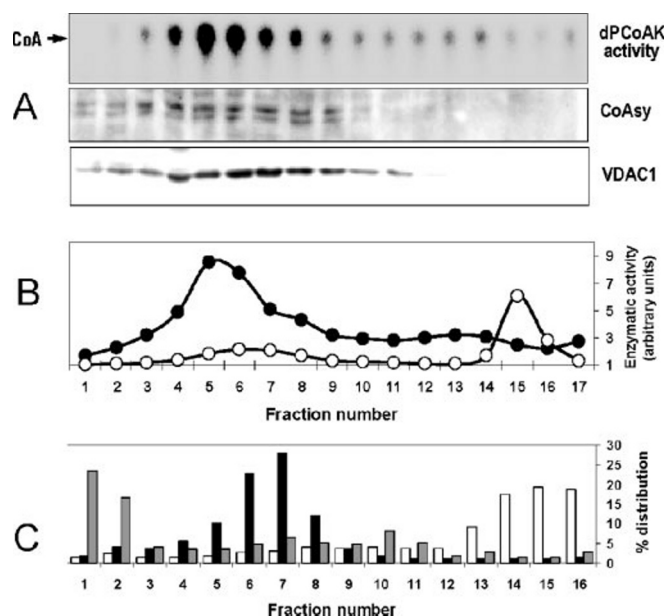
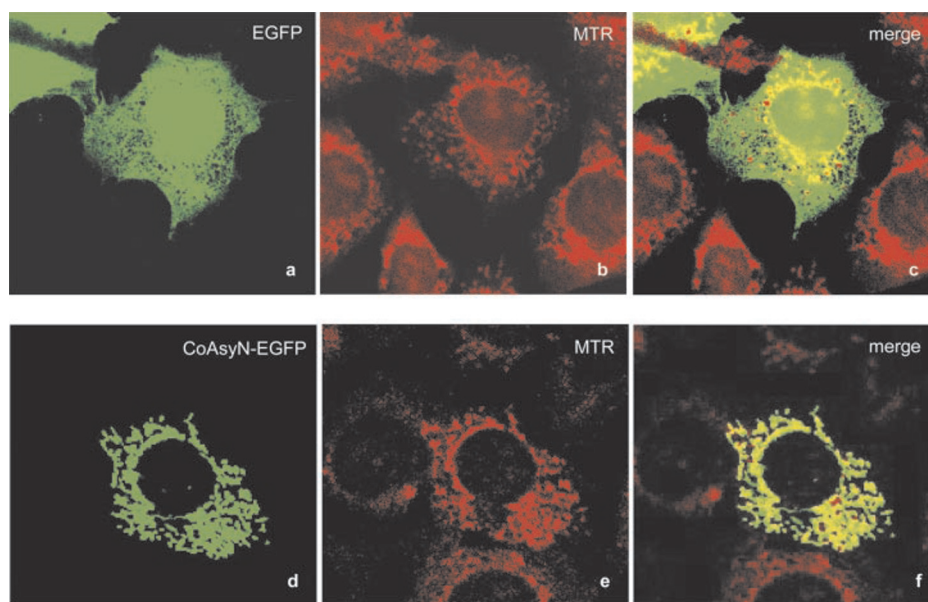


FIG. 4. Subcellular fractionation reveals that endogenous CoAsy is associated with mitochondrial fractions. *A*, CoAsy co-fractionates with VDAC1, a mitochondrial outer membrane protein. A light mitochondrial fraction isolated from MCF-7 cells by differential centrifugation was fractionated in 10–30% OptiPrep gradient. Fractions from the gradient were unloaded and numbered from the top. The dPCoAK activity was measured as described under “Experimental Procedures” using 5 μ l from each fraction. Reaction products were separated by thin layer chromatography and visualized by autoradiography. Equal amounts of indicated fractions were resolved by SDS/PAGE and immunoblotted with antibodies against CoAsy or VDAC1. *B*, CoAsy is not present in fractions containing peroxisomes. Distribution of dPCoAK (●) and catalase (○) activities in fractions from the 10–30% OptiPrepTM gradient. Catalase activity was measured as recommended by OptiPrep manufacturer’s protocol using 25 μ l from each fraction in the assay. *C*, the distribution of organelles from the light mitochondrial pellet in preformed OptiPrep 10–30% gradient according to manufacturer’s protocol. The activities of succinate dehydrogenase (black columns), catalase (white columns), and β -galactosidase (gray columns) correspond to mitochondria, peroxisomes, and lysosomes, respectively.

Furthermore, we analyzed CoAsy and catalase (a marker of peroxisomes) activities in obtained fractions. As shown in Fig. 4*B*, the dPCoAK activity of CoAsy is present in the same fractions as VDAC1, whereas catalase activity appears in frac-

tions that correspond to peroxisomes according to the OptiPrep application manual (Fig. 4*C*). A similar pattern of distribution of CoAsy, VDAC1, and catalase was obtained with the use of the heavy mitochondrial fraction (data not shown). These results demonstrate that native CoAsy co-fractionates with mitochondria.

CoAsy Localizes to the Mitochondrial Outer Membrane—To determine the submitochondrial topology of CoAsy, purified mitochondria were subjected to limited proteolysis and extraction under alkaline conditions and with 3 M urea. To ascertain whether CoAsy is exposed on the mitochondrial surface and is sensitive to proteolysis, purified mitochondrial fractions were treated with proteinase K. The reaction products were resolved by SDS-PAGE and immunoblotted with CoAsy, VDAC1, and Mn-SOD antibodies. As shown in Fig. 5*A*, mitochondria-associated CoAsy is significantly more sensitive to protease treatment than VDAC1, which forms mitochondrial pores and is only partially exposed on the surface.

Extraction under neutral conditions indicated that CoAsy, VDAC1, and Mn-SOD (as a matrix marker) remain associated with mitochondria (Fig. 5*B*). The results of an extraction assay with 3 M urea showed that VDAC1 and Mn-SOD are resistant to this treatment, whereas a small proportion of CoAsy is extracted from mitochondria. When the mitochondrial suspension was treated with 0.1 M Na_2CO_3 , pH 11, the amount of CoAsy associated with mitochondria was significantly reduced, but the amount of VDAC1 or Mn-SOD was not altered. These data suggest that protein-protein interactions may also contribute to the association of CoAsy with the mitochondrial outer membrane (MOM).

Taken together, these results indicate that CoAsy is a membrane-associated protein that is anchored to the MOM through its N-terminal region, exposing both enzymatic domains to the cytosol. In addition, protein-protein interactions seem to be involved in stabilizing the association of CoAsy with the membranes.

Growth Factors and Metabolic Regulators Do Not Alter Subcellular Localization of CoAsy—A number of cellular proteins have been shown to interact with mitochondria in a regulated manner. Therefore, it was interesting to investigate whether growth factors and activators of cellular metabolism can modulate subcellular localization of CoAsy. NIH3T3 cells were transfected with Myc-CoAsy, starved for 24 h in medium without donor calf serum and stimulated with insulin-like growth

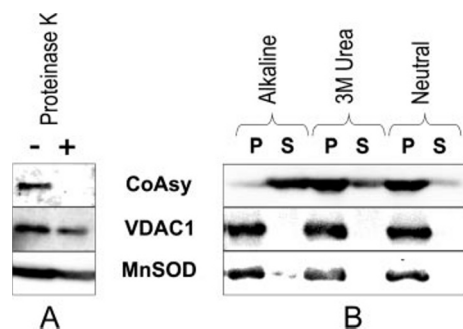


FIG. 5. CoAsy is localized on the outer mitochondrial membrane. A, mitochondria-associated CoAsy is sensitive to protease treatment. The light mitochondrial fraction (100 μ g of proteins) was treated with or without proteinase K for 30 min at room temperature. The samples were resolved by SDS-PAGE and analyzed by immunoblotting. B, sensitivity of CoAsy to extraction with 3 M urea or under alkaline conditions. The light mitochondrial fraction (100 μ g of proteins) was extracted under neutral (isotonic buffer) or alkaline (0.1 M Na_2CO_3 , pH 11) conditions or in the presence of 3 M urea. The insoluble pellet (P) and the supernatant (S) of these extractions were resolved by SDS-PAGE and analyzed by immunoblotting with CoAsy, VDAC1 (mitochondrial outer membrane marker), and Mn-SOD (mitochondrial matrix marker) antibodies.

factor, EGF, phorbol 12-myristate 13-acetate, or donor calf serum. Subcellular localization of Myc-CoAsy was analyzed by confocal-immunofluorescent microscopy using anti-Myc epitope antibodies. The Myc-CoAsy signal was found exclusively on mitochondria, and its localization did not alter in cellular responses to insulin-like growth factor, EGF, donor calf serum, or phorbol 12-myristate 13-acetate (data not shown).

Modulation of CoAsy Activity by Phospholipids—The activity of many membrane or membrane-associated proteins is regulated by phospholipids. Studies of the lipid composition of the MOM in mammalian cells showed that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the main components (35). Considering this, we initially investigated the effect of these two phospholipids on the PPAT and dPCoAK activities of CoAsy. In this study, we used recombinant CoAsy derived from a baculoviral expression system. The data presented in Fig. 6A reveal that both PPAT and dPCoAK activities of CoAsy are strongly activated by PC- and PE-containing vesicles. We observed 21- and 9-fold activation of dPCoAK activity by 10 mM of PE and PC, respectively. The PPAT activity is enhanced ~6- and 2-fold by the same concentration of PE and PC, respectively. The outcome of this study was reproduced in four independent experiments. In addition, we tested the effect of phosphatidylserine and phosphatidylinositol, prepared with 10% PC to increase the stability of the vesicles. Addition of these phospholipids into PC-based vesicles had only a small stimulatory effect on PPAT and dPCoAK activities of CoAsy (data not shown). It is important to note that the activation of CoAsy by phospholipids is concentration dependent. Maximum activation of PPAT activity was observed with 2.5 mM PC/PE, whereas dPCoAK activity did not reach a plateau even with 20 mM PC/PE (Fig. 6B). This finding demonstrates, for the first time, that phospholipids, such as PC and PE, are potent modulators of CoAsy activity *in vitro*.

DISCUSSION

The CoA biosynthetic pathway was deciphered more than 20 years ago. The enzymes mediating the five universal steps in CoA biosynthesis have been purified from many sources and extensively characterized. However, molecular cloning of corresponding cDNAs has been accomplished only recently. The availability of molecular reagents that have evolved from these studies provided the necessary tools for the elucidation of regulatory mechanisms governing CoA biosynthesis. The focus of

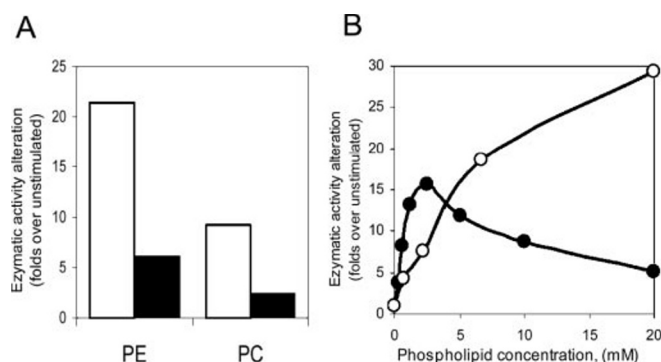


FIG. 6. Activation of CoAsy by phospholipids. A, PC and PE activate both PPAT and dPCoAK activities of CoAsy. Affinity-purified preparations of CoAsy obtained from a baculovirus expression system were used in this study. The assays were performed as described under "Experimental Procedures" using 0.1 μ g of recombinant protein in the presence or absence of 10 mM phospholipids. Reaction products were resolved by paper chromatography. The position of radiolabeled products and enzymatic activity alteration were determined using a filmless autoradiographic analysis system (Bio-Rad). PPAT and dPCoAK activities of CoAsy are given as black and white columns respectively. y-axis value represents a ratio between phospholipid treated and non-treated enzyme activities. B, concentration-dependent stimulation of PPAT and dPCoAK activities of CoAsy by phospholipids. The assay was performed in the same way as described in above, except that vesicles were prepared from a 1:1 mixture of PC and PE. ●, PPAT activities of CoAsy; ○, dPCoAK activities of CoAsy. y-axis value represents a ratio between phospholipid treated and non-treated enzyme activities. x-axis represents the concentration of phospholipids in the reaction mixture.

this study was on the analysis of subcellular localization and regulation of CoAsy, the enzyme that mediates the last two stages of CoA biosynthesis.

The compartmentalization of the CoA biosynthetic machinery has been the subject of many studies. It has been demonstrated that the first three enzymes of the CoA biosynthetic pathway are cytosolic proteins. To date, subcellular localization of CoAsy has not been clearly defined. Both PPAT and dPCoAK activities of CoAsy were reported to be present in the cytosol, mitochondrial outer membrane and mitochondrial matrix (3, 25, 26). The data presented in this study clearly demonstrate that CoAsy is localized on mitochondria. A panel of deletion mutants was used to demonstrate that the conserved hydrophobic stretch at the N terminus anchors CoAsy to mitochondria. Based on protease sensitivity assay and extraction of CoAsy from purified mitochondria, we propose that the N-terminal conserved hydrophobic stretch, and perhaps protein-protein interactions, anchor the enzyme on the mitochondrial outer membrane. Moreover, we provide evidence that CoAsy is not localized on peroxisomes, which are, together with mitochondria, the main stores of cellular CoA.

The biosynthesis of CoA on the MOM may facilitate its transport into mitochondria or the generation of CoA-thioesters. The existence of a specific CoA transporter on the mitochondrial inner membrane has been reported (36). It was shown that the transport of CoA into mitochondria is driven by the membrane potential, which is stimulated by substrates of the tricarboxylic acid cycle, such as succinate, malate, and α -ketoglutarate. It is important to note that the pores on the mitochondrial outer membrane are big enough for CoA to translocate freely into the inter membrane space.

Several CoA- or CoA thioester-modifying enzymes are associated with the MOM. For example, long chain acyl-CoA synthase uses CoA and long chain fatty acids to generate acyl-CoA (37). Production of acyl-CoA is the first step in a series of events leading to the degradation of fatty acids by β -oxidation. Long chain acyl-CoA synthase was found in complex with VDAC (38, 39), and it is possible that anchoring of CoA synthase to the

outer mitochondrial membrane could be facilitated by specific interactions with VDAC or VDAC-associated proteins. Another example of a MOM-associated protein is acetyl-CoA carboxylase 2, which catalyzes the formation of malonyl-CoA from acetyl-CoA (40). Similar to CoA synthase, acetyl-CoA carboxylase 2 is targeted into the MOM in the $\text{NH}_{2\text{in}}\text{--COOH}_{\text{out}}$ orientation via a unique N-terminal hydrophobic region.

So far, sequence determinants necessary for targeting of cellular proteins to the MOM have not been clearly defined. It has been proposed that proteins with a moderately hydrophobic stretch and adjacent positively charged amino acids favor targeting to the MOM (24, 41). In contrast, proteins with highly hydrophobic stretch and without net positive charges within the flanking region are targeted to the ER, the Golgi, and the plasma membranes. We are currently testing by site-directed mutagenesis and confocal microscopy whether these criteria apply for CoAsy.

The regulation of CoA synthase activity is not well understood. No physiological regulators of CoAsy have been identified so far. We demonstrate in this study that PE and PC, the main components of the MOM, are potent activators of CoAsy *in vitro*. We propose that binding of 4'PP to the PPAT domain induces a conformational change, which primes CoAsy for activation by membrane phospholipids. The interaction between CoAsy and phospholipids may remove a conformational constraint on both the PPAT and dPCoAK activities. The region of CoAsy involved in lipid activation remains to be identified. Our preliminary data indicate that sequences within the dPCoAK domain mediate phospholipid-induced activation of CoA synthase.² Overall, these findings indicate that the last two stages of CoA biosynthesis take place on the mitochondrial outer membrane and that the activity of CoA synthase is regulated by phospholipids.

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² A. Zhyvoloup, unpublished observations.