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Molecular Cloning of CoA Synthase

THE MISSING LINK IN CoA BIOSYNTHESIS*[S]

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Coenzyme A functions as a carrier of acetyl and acyl groups in living cells and is essential for numerous biosynthetic, energy-yielding, and degradative metabolic pathways. There are five enzymatic steps in CoA biosynthesis. To date, molecular cloning of enzymes involved in the CoA biosynthetic pathway in mammals has been only reported for pantothenate kinase. In this study, we present cDNA cloning and functional characterization of CoA synthase. It has an open reading frame of 563 aa and encodes a protein of ~60 kDa. Sequence alignments suggested that the protein possesses both phosphopantetheine adenylyltransferase and dephospho-CoA kinase domains. Biochemical assays using wild type recombinant protein confirmed the gene product indeed contained both these enzymatic activities. The presence of intrinsic phosphopantetheine adenylyltransferase activity was further confirmed by site-directed mutagenesis. Therefore, this study describes the first cloning and characterization of a mammalian CoA synthase and confirms this is a bifunctional enzyme containing the last two components of CoA biosynthesis.

Coenzyme A (CoA)[1] is the principal acyl and acetyl group carrier in cells and participates in the metabolism of fatty acids, carbohydrates, and amino acids (1–3). CoA is also involved in the regulation of several key reactions in intermediary metabolism. It is estimated that about 4% of all cellular enzymes utilize CoA or its thioester derivatives as substrates. The biosynthesis of CoA in mammalian cells occurs in five steps, which utilize pantothenate (vitamin B_5), ATP, and cysteine (4). In the first step, pantothenic acid is phosphorylated to 4′-phosphopantothenic acid in a reaction mediated by pantothenate kinase. This is a rate-limiting step in CoA biosynthesis, and the activity of pantothenate kinase is strongly inhibited by coenzyme A and all of its acyl esters (5–7). The product of the first reaction is then converted to 4′-phosphopantothenoylcysteine, which is subsequently decarboxylated to 4′-phosphopantotheine. The 4′-phosphopantothenoylcysteine synthase and phosphopantothenoylcysteine decarboxylase catalyze these two reactions, respectively. Another rate-limiting step in this biosynthetic pathway involves the conversion of 4′-PP to dPCoA by 4′-phosphopantotheine adenylyltransferase. Dephospho-CoA kinase phosphorylates the 3′-hydroxyl group of the ribose ring of dPCoA in the final stage of CoA biosynthesis.

The tissue level of CoA is regulated by various extracellular stimuli, including hormones, nutrients, and cellular metabolites. It has been shown that insulin, glucose, fatty acids, pyruvate, and ketone bodies inhibit CoA biosynthesis, while glucocorticoids and glucagon, as well as drugs such as clofibrate, increase tissue concentration of CoA (8–12). Altered homeostasis of CoA has been observed in diverse disease states, such as diabetes, starvation, alcoholism, Reye syndrome, medium-chain acyl CoA dehydrogenase deficiency, vitamin B_{12} deficiency, hypertension, and certain types of tumors (13–19).

Enzymatic activities responsible for each step of CoA biosynthesis have been purified from various mammalian sources and characterized. However, these studies have not been extended into protein sequence analysis and cDNA cloning of enzymes involved in the pathway of CoA biogenesis. Recently, bioinformatic studies led to the identification and characterization of a gene and a cDNA coding for mammalian pantothenate kinase (20, 21). Here, we report molecular cloning of CoA synthase, which encodes a protein of 563 amino acids. Sequence alignments, mutational analysis, and biochemical characterization indicated that CoAsy possesses two enzymatic domains, which mediate the last two steps in CoA biosynthesis: conversion of 4′-PP into dPCoA and subsequently into CoA.

EXPERIMENTAL PROCEDURES

Cell Cultures and Antibodies—HEK293 cell line was purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Anti-Myc (9E-10) monoclonal antibody was purchased from Santa Cruz. A His-tag fusion protein, containing the C-terminal domain of CoAsy (His-dCoAK) was used to raise specific polyclonal antibodies. Immunoreactive sera were affinity-purified on an Actigel matrix containing His-dPCoAK fusion protein.

Yeast Two-hybrid Screen—The DupLEX-A yeast two-hybrid system was used in this study (OriGENE Technologies). The pEG202/S6Kα "bait" construct was created by standard cloning techniques and used to screen a mouse embryo cDNA library. Autoactivation assay, testing for nuclear localization of the bait fusion protein, selection of positive

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[1] The abbreviations used are: CoA, coenzyme A; CoAsy, CoA synthase; 4′-PPA, 4′-phosphopantothenic acid; PPAT, phosphopantethein adenylyltransferase; 4′-PP, 4′-phosphopantetheine; dPCoA, dephospho-CoA; dPCoAK, dephospho-CoA kinase; NTA, nitrilotriacetic acid; DTT, dithiothreitol; S6K, S6 kinase.

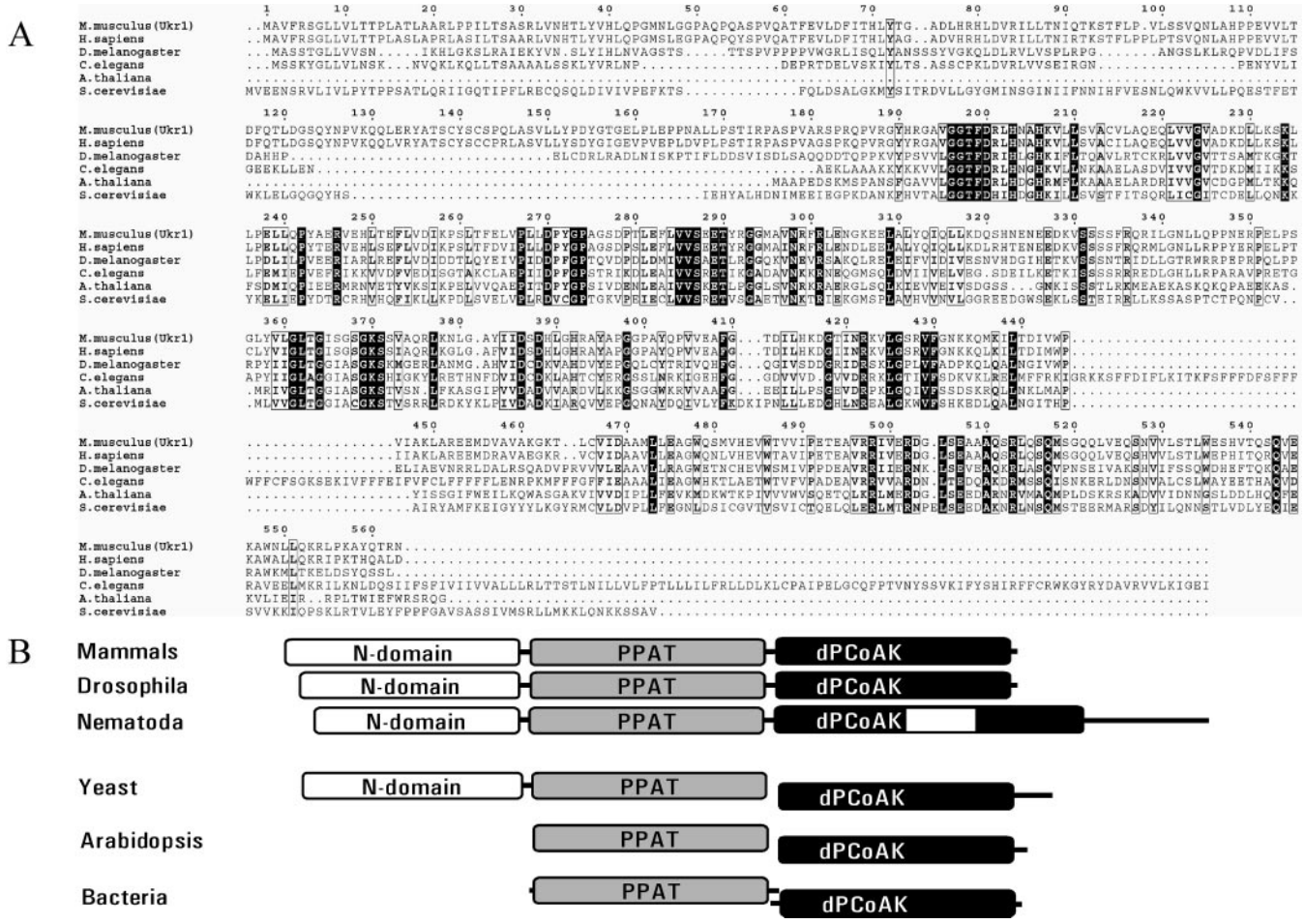

FIG. 1. **Multiple sequence alignments and domain structure of Ukr1.** *A*, alignment of the predicted amino acid sequence of Ukr1 with homologous sequences derived from *H. sapiens*, *D. melanogaster*, *A. thaliana*, and *S. cerevisiae*. Sequences were aligned using ClustalW. The data base sequence numbers are as follows: *H. sapiens* (GenBank™ accession number AF453478), *D. melanogaster* (GenBank™ accession number AAF50749), *C. elegans* (GenBank™ accession number AAK29952), *A. thaliana* (GenBank™ accession numbers AAD15511 and AAD15601) and *S. cerevisiae* (GenBank™ accession numbers NP011793 and NP010482). Identical amino acids are shown on *dark background*, while conserved amino acids are *framed*. *B*, domain organization of Ukr1/CoA synthase and its homologues from various organisms. In *C. elegans*, *A. thaliana*, *S. cerevisiae*, and prokaryotes PPAT and dPCoAK domains reside on different proteins. The hydrophobic insert in *C. elegans* dPCoAK domain is shown as a *white box*. The N-terminal extension is only present in eukaryotes.

clones, and mating assay were performed as recommended by the manufacturer.

Northern and Western Blot Analysis—A mouse tissue mRNA blot (a generous gift from Dr. V. Buchman) was hybridized with an 850-bp cDNA fragment of CoAsy. The probe was labeled using the Readiprime™II random prime labeling kit from Amersham Biosciences. Chicken β-actin cDNA probe was used as a control.

Homogenates of adult rat tissues and cell lines were prepared in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM EDTA, and a mixture of protease inhibitors (Roche Molecular Diagnostics). A portion of total lysate (40 μg) from each tissue and cell line was separated by SDS-PAGE and immunoblotted with affinity-purified anti-CoAsy antibodies.

Plasmid Construction and Expression Studies—Full-length CoAsy was amplified by PCR and cloned into pcDNA3.1 vector (Invitrogen) in frame with the N-terminal Myc-tag epitope. The dPCoAK domain was cloned into pET23d plasmid (Novagene) in-frame with His-tag sequences, located at the N terminus. Expression and affinity purification of His-dPCoAK was carried out in BL21 DE3 cells and on NTA-agarose, respectively. Transient transfection of HEK293 cells was performed using Polyfect under conditions recommended by the manufacturer (Qiagen). Immunoprecipitation assays and Western blot analysis were carried out as described previously (22).

QuikChange site-directed mutagenesis kit (Stratagene) was used to generate a point mutation in the PPAT domain (His^{203} to Ala). All constructs were verified by DNA sequencing.

Analysis of CoA Synthase Activities—The PPAT activity of CoAsy was measured as described below. The immunoprecipitates, containing Myc-CoAsy were mixed with 0.2 mM 4′-PP, 0.25 mM ATP, 0.5 μCi of [γ-^{32}P]ATP, in BB1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1.5 mM DTT). The reaction mixture was incubated at 25 °C for 30 min. Reaction products were separated by descending paper chromatography using Whatman 3MM paper and developing system containing isobutyric acid:0.5 N ammonium hydroxide (100:60) and 1 mM EDTA (23). A phosphoimager system (Bio-Rad) was used to identify the position of radiolabelled products.

Dephospho-CoA kinase activity of immune complexes or recombinant His-dPCoAK was assayed at 25 °C for 30 min in buffer containing 150 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1.5 mM DTT, 0.2 mM dPCoA, 0.25 mM ATP, 0.5 μCi of [γ-^{32}P]ATP. The reaction products were separated and analyzed in the same way as described above for the PPAT assay.

Both PPAT and dPCoAK activities were also measured spectrophotometrically using enzymatic systems as described previously (24).

RESULTS

Using the full-length coding sequence of ribosomal S6 kinase αII (S6KαII) as bait in a yeast two-hybrid screen of a mouse embryo cDNA library, we isolated 16 positive clones, which were confirmed by mating assay. Restriction analysis and DNA sequencing revealed that 13 clones contained the same insert. One of these cDNA clones, designated Ukr1, was fully sequenced and found to have an open reading frame that translated into a protein of 563 amino acids (Fig. 1). The deduced amino acid sequence has a calculated molecular weight of 62,023. Sequence alignments indicated that the central region of Ukr1 shows high homology to phosphopantetheine adenylyl-

transferases, which belong to the superfamily of cytidylyltransferases (Supplementary data, Fig. 1*A*). The C-terminal region is highly homologous to the catalytic domain of dephospho-CoA kinases (Supplementary data, Fig. 1*B*). The N terminus of Ukr1 does not exhibit signatures of any known domains or motifs. The presence of PPAT and dPCoAK domains strongly indicated that Ukr1 cDNA might encode a protein involved in the biosynthesis of CoA. This was an interesting observation, as there is a previous report describing purification of an enzyme from pig liver possessing both PPAT and dPCoAK activities (25). This bi-functional enzyme with a molecular mass of ~57 kDa was termed CoA synthase, as it had the potential to mediate the final stages of CoA biosynthesis. As the protein was never sequenced and the gene was not identified, the hypothesis of a bifunctional CoA synthase was never formally proven. Based on the observation that Ukr1 contained domains predicted to have both PPAT and dPCoAK activities combined with the fact that the Ukr1 gene product has a predicted molecular mass of 62 kDa, we hypothesized that Ukr1 cDNA encoded CoA synthase.

Bioinformatic analysis revealed that homologues of mouse Ukr1/CoAsy are present in *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Saccharomyces cerevisiae* and exhibit 87, 33, 28, 29, and 23% homology at the protein level, respectively (Fig. 1*A*). To our knowledge, none of the gene products of these data base entries have been biochemically characterized nor shown to possess PPAT and/or dPCoAK activities. It is important to note that in prokaryotes and lower eukaryotes PPAT and dPCoAK activities reside on different proteins (Fig. 1*B*). The merger of both enzymatic activities in higher eukaryotes suggests the existence of a novel, more recently evolved mechanism in the regulation of CoA biosynthesis. Moreover, the N-terminal extension is only present in eukaryotes and might contain some regulatory sequences. Interestingly, the *C. elegans* homologue contains a highly hydrophobic insert in the middle of dPCoAK domain and a hydrophobic C-terminal extension.

The expression level of CoAsy in tissues and cell lines was examined by Northern and Western blot analysis. Probing of total RNA from mouse tissues with a radioactively labeled fragment of CoAsy revealed a major transcript of 2.3 kb and two minor bands at around 1.7 and 2 kb (Fig. 2*A*). The highest expression levels were found in kidney and liver, whereas colon, lung, intestine, and spleen showed the lowest levels of transcripts. The probe of chicken β-actin cDNA was used as a control (Fig 2*A*, *lower panel*).

To analyze expression of CoAsy at the protein level, the C-terminal region of CoAsy containing the dPCoAK domain was expressed in bacteria as His-tag fusion protein (His-dPCoAK). Recombinant protein was purified by affinity chromatography on NTA-agarose and used to generate polyclonal antibodies. Affinity-purified antibodies specifically recognized His-dPCoAK and the full-length Myc-CoAsy in Western blotting and immunoprecipitation experiments (Supplementary data, Fig. 2). When rat tissue lysates were immunoblotted with anti-CoAsy antibody, a major immunoreactive band was observed at around 60 kDa, which was slightly lower than recombinant Myc-CoAsy expressed in HEK293 cells. CoAsy expression is high in kidney, liver, and brain, whereas ovaries and lung express low levels of the protein (Fig. 2*B*). There is a good correlation between CoAsy tissue expression patterns at the RNA and protein levels. Immunoblotting also revealed that CoAsy was present in a wide range of cell lines including 3T3-L1 adipocytes and J774.4 macrophages (Fig. 2*C*). Therefore we conclude that CoAsy is a widely distributed enzyme, but particularly significant levels of expression are observed in

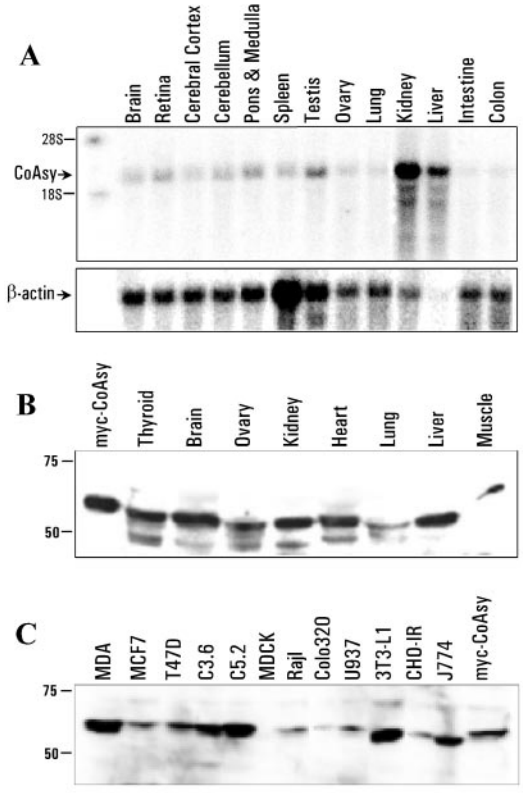

FIG. 2. **Expression of CoAsy in cell lines and tissues.** *A*, Northern blot analysis of CoAsy expression in mouse tissues. Details of the specific probes and blotting conditions are described under "Experimental Procedures." The blot was stripped and reprobed for β-actin expression (*lower panel*). Immunoblot analysis of CoAsy expression in rat tissues (*B*) and various cell lines (C). The antigen-antibody complexes were detected using enhanced chemiluminescence and fluoroimaging system (Bio-Rad).

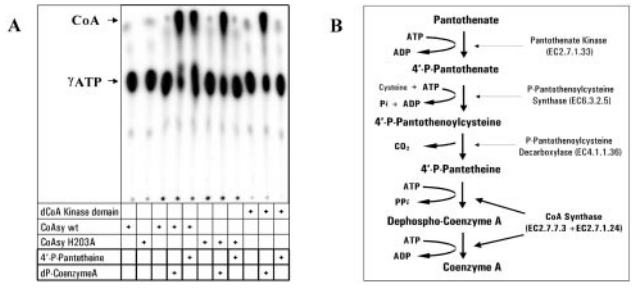

FIG. 3. **CoA synthase mediates the last two steps in CoA biosynthesis.** *A*, Analysis of PPAT and dPCoAK activities of CoAsy. Affinity-purified His-dPCoAK (0.5 μg) or immune complexes containing Myc-CoAsy expressed in HEK293 cells were assayed for PPAT and dPCoAK activities as described under "Experimental Procedures." *B*, schematic presentation of the CoA biosynthetic pathway.

tissues exhibiting elevated lipid metabolism such as heart, liver, and fat.

To determine whether the CoAsy cDNA encodes a protein possessing the predicted enzymatic activities, Myc-tagged CoAsy was expressed in HEK293 cells and recombinant protein immunoprecipitated with anti-Myc antibody. Immune complexes were divided and assayed for PPAT and dPCoAK activities as described under "Experimental Procedures." When dPCoA was used as a substrate, the appearance of radioactively labeled CoA was detected by descending paper chromatography (Fig. 3*A*). Bacterial preparations of His-dPCoAK domain were also tested in this assay and found to possess dPCoAK activity (Fig. 3*A*). To test the presence of PPAT activ-

ity, immunoprecipitated Myc-CoAsy was incubated in a reaction mixture containing 4′-PP as the substrate. To convert 4′-PP to CoA, both the PPAT and dPCoAK activities are required (Fig. 3*B*). The production of radiolabeled CoA in this assay (Fig. 3*A*) confirmed that CoAsy does indeed possess both activities. Commercial preparations of cold CoA and 4′-PP were used as standards. Furthermore, PPAT and dPCoAK activities of CoAsy were also confirmed using enzymatic systems and spectrophotometric analysis as described under "Experimental Procedures" (data not shown).

The crystal structure of bacterial PPAT in complex with 4′-PP has been determined recently (26). The structure allowed the identification of amino acid residues, which are essential for substrate recognition and catalytic activity. Based on these structural studies and mutational analysis of critical amino acid residues in the catalytic pocket of cytidylyltransferases (27), we mutated His^{203} to alanine in the PPAT domain of CoAsy. Transient expression studies in HEK293 cells followed by immunoprecipitation and an *in vitro* PPAT assay unambiguously demonstrated that the Myc-CoAsy-H203A mutant is not capable of generating CoA when 4′-PP is used as a substrate (Fig. 3*A*). At the same time, dPCoAK activity of the Myc-CoAsy-H203A mutant remains unaltered.

Therefore, based on sequence alignments and biochemical characterization, we can conclude that the Ukr1 cDNA encodes the missing link in CoA biosynthesis, the bifunctional enzyme CoA synthase (Fig. 3*B*). Since CoAsy was identified in a yeast two-hybrid screen as an S6K-binding partner, we are currently investigating the specificity of this interaction in mammalian cells and its functional consequences.

DISCUSSION

Vitamin B_5 is a key component in the biosynthesis of CoA. It is readily available from diverse dietary sources, a fact that is underscored by the difficulty encountered in attempting to induce pantothenate deficiency. Vitamin B_5 deficiency has not been linked with any particular disease, but results in generalized malaise clinically. It has been demonstrated that tissue CoA levels are not significantly altered in pantothenate deficiency, suggesting that cells are equipped to preserve their pantothenate content, possibly by a recycling mechanism for utilizing pantothenate obtained from the degradation of pantothenate-containing molecules.

Biosynthesis of CoA is a universal pathway, conserved from prokaryotes to mammals. Multistep biosynthesis of CoA and the enzymes involved in this process are shown in Fig. 3*B*. This study reports the molecular cloning and biochemical characterization of mammalian CoA synthase, previously a gap in the genetics of the mammalian CoA biosynthetic pathway. The presence of PPAT and dPCoAK domains in the predicted amino acid sequence of murine CoAsy strongly suggested its involvement in the biosynthesis of CoA. Expression studies in HEK293 cells provided evidence that recombinant CoAsy possesses both PPAT and dPCoAK activities. These findings were further supported by mutational studies and biochemical analysis of bacterially expressed dPCoAK domain. Interestingly, *D. melanogaster* and *C. elegans* have homologues of mammalian CoAsy, while in lower eukaryotes, such as *S. cerevisiae* and bacteria, the PPAT and dPCoAK activities reside on different proteins. These differences suggest the existence of distinct modes of regulation of CoA biosynthesis. Indeed, the CoA-synthesizing complex in *S. cerevisiae* was found to have a molecular mass around 400 kDa, while in higher eukaryotes, the existence of such a complex has not been reported (23). The compartmentalization of the CoA biosynthetic pathway is poorly understood in mammals. Since mitochondria and peroxisomes contain the greatest concentrations of CoA, it has been proposed that the last enzymes in the pathway are located inside these compartments (28). Others report that mitochondria can transport CoA into the matrix, implying that all CoA synthesizing enzymes are cytosolic proteins (29). Analysis of subcellular localization of CoAsy supports the latter hypothesis, as CoAsy is located predominately in the cytoplasm.[2]

Metabolic labeling experiments have revealed that both pantothenate and 4′-PP accumulate in the cell, suggesting that pantothenate kinase and PPAT catalyze rate-limiting steps in the pathway (30). Therefore, it is apparent that the PPAT activity of CoAsy might be regulated in cellular responses to extracellular stimuli or environmental changes. The availability of the molecular reagents that have evolved from this study will allow us to study regulatory mechanisms governing the final stages of CoA biosynthesis.

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[2] A. Zhyvoloup, unpublished data.

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