Feedback Regulation of Murine Pantothenate Kinase 3 by Coenzyme A and Coenzyme A Thioesters

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Pantothenate kinase catalyzes a key regulatory step in coenzyme A biosynthesis, and there are four mammalian genes that encode isoforms of this enzyme. Pantothenate kinase isoform PanK3 is highly related to the previously characterized PanK1β isoform (79% identical, 91% similar), and these two almost identical proteins are expressed most highly in the same tissues. PanK1β and PanK3 had very similar molecular sizes, oligomeric form, cytoplasmic cellular location, and kinetic constants for ATP and pantothenate. However, these two PanK isoforms possessed distinct regulatory properties. PanK3 was significantly more sensitive to feedback regulation by acetyl-CoA (IC50 1 μM) than PanK1β (IC50 10 μM), and PanK3 was stringently regulated by long-chain acyl-CoA (IC50 2 μM), whereas PanK1β was not. Domain swapping experiments localized the difference in the two proteins to a 48-amino-acid domain, where they are the most divergent. Consistent with these more stringent regulatory properties, metabolic labeling experiments showed that coenzyme A (CoA) levels in cells overexpressing PanK3 were lower than in cells overexpressing an equivalent amount of PanK1β. Thus, the distinct regulatory properties exhibited by the family of the pantothenate kinases allowed the rate of CoA biosynthesis to be controlled by regulatory signals from CoA thioesters involved in different branches of intermediary metabolism.

Pantothenate is the essential precursor for CoA, which is a cofactor for a multitude of metabolic reactions including the oxidation of fatty acids, carbohydrates, pyruvate, lactate, ketone bodies, and amino acids, as well as many synthetic reactions. PanK catalyzes the first committed step and is the rate-controlling enzyme in CoA biosynthesis in bacteria (1) and mammals (2). PanK expression levels define the upper threshold of the cellular CoA content (3, 4), and PanK biochemical activity is feedback-regulated by CoA and/or CoA thioesters (3, 5–8), providing a mechanism to coordinate the rate of CoA synthesis with metabolic demand. Loss of feedback regulation by mutation at a single residue results in runaway CoA production (9). A notable exception to this rule is the PanK from Staphylococcus aureus, which lacks CoA feedback regulation (10), consistent with a role for CoA as the primary thiol in the disulfide redox system in this organism, as well as being the major acyl-group carrier (11–13). In mammals, PanK activity and/or CoA content are altered in response to metabolic state (14–18), insulin (2), glucagon or glucocorticoids (19, 14, 19–22), or diabetes (23, 24). CoA is found in all cellular compartments, but the highest concentrations of CoA exist in mitochondria and peroxisomes (25, 26). Mitochondrial CoA is used as a cofactor in the tricarboxylic acid cycle and fatty acid β-oxidation, and the concentrations of CoA and its thioesters regulate the rates of these processes (27). Peroxisomes play a major role in very long chain fatty acid β-oxidation and also have high concentrations of CoA (28, 29), most of which appears to be bound to matrix components (30).

The discovery of multiple isoforms of PanK encoded by four genes in humans (31–34) and mice (5, 35) suggests a complexity associated with diet-, drug-, or disease-induced responses of PanK expression and activity. All PanK isoforms have a common catalytic core that is defined by the primary structures of PanK1β and PanK3 (see Fig. 1). PanK1α and PanK2 possess amino-terminal extensions to this core, which, in the case of PanK1α, alters the sensitivity of the protein to feedback inhibition (5), and in the case of PanK2, specifies its subcellular localization to the mitochondria (33, 36, 37). PanK4 has a long carboxyl-terminal extension of unknown function. PanK1α and PanK1β arise from alternate initiation exons in the Pan1k gene (5, 32). Human PANK1α and -1β proteins are located in the cytoplasm, and PANK1α mRNA expression and activity are stimulated by bezafibrate administration, resulting in elevated CoA (32). Mouse PanK1α and -1β isoforms are most highly expressed in liver and have distinct regulatory properties and are differentially controlled by CoA and acetyl-CoA (5). The human PANK2 gene encodes a precursor protein that undergoes sequential cleavage at two positions to produce a final product localized to the mitochondria (33, 36, 37). The inherited PanK-associated neurodegenerative disorder was recently linked to mutations in the human PANK2 gene (31). PanK-associated neurodegenerative disorder patients have a pathological accumulation of iron in the basal ganglia and a combination of motor symptoms in the form of dystonia, dystarthis, intellectual impairment, and gait disturbance (38). Interestingly, the mouse knock-out of the Pank2 gene gives rise to several phenotypes including retinal degeneration; however, the iron accumulation and dystonia associated with human PANK2 mutations are not observed (39). Little is known about the PanK3 and PanK4 species beyond the identification of their cDNAs and the RNA expression pattern. The PANK3 gene is most highly expressed in liver, whereas the PANK4 transcript is highest in muscle (31).

The focus of this work is to characterize the biochemical properties of PanK3 in comparison with the known PanK1β isozyme. PanK3 and PanK1β have very highly related primary sequences (see Fig. 1), and northern blot analysis shows that the PanK1 and PanK3 isoforms are most highly expressed in liver (3, 36). On the surface, there is no obvious explanation for why there are two apparently identical proteins expressed in the same tissue. The co-expression of these isoforms in liver suggested that they may play different roles in diet- and drug-induced alterations in CoA levels or in the regulation of intermediary metabolism. Our work revealed that the regulation of PanK3 was dis-
tinctly different from previously characterized isoforms in eukaryotes. PanK3 was significantly more sensitive to feedback regulation by CoA and CoA thiosteres than the other isozymes that are highly expressed in liver, PanK1α, and PanK1β.

**EXPERIMENTAL PROCEDURES**

**Preparation of Pantothenate Kinases**—The mouse PanK1β expression vector was described previously (5). The mouse cDNAs homologous to the human PanK1β (5) and PanK3 proteins (31) were cloned into pcDNA3.1(−) (Invitrogen) to generate plasmids pRC63 and pPJ218, respectively. The two PanK chimeras, namely, PanK3-β3-1β and PanK1β-3-1β, were constructed using overlap extension PCR, in which the sequences encoding the 48-residue region indicated in Fig. 1 were replaced in either PanK1β or PanK3 by the corresponding sequence of the other isoform, yielding plasmids pKM59 and pKM58, respectively. Both strands of the chimeric cDNAs were completely sequenced and checked correct.

The PanK proteins were overexpressed in HEK 293T cells (from Dr. Suzanne Baker, St. Jude Children’s Research Hospital) cultured in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum (Atlanta Biologicals) following transfection with FuGENE 6 according to the manufacturer’s recommendations (Roche Applied Science). Cells were collected 48 h after transfection, washed with phosphate-buffered saline, and resuspended in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM NaF, and 5 μg/ml leupeptin). After incubation on ice for 1 h, cells were lysed by sonication. The lysate was centrifuged at 5,000 × g for 5 min at 4 °C to remove the unbroken cells and nuclei. The supernatant was stored in 50% glycerol at −20 °C. Protein concentrations were determined by the method of Bradford (40) using γ-globulin as a standard.

The whole liver of a 4-month-old male C57BL6/J mouse was harvested and homogenized in 2.5 ml of 20 mM potassium phosphate buffer containing 1 mM ATP. The total homogenate was centrifuged at 20,000 × g for 45 min at 4 °C. The resultant supernatant was dialyzed overnight against the homogenization buffer at 4 °C. The protein concentration in the dialyzed liver lysate was determined by the Bradford assay (40).

**Western Blot Detection of PanK Protein**—A peptide SKDNYKRVT-GTSLGC was synthesized and coupled to keyhole limpet hemocyanin by the Hartwell Center for Biotechnology, St. Jude Children’s Research Hospital, and was sent to Rockland Inc. (Gilbertsville, PA) for raising rabbit polyclonal antiseraum against all PanK isoforms except PanK4. Affinity purification of the polyclonal antibody was performed as described previously (5, 41). HEK 293T cells were transfected and lysed using hypotonic lysis buffer as described above. Aliquots of the crude cell lysate from transfected cells were fractionated by SDS-PAGE on 10% NuPAGE® Novex Bis-Tris gels (Invitrogen). The separated proteins were electroblotted onto a polyvinylidene difluoride membrane, and the PanK isoforms were detected by PanK-specific antibody at a dilution of 1:500 (stock is 1.4 mg/ml), which was incubated with the membrane for 1 h at room temperature. Horseradish peroxidase-labeled anti-rabbit IgG was used as the secondary antibody and was diluted to 1:5,000 prior to incubation with the membrane for 1 h at room temperature. The blots were washed five times with 100 ml of Tris-buffered saline-Tween 20 and developed using the ECF detection reagents according to the manufacturer’s instructions (Amersham Biosciences). The fluorescence signal was detected using a Typhoon 9200 (Amersham Biosciences) and analyzed with ImageQuant 5.2 (Amersham Biosciences).

**Pantothenate Kinase Assays**—Briefly, the standard pantothenate kinase assays (5, 9) contained D-[1-14C]pantothenate (90 μM; specific activity 55 mCi/mmol; Amersham Biosciences), ATP (2.5 mM, pH 7.0), MgCl2 (10 mM), Tris-HCl (0.1 M, pH 7.5), and the indicated amount of cell extract or column fraction containing PanKs in a total volume of 40 μl. The mixture was incubated at 37 °C for 10 min. The reaction was stopped by adding 4 μl of 10% (v/v) acetic acid to the mix, and then 40 μl of the mixture was deposited onto a Whatmann DE81 ion-exchange filter disk that was washed in three changes of 1% acetic acid in 95% ethanol (25 ml/disk) to remove unreacted pantothenate. 4′-Phospho-pantothenate was quantitated by counting each dried disk in 3 ml of scintillation fluid.

**Metabolic Labeling**—HEK 293T cells were transfected with either a control plasmid without an insert (pcDNA3.1) or plasmids expressing either PanK1β (pRC63) or PanK3 (pPJ218) and incubated for 18 h. The medium was then changed to pantothenate-free Dulbecco’s modified Eagle’s medium containing 10% dialyzed calf serum and 2 μM [1-14C]pantothenate. The cells were labeled for 4 or 8 h, harvested, and washed twice with phosphate-buffered saline. The cell pellet was lysed by sonication in 20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 5 mM EDTA, 50 mM NaF. Cell debris was removed by centrifugation and aliquots were removed for determination of protein and total [1-14C]pantothenate incorporated into the cell. The identity of the intracellular metabolites was determined by fractionation of the cell lysates by thin-layer chromatography on Silica Gel H plates developed with butanol:acetic acid:water (5:2:3) using the standards described previously (1). Sections (0.5 cm) were scraped from the plate, and the radioactivity quantitated using a liquid scintillation counter.

**RT-PCR Analysis**—Total RNA was isolated from male mouse liver using TRIzol reagent according to the manufacturer’s instructions. Pelleted RNA was resuspended in nuclease-free water, digested with DNase I to remove any contaminating genomic DNA, aliquoted, and reprecipitated in ethanol and stored at −20 °C. Reverse transcription was performed using SuperScript™ II RNase H− reverse transcriptase (Invitrogen), the RNA template, and random primers to make the corresponding cDNAs. For conventional RT-PCR, multiplex PCR amplification and detection were performed as described previously (42) using isoform-specific primers to confirm the absence or presence of expression. Quantitative real-time PCR of the PanK isoforms was performed using the ABI Prism® 7700 sequence detection system using primers

### Table ONE

| Gene     | Forward primer (5′-3′)                  | Reverse primer (5′-3′)                  | Probe (5′-3′)   |
|----------|----------------------------------------|----------------------------------------|----------------|
| PanK1α   | CTGCGGAGAGGATGAGACT                    | CCACCATATCCATACAAA                     | AACGCCGCATCCA  |
| PanK1β   | GGGGCGTCTAATGACCCCTTTAA               | CCACCATATCCATACAAA                     | TGGCAGAAAGAACCC |
| PanK1    | ATGACTGCGTTCCTCATACAA                 | TGGGACCCCTCCAAATT                     | CCGTATGGAGGGGAC |
| PanK2    | TGGGCGTCTAATGACCCCTTTAA               | TCTCATACATTTGCACGAGAAG                | TGGACCTCCACCAGCAGTAC |
| PanK3    | CTACAGGACCCGCTCATCAT                  | CTTGCGTCACAGGACAGA                    | AAGCTGACGAAACCCGAGC |
| PanK4    | GAAAGCGGCGCTGCACTAA                   | CCGCTCTTCGACCTGATAGA                  | CTTTTCAGAGGTCAGGCTC |

*All probes used the FAM reporter and BHQ1 as quencher.*
and probes listed in TABLE ONE. The Taqman Rodent glyceraldehyde-3-phosphate dehydrogenase control reagent (Applied Biosystems) was the source of the primers and probe for quantitating the control glyceraldehyde-3-phosphate dehydrogenase mRNA. RNA was isolated from three mouse livers, and each RNA sample was quantified in quintuplicate. All of the real-time values were compared using the CT method, in which the amount of target RNA ($2^{-\Delta CT}$) was normalized to the PanK1α reference ($\Delta C_T$), which was set as the calibrator at 1.0.

**RESULTS**

**Expression and Activity of PanK3—PanK1β and PanK3 are highly related proteins encoded by separate genes. These two enzymes are 79.2% identical and 91.4% similar (Fig. 1). The predicted monomer molecular sizes are 41.6 kDa for PanK1β and 41.1 kDa for PanK3. The two full-length cDNAs were cloned into the pcDNA3.1(—) expression vector and transfected into 293T cells. Both proteins were nearly identical under the standard assay conditions. These comparisons were facilitated by immunoblotting with the anti-pantothenate kinase antibody, which recognizes an epitope common to both PanK3 and PanK1β. PanK activity was normalized to the amount of PanK protein expression based on the quantification of the western blot signal as described under “Experimental Procedures.”**

**Kinetic Constants and Oligomeric State of PanK3 and PanK1β—**One possible difference in these two proteins may be their fundamental kinetic constants for ATP and pantothenate; therefore, we determined these values for both of the proteins. PanK3 had a $K_{m}$ for ATP of 112 ± 27 µM and a $K_{m}$ for pantothenate of 9.5 ± 1 µM. Under the same experimental conditions, PanK1β exhibited a $K_{m}$ for ATP of 87 ± 31 µM and a $K_{m}$ for pantothenate of 5.7 ± 0.5 µM. These data illustrated that these fundamental catalytic properties of the two enzymes were very similar.

We also examined the Stokes radii of the two PanK isoforms to determine whether there was a difference in their oligomeric states (Fig. 2A). Although the data do not provide unequivocal information on the multimeric state of these enzymes, the other pantothenate kinases are dimers (6, 10, 36), and this appears to be the most common functional state. These values for both of the proteins. PanK3 had a $K_{m}$ for pantothenate of 5.7 µM and a $K_{m}$ for ATP of 112 µM. These data illustrated that these fundamental catalytic properties of the two enzymes were very similar.

**Feedback Regulation of PanK3—**A hallmark feature of pantothenate kinases is that they are negatively regulated by CoA and/or its thioesters. Therefore, we performed a series of experiments with different CoA thioesters to determine whether PanK3 and PanK1β differed in their regulatory properties (Fig. 3). PanK1β was inhibited by acetyl-CoA ($IC_{50} = 10 \mu M$) and modestly by malonyl-CoA ($IC_{50} > 100 \mu M$) (Fig. 3A). In contrast, PanK1β was refractory to feedback inhibition by both CoA and palmitoyl-CoA. The regulatory properties of PanK3 were clearly different (Fig. 3B). PanK3 was more sensitive to acetyl-CoA ($IC_{50} = 1 \mu M$) and malonyl-CoA ($IC_{50} = 8 \mu M$) and was modestly inhibited by free CoA ($IC_{50} > 100 \mu M$). The most striking difference between the two proteins was the stringent regulation of PanK3 by palmitoyl-CoA ($IC_{50} = 2 \mu M$), which was in sharp contrast to the inability of palmitoyl-CoA to affect the activity of PanK1β.

**Expression of PanK3 and PanK1 mRNA in Mouse Liver—**Northern blot analysis of mouse tissues showed that both PanK3 and PanK1 were most abundantly expressed in both liver and kidney (3, 31). Thus, these two isoforms were most abundantly expressed in the same tissues, but the available data do not provide information about the relative levels of PanK1 and PanK3 expression in a single tissue. Liver apparently has the highest expression of both isoforms, and we performed real-time RT-PCR to quantify the levels of the PanK isoform mRNAs in this tissue (Fig. 4A). Clearly, the PanK1α isoform was the most highly expressed in male mouse liver. PanK1β and PanK3 were present at approximately the same level and were half as abundant as PanK1α. These data suggested that about 25–30% of the pantothenate kinase protein in liver cytosol was PanK3 and 70–75% was PanK1α + PanK1β and that the PanK1α:PanK1β:PanK3 ratio was 2:1:1. PanK2 was expressed at about 18% the level of PanK1. The PanK2 isoform is associated with mitochondria (33, 36) and was therefore not located in the same cellular compartment as PanK1 and PanK3. PanK4 was expressed at the lowest level, and the function and location of this isoform are unknown. These data showed that the PanK1α, PanK1β, and PanK3 isoforms were the most abundant PanKs expressed in male mouse liver cytosol.

We took advantage of the fact that PanK3 was refractory to CoA inhibition to design an experiment with mouse liver cytosol to estimate the percentage of total PanK activity attributable to this isoform. CoA was titrated into the PanK assay using mouse liver cytosolic extracts.
until the inhibition of activity was complete (Fig. 4B). Approximately 25% of the total liver PanK activity was refractory to CoA inhibition. Since PanK1α and PanK3 are inhibited by free CoA, the CoA-independent activity was attributed to the amount of PanK1β in the extract. Thus, this biochemical experiment sets the level of PanK1β as 25% of the total PanK, a finding that is consistent with the PanK1β mRNA abundance determined by quantitative RT-PCR (Fig. 4A).

**Control of Cellular CoA Levels by PanK3 Expression**—We performed a steady-state metabolic labeling experiment to determine whether the feedback regulation of PanK3 was also more stringent than PanK1β in vivo. Plasmids expressing either the PanK3 (pPJ218) or PanK1β (pRC63) cDNAs or a control plasmid without an insert (pcDNA3.1(−)) were transfected into 293T cells, and 18 h later, the cultures were labeled with 2 μM [1-14C]pantothenate. Transfected cells were labeled to equilibrium, which was achieved by 4 h. The cells were harvested, and the effect of PanK expression on CoA levels was determined by chromatographic analysis of the intracellular pantothenate-derived metabolites. An example of this analysis at the 8 h time point is shown in Fig. 5. In the control cells, pantothenate was the major intracellular metabolite, with CoA and 4′-phosphopantetheine comprising 8.1 and 5.3% of the pool, respectively (Fig. 5A). This result was anticipated (3) and illustrated that the utilization of pantothenate was the limiting step. Expression of PanK1β led to the elimination of intracellular pantothenate and the accumulation of CoA (73.5%) and 4′-phosphopantetheine (26.5%), illustrating that increased levels of PanK1β drove the accumulation of intracellular CoA (Fig. 5B). The cells transfected with the PanK3 construct had an intermediate metabolic phenotype (Fig. 5C). Pantothenate remained a significant component of the intracellular pool (50.6%), but
CoA was also appreciably elevated to 42.7% of the total pool. When these alterations in pool sizes at 4 and 8 h were averaged and normalized to cellular protein, there were distinctly different metabolite pool sizes in cells expressing the two proteins. PanK1β expression increased the total pantothenate uptake 4-fold, and the intracellular CoA levels increased 33-fold (Fig. 6). Concomitant with the elevation of CoA, 4′-phosphopantetheine increased 24-fold in the PanK1β-transfected cells. In contrast, total pantothenate incorporation into cells expressing PanK3 increased 1.5-fold but only resulted in a 7.6-fold increase in CoA (Fig. 6). The 4′-phosphopantetheine increased a more modest 1.8-fold. Significantly, the levels of intracellular pantothenate in PanK3-transfected cells were the same as in the controls, whereas pantothenate was absent from the PanK1β-transfected cell population. This observation suggested that the total intracellular CoA was limited by pantothenate availability in the PanK1β-expressing cells, but in the PanK3-expressing cells, the CoA content was limited by the activity of PanK3. These marked differences were not due to differences in the level of protein expression as judged by western blot analysis (Fig. 6, inset), but rather, were attributed to the more stringent feedback regulation of the PanK3 isoform.

Structural Determinants of Feedback Regulation—The comparison of the primary sequences of PanK1β and PanK3 indicated that they were most divergent in a 48-residue-amino-acid patch located within the...
amino-terminal domain (Fig. 1). We investigated whether this region contributed to the different regulatory properties of the proteins by generating and analyzing the catalytic and regulatory properties of chimeric proteins, in which the 48-residue region was swapped between the two proteins (Fig. 7). Both chimeras were expressed and had specific activities that were about 50% of their wild-type counterparts. The PanK1β-3-1β chimera gained the ability to be inhibited by palmitoyl CoA (Fig. 7A), whereas the PanK3-1β-3 chimera lost its sensitivity to this regulatory ligand (Fig. 7B). PanK1β and PanK3 also differ significantly in their response to acetyl-CoA (Fig. 3); therefore, we also examined the response of the chimeras to this ligand. The PanK1β-3-1β chimera was more sensitive to acetyl-CoA inhibition than PanK1β (Fig. 7C), and the PanK3-1β-3 chimera became more resistant to acetyl-CoA (Fig. 7D). These data illustrated that the 48-residue domain influenced the ability of both palmitoyl- and acetyl-CoA to block PanK activity.

**DISCUSSION**

The major cytosolic pantothenate kinase isoforms, PanK1α, PanK1β, and PanK3, differ significantly in their sensitivity to feedback inhibition by CoA thioesters, thus allowing CoA biosynthesis to receive regulatory signals from different branches of intermediary metabolism. PanK1β is insensitive to inhibition by CoA and palmitoyl-CoA but is inhibited by
acetyl-CoA (IC₅₀ = 10 μM). PanK1α responds to higher concentrations of CoA (IC₅₀ = 80 μM) and lower concentrations of acetyl-CoA (IC₅₀ = 5 μM) and palmitoyl-CoA (IC₅₀ = 30 μM). PanK3 was more sensitive to CoA thioester regulation than either of the PanK1 isoforms, and acetyl-CoA (IC₅₀ = 1 μM) and palmitoyl-CoA (IC₅₀ = 2 μM) were equipotent regulators. Like PanK1α, PanK3 responded weakly to CoA (IC₅₀ > 100 μM). The significance of the regulatory properties of PanK3 and PanK1β determined in vitro became apparent in vivo, both in cultured cells and in liver. CoA biosynthesis in cells following enforced expression of either of these two isoforms reflected their sensitivities to feedback inhibition. Although PanK3 expression increased the intracellular CoA levels, it was not nearly as effective as the expression of a similar amount of PanK1β, reflecting the in vitro observation that PanK3 activity is more stringently controlled by components of the CoA pool (Figs. 5 and 6). In male liver cytosolic extracts, ~25% of the total PanK activity is refractory to inhibition by CoA (Fig. 4B), corresponding to the relative expression level of PanK1β (Fig. 4A), which is insensitive to CoA inhibition (Fig. 3A). Quantitative RT-PCR analysis of male mouse liver mRNA indicates that the ratio of PanK1α:PanK1β:PanK3 is 2:1:1 (Fig. 4A). PanK1β and PanK3 are nearly identical in primary sequence, are most highly expressed in liver (3, 31), and are located in the same cellular compartment. The different biochemical regulation of PanK1β and PanK3 provided an explanation for their co-existence in the same tissue but also on the nutritional or pharmacological state and perhaps on gender. The composition of the homogeneous growth media used in culture models may also influence the cells to express a constellation of PanK isoforms different from the tissues from which they were derived.

Acknowledgments—We thank Daren Hemingway, Pam Jackson, Karen Miller, and Jina Wang for expert technical assistance.

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