Cloning and Characterization of a Eukaryotic Pantothenate Kinase Gene (panK) from Aspergillus nidulans*

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Pantothenate kinase (PanK) is the key regulatory enzyme in the CoA biosynthetic pathway. The PanK gene from Escherichia coli (coa) has been previously cloned and the enzyme biochemically characterized; highly related genes exist in other prokaryotes. We isolated a PanK cDNA clone from the eukaryotic fungus Aspergillus nidulans by functional complementation of a temperature-sensitive E. coli PanK mutant. The cDNA clone allowed the isolation of the genomic clone and the characterization of the A. nidulans gene designated panK. The panK gene is located on chromosome 3 (linkage group III), is interrupted by three small introns, and is expressed constitutively. The amino acid sequence of A. nidulans PanK (aPanK) predicted a subunit size of 46.9 kDa and bore little resemblance to its bacterial counterpart, whereas a highly related protein was detected in the genome of Saccharomyces cerevisiae. In contrast to E. coli PanK (bPanK), which is regulated by CoA and to a lesser extent by its thioesters, aPanK activity was selectively and potently inhibited by acetyl-CoA. Acetyl-CoA inhibition of aPanK was competitive with respect to ATP. Thus, the eukaryotic PanK has a distinct primary structure and unique regulatory properties that clearly distinguish it from its prokaryotic counterpart.

Pantothenate kinase (PanK)1 (ATP:D-pantothenate 4'-phosphotransferase, EC 2.7.1.33) catalyzes the first committed step in the universal biosynthetic pathway leading to CoA. Phosphopantetheine is metabolized rapidly to CoA (for review, see Ref. 1), which participates as an acyl group carrier in the tricarboxylic acid cycle, fatty acid metabolism, and numerous other reactions of intermediary metabolism (2). The 4'-phosphopantetheine portion of CoA is an essential prosthetic group in a number of enzyme systems including the acyl carrier protein components of bacterial and eukaryotic fatty acid synthases (3), citrate lyase (4), ferrichrome synthetase from Aspergillus quadricinctus (5), and malonate decarboxylase of Malonomonas rubra (6). 4'-Phosphopantetheine is also required for δ-(L-α-aminoacipityl)-L-cysteynly-D-valine synthetase, the first enzyme for penicillin biosynthesis in fungi including Aspergillus nidulans (7). Escherichia coli is capable of de novo pantothenate biosynthesis, and a sodium-dependent permease actively transports pantothenate into the cell in both bacteria (8–10) and mammals (11, 12). However, metabolic labeling experiments in E. coli (13) and rat heart (14, 15) show that the utilization, rather than the supply, of pantothenate controls the rate of CoA biosynthesis. In fact, E. coli produces 15-fold more pantothenate than is required for maintaining the intracellular CoA level (13). This excess pantothenate is excreted into the medium.

E. coli mutants with temperature-sensitive bPanK activity are also temperature-sensitive for CoA biosynthesis and growth (16). The bPanK gene of E. coli (coaA) was cloned by functional complementation and found to be identical to a previously sequenced temperature-sensitive allele called rts (17–19). E. coli bPanK is a homodimer of 36 kDa subunits which exhibits highly positive cooperative ATP binding and utilizes a sequential ordered mechanism with ATP as the leading substrate (20). CoA and its thioesters inhibit bPanK activity by competitive binding to the ATP site (20, 21). Nonesterified CoA is the most potent inhibitor of bPanK in vitro and in vivo, whereas acetyl-CoA is about 20% as effective as CoA (21). CoA and CoA thioesters also inhibit mammalian (14, 22, 23) and plant (24) PanK enzymes. Although both CoA and acetyl-CoA are reported to inhibit these enzymes, in general, acetyl-CoA is more effective than CoA.

The goal of the present study was to extend the molecular and biochemical characterization of PanK to eukaryotic cells. Homologs of E. coli bPanK protein and the coaA gene (accession no. M90071) are clearly detected in the genomes of Hemophilus influenzae (accession no. U32746), Mycobacterium tuberculosis (TIGR gmt7548), Vibrio cholerae (TIGR GVCCS17R), Streptococcus pyogenes (OUACGT Contig282), and Bacillus subtilis (accession no. D84432) using standard search and sequence alignment tools. In contrast, a similar search of the Saccharomyces cerevisiae genome data base did not reveal the presence of a predicted protein with significant sequence similarity to the E. coli bPanK protein or the nucleotide sequence of the coaA gene. Also, sequences related to bPanK could not be

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1 The abbreviations used are: PanK, pantothenate kinase; aPanK, A. nidulans pantothenate kinase; yPanK, Saccharomyces cerevisiae pantothenate kinase; bPanK, Escherichia coli (bacterial) pantothenate kinase; kb, kilobases; PCR, polymerase chain reaction; IPTG, isopropyl-thio-β-D-galactopyranoside.
identified in the mammalian expressed sequence-tagged data base. These results indicated that eukaryotic cells possess a PanK with a distinctly different primary structure and dissimilar regulatory properties compared with its prokaryotic counterpart.

EXPERIMENTAL PROCEDURES

Materials—Sources of supplies were: American Radiolabeled Chemicals, (B-1-14C)phosphopantothenate (specific activity, 55 mCi/mmol); Appligene, pUC18; Bio-Rad, Bradford dye-binding protein assay solution; Boehringer Mannheim, Klenow fragment; Analytech Inc., 250-µm silica Gel H plates; Bio 101, GeneClean II kit; NEN Life Science Products, D-[1-35S]pantothenate (specific activity, 54.5 mCi/mmol) and [32P]dCTP, dATP, dGTP, and Klenow fragment. Southern blots were hybridized with the probe. Blots were washed at 23 °C in 0.1 M sodium citrate. One of the four positive cosmids, W19B06 (34C5), was identified in the mammalian expressed sequence-tagged data base. These results indicated that eukaryotic cells possess a PanK with a distinctly different primary structure and dissimilar regulatory properties compared with its prokaryotic counterpart.

Eukaryotic panK Gene from A. nidulans

Materials—Sources of supplies were: American Radiolabeled Chemicals, (B-1-14C)phosphopantothenate (specific activity, 55 mCi/mmol); Appligene, pUC18; Bio-Rad, Bradford dye-binding protein assay solution; Boehringer Mannheim, Klenow fragment; Analytech Inc., 250-µm silica Gel H plates; Bio 101, GeneClean II kit; NEN Life Science Products, D-[1-35S]pantothenate (specific activity, 54.5 mCi/mmol) and [32P]dCTP, dATP, dGTP, and Klenow fragment. Southern blots were hybridized with the probe. Blots were washed at 23 °C in 0.1 M sodium citrate. One of the four positive cosmids, W19B06 (34C5), was identified in the mammalian expressed sequence-tagged data base. These results indicated that eukaryotic cells possess a PanK with a distinctly different primary structure and dissimilar regulatory properties compared with its prokaryotic counterpart.
the cosmids. The two genomic DNA fragments were separated, purified, and subcloned into pUC18 that had been digested with XbaI. Hybridization of the two genomic DNA fragments with the A. nidulans phagemid ASTA1999 cDNA fragment identified the 5.1-kb clone (plasmid pSTA2000) containing the genomic DNA corresponding to the gene designated panK.

DNA Sequencing—The genomic DNA (pSTA2000) and cDNA (ASTA1999) sequences were determined on both strands by automated DNA sequencing using an Applied Biosystems 373A automated fluorescent sequencing apparatus and a PRISM Ready Reaction dideoxy DNA sequencing using an Applied Biosystems 373A automated fluorescent sequencing apparatus and a PRISM Ready Reaction dideoxy terminator cycle sequencing kit (Applied Biosystems) with primers at a concentration of 10 μM and 0.5 μg of plasmid DNA, both of which were assembled using Sequencher (Gene Codes Corp.). The cDNA sequence of panK was verified independently by automated DNA sequencing at the Molecular Resource Center of St. Jude Children’s Research Hospital.

Expression Analysis— Cultures of the wild-type A. nidulans strain GOS1 (carrying the biotin auxotrophic marker biaI) were grown at 30 °C for 16 h in liquid minimal medium containing 5 mM ammonium tartrate or 10 mM sodium nitrate as sole nitrogen source (27) with or without a final concentration of 1 μg/ml pantothenate. Total RNA was extracted from mycelium as described previously (28) and mRNA prepared using a Quick Prep mRNA purification kit. Northern blot analysis was carried out as described previously (28) using a 2-kb BamHI fragment of pSTA2000.

Subcloning of A. nidulans panK cDNA and Expression of aPanK in E. coli—The panK cDNA gene from Aspergillus was amplified from phagemid ASTA1999, encoding apanK. The forward primer created a novel restriction site for NdeI at the amino-terminal methionine and removed an internal BamHI site (5′-GTGATATGTCGAGCTACTGCTA3′). The reverse primer introduced a BamHI site downstream of the stop codon (5′-AGATCCCGTTGCGCCCTATGCTAT3′). A polymerase chain reaction (PCR) was performed using Advantage cDNA polymerase mix (CLONTECH), and the product was ligated into the TA cloning vector pCR2.1 (Invitrogen). The ligation mixture was transformed into E. coli One Shot cells (Invitrogen). After overnight growth, plasmid was isolated from the ampicillin-resistant population of cells and digested with NdeI and BamHI, and the appropriate fragment was gel purified by QIAquick (Qiagen). The purified fragment was ligated into NdeI and BamHI digested pET-15b (Novagen) treated with calf intestinal alkaline phosphatase. This ligation mixture was used to transform E. coli strain BL21(DE3) (Novagen), and ampicillin-resistant transformants were screened for the correct insertion by PCR.

Construction of bPanK Expression Vector—The E. coli coaA gene encoding bPanK was amplified by PCR from pWS7-13-2. A forward primer (5′-CATATGATATAAAAGATCAAACG-3′) introduced a NdeI site at the first translational start and an additional internal EcoRI site (5′-GAATTCGAGCTACTGCTA3′) introduced an internal BamHI binding site to reduce the occurrence of shorter transcripts. A reverse primer (5′-GGATCCGAGCTATCCGCCTCCGCAAA-3′) added a BamHI site for subcloning. The PCR was performed using the Advantage cDNA polymerase mix (CLONTECH), and the product was ligated into pCR2 (Invitrogen).

The ligation mixture was transformed into One Shot cells (Invitrogen). After overnight growth on ampicillin selection medium, plasmid DNA was isolated from a mixture of cells and digested with NdeI and BamHI. The appropriate fragment was gel purified and isolated by QIAquick (Qiagen). The purified DNA fragment was ligated into NdeI- and BamHI-digested pET-15b (Novagen) treated with calf intestinal alkaline phosphatase. This ligation mixture was used to transform strain BL21(DE3) (Novagen), and transformants were screened for ampicillin resistance and by PCR for the presence of the coaA insert in the plasmid vector. Single colonies were isolated and cultured to mid-log phase, frozen at −70 °C in the presence of 7% dimethyl sulfoxide, and screened for overexpression of bPanK protein by SDS-polyacrylamide gel electrophoresis after IPTG induction. Plasmids were recovered from several clones that overexpressed the His-tagged protein of the appropriate molecular size. These plasmids were each transformed into strain DH5α for subsequent plasmid purification and DNA sequencing.

**Fig. 2.** Expression of the panK gene and analysis of the purified aPanK protein. Panel A, analysis of mRNA expression from the panK gene by Northern blot. mRNA was isolated from A. nidulans cells grown in A. nidulans minimal medium (7) with 5 mM ammonium tartrate as the sole source of nitrogen and containing 1 μg/ml pantothenate. RNA was hybridized with a 32P-labeled probe prepared from the 2-kb BamHI fragment of pSTA2000. Molecular size markers (in kb) were indicated on the left. Panel B, analysis of the purified aPanK His-Tag fusion protein by SDS-gel electrophoresis. The His-Tag aPanK fusion protein was expressed from plasmid pET15b/aPanK and was purified by metal ion chelate affinity chromatography from E. coli cell extracts as described under “Experimental Procedures.” Molecular mass markers (in kDa) are indicated on the right.

**Fig. 3.** Alignment of the predicted amino acid sequences of aPanK, bPanK, and yPanK. The predicted amino acid sequence of aPanK determined in this study was compared with the predicted sequence of the Ydr531w gene (accession no. YDR531W) identified in the S. cerevisiae genome by a sequence similarity search using the aPanK sequence. aPanK is also compared with E. coli bPanK (the product of the coaA gene). Highlighted residues are those that are identical between the aPanK protein and either yPanK or bPanK. The lysine residue highlighted with white on black indicates the location of Lys-101, the ATP binding site on yPanK (20) and its alignment with Lys-141 in aPanK and Lys-85 in yPanK. In the percent identity calculations, similar amino acid groups were defined as follows: P, A, G, S, T; Q, N, E; D; H, K, R; C, V, L, I, M; and F, Y, W.
**RESULTS**

Isolation of a Eukaryotic PanK by Functional Complementation—A cDNA expression library was prepared as described under “Experimental Procedures” from wild-type *A. nidulans*, prototrophic strain G1071. The library was transformed into the *E. coli* strain ts9, which carried a conditionally defective *rts* allele (25) and exhibited poor growth at 37 °C. Selection for growth of the transformants at 37 °C yielded colonies that harbored library phagemids that functionally complemented the defect. Three phagemids were purified and retransformed into either *E. coli* strains ts9 or DV73. Strain DV73 harbored the defective *coaA15* allele, which expressed a temperature-sensitive bPanK (16). The *coaA* and *rts* genes are allelic (17). Phagemid designated λSTA1999 complemented the temperature-sensitive growth defect in both bacterial mutants on rich medium, albeit yielding slightly smaller colony diameters than the control colonies arising from transformation with the positive control plasmid, pWS7-13-2, expressing the *E. coli* *coaA* gene. Limited growth of strain DV73 (*coaA15* (Ts)) on rich medium at 42 °C is often observed because of a large preexisting CoA pool coupled with the high level of amino acid supplementation (16, 30). Therefore, transformation of strain DV73 with phagemid λSTA1999 was repeated, and ampicillin-resistant colonies were selected at the permissive temperature, 30 °C. Subsequently, 48 colonies were scored for the temperature-dependent growth phenotype on glucose-minimal medium at 42 °C. All 48 colonies grew at the nonpermissive temperature, verifying that complementation was not caused by reversion of the host strain phenotype. These data clearly indicated that the *A. nidulans* cDNA expressed from phagemid λSTA1999 encoded the functional equivalent of an active pantothenate kinase.

The *Aspergillus* cDNA insert in phagemid λSTA1999 was used to screen a bank of genomic clones representing *A. nidulans* chromosomes I–VIII obtained from the Fungal Genetic Stock Center as described under “Experimental Procedures.” Genomic DNA was blotted onto membranes and hybridized with a **P**-labeled probe derived from the 450-bp pair *KpnI* / *SpeI* fragment of phagemid λSTA1999. Positive cosmids from the first screen were identified as W19B06, W21A12, W21H08, W23E02, W23D11, W24H12, and W24H03 from chromosome III. Cosmid W19B06 (34C5) was digested with a panel of restriction enzymes, and the fragments were separated by agarose gel electrophoresis and blotted onto membranes. Hybridization with the 450-bp probe λSTA1999 probe signaled a region of the gel containing a 5.0/5.1-kb doublet from an *XbaI* digest. Purification of the two DNA bands and reprobing identified the 5.1-kb fragment as containing the genomic sequences for the cDNA insertion in phagemid λSTA1999. The gene for *A. nidulans* pantothenate kinase (aPanK) was designated *panK*, and the genomic fragment containing this gene was subcloned to yield plasmid pSTA2000.

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**TABLE I**

| Purification step | Volume | Protein content | Activity | Specific activity | Yield | Purification |
|------------------|--------|----------------|----------|------------------|-------|--------------|
| Lysate           | 47 m   | 130.7 m        | 36.5     | 0.279            | 100   | 1            |
| Eluate           | 50 m   | 10.6 m         | 15.9     | 1.507            | 44    | 5.4          |

*1 unit = μmol/min.*

**TABLE II**

| NTP | % activity |
|-----|------------|
| ATP | 100.0 *a* |
| GTP | 72.4       |
| CTP | 60.6       |
| TTP | 36.0       |

*1 mM NTP plus 2.5 mM Mg<sup>2+</sup>.

**TABLE III**

| Divalent cation | % activity |
|-----------------|------------|
| Magnesium (MgCl<sub>2</sub>·6H<sub>2</sub>O) | 100.0 <sup>b</sup> |
| Calcium (CaCl<sub>2</sub>·2H<sub>2</sub>O) | 1.2       |
| Cobalt (CoCl<sub>2</sub>·6H<sub>2</sub>O) | 31.3      |
| Manganese (MnCl<sub>2</sub>·4H<sub>2</sub>O) | 47.7      |
| Zinc (ZnCl<sub>2</sub>) | 2.1       |

*400 μM cation plus 400 μM ATP.

*0.6 μmol/min/mg.*
Fig. 4. Kinetic characterization of aPanK. Homogeneous aPanK was assayed for activity with various concentrations of pantothenate (4–62.5 μM) in the presence of 20 (●), 80 (○), or 320 (□) μM ATP (panel A). aPanK was also assayed for activity with various concentrations of ATP (20–320 μM) in the presence of 16 (●), 32 (○), or 64 (□) μM pantothenate (panel B). Double reciprocal plots of the initial velocities indicate a $K_m$ of 60 μM for pantothenate and a $K_m$ of 145 μM for ATP at saturating concentrations of the second substrate.

Fig. 5. Kinetic analysis of inhibition by acetyl-CoA. Homogeneous aPanK was assayed for activity with various concentrations of pantothenate (4–62.5 μM) without inhibitor (○) or in the presence of 20 (●), 40 (□), or 80 (■) μM acetyl-CoA (panel A). aPanK was also assayed for activity with various concentrations of ATP (20–320 μM) without inhibitor (○) or in the presence of 5 (●), 16 (□), or 32 (■) μM acetyl-CoA (panel B). Double reciprocal plots indicated that inhibition of aPanK by acetyl-CoA was noncompetitive with respect to pantothenate and competitive with respect to ATP.

Structure and Expression of the panK Gene—The DNA sequence of the panK cDNA and a 2115-base pair stretch of plasmid pSTA2000 encompassing the panK gene was determined on both strands (Fig. 1). The panK gene contained a single open reading frame interrupted by three short introns typical of those found in fungi (31). The positions of the introns were confirmed by comparison of the genomic and cDNA sequences. Although we have not directly determined the amino-terminal protein sequence for aPanK, the putative translational start is the first methionine codon of the open reading frame. There was an in-frame stop codon located 7 codons upstream of the predicted methionine start codon. The size of the expressed aPanK protein (see below) confirms this Met as the start site as the next methionine residue is located 154 amino acids downstream. The site of polyadenylation is approximately 508 nucleotides downstream from the translational stop codon and is indicated by the arrow in Fig. 1. However, the precise polyadenylation acceptor could not be determined simply by comparison of the genomic and cDNA sequences because there is a stretch of 6 A nucleotides in the genomic sequence at this point. An AATAAA polyadenylation signal is located 21 nucleotides upstream from the proposed polyadenylation site.

Northern blot analysis of mRNA prepared from A. nidulans indicated that the panK gene was transcribed as a single mRNA (Fig. 2A). The apparent size of the panK transcript, 1.85 kb, was consistent with the size predicted from the analysis of the genomic and cDNA sequences (Fig. 1). The transcript occurred in approximately the same abundance in cells grown with either ammonium or nitrate as the nitrogen source, in the presence or absence of pantothenate, and the level of panK transcript was much lower that that of A. nidulans actin (data not shown).

Comparison of the Protein Sequences of aPanK, yPanK, and bPanK—The predicted protein sequence of aPanK consisted of 420 amino acids with a predicted molecular mass of 46.9 kDa. This sequence was used to perform a similarity search against the S. cerevisiae genome data base. This search identified a predicted open reading frame (Ydr531w) that consisted of 367 amino acid residues with a predicted molecular mass of 40.9 kDa. The Ydr531w open reading frame was 44.8% identical and 60.2% similar to the aPanK sequence (Fig. 3). Based on this strong similarity the Ydr531w open reading frame is predicted to encode the yeast pantothenate kinase (yPanK). The major difference between aPanK and yPanK was in the amino-terminal domain, which was significantly longer in aPanK. We detected only a single PanK isoform in the S. cerevisiae genome.

The similarity between aPanK and the previously described bPanK from E. coli was far less striking (Fig. 3). bPanK is a protein composed of 316 amino acids with a molecular mass of 36.4 kDa. bPanK was 16.2% identical and 32.9% similar to aPanK. These calculations were based on the introduction of several significant gaps in the bPanK sequence to align the smaller bacterial protein with the larger aPanK. Nonetheless, the comparison between bPanK and aPanK/yPanK points to the location of the ATP binding site in aPanK/yPanK. Lysine 101 is a critical residue in bPanK required for the binding of both the ATP substrate and the CoA regulators to the enzyme (20). The Lys-101 residue in bPanK corresponds to Lys-141 in aPanK and Lys-85 in yPanK as indicated in Fig. 3, suggesting that these lysine residues may be involved in nucleotide binding in the aPanK and yPanK proteins.

The Aspergillus panK Gene Encodes a Functional PanK—The functional complementation by phagemid STA1999 coupled with the cDNA/genomic DNA sequence analysis strongly indicated that the Aspergillus panK gene encoded a functional PanK. This point was tested by assaying extracts from strain...
FIG. 6. Inhibition of aPanK or bPanK activities by acetyl-CoA or CoA. Homogeneous preparations of aPanK (panel A) or bPanK (panel B) were assayed for activity at 1 mM ATP and 45.5 μM (1 μCi of [1-14C]pantothenate) with various concentrations (8–128 μM) of free CoA, acetyl-CoA, or malonyl-CoA as indicated. The specific activity of aPanK was 2.33 μmol/min/mg and of bPanK was 2.25 μmol/min/mg in these assays.

DV73 (coaA15(Ts)) transformed with phagemid λSTA1999 for PanK activity. Because strain DV73 had a temperature-sensitive bPanK (16), extracts from strain DV73 transformed with the empty control plasmid possessed a low background PanK specific activity. Transformation of strain DV73 with phagemid λSTA1999 resulted in a dramatic increase in PanK activity in the soluble fractions of the cells (data not shown). These results were consistent with the complementation studies and demonstrated that the protein expressed by phagemid λSTA1999 was a functional PanK.

Characterization and Kinetic Analysis of aPanK—The biochemical properties of aPanK were examined in more detail after the cloning of the aPanK cDNA into the pET-15b expression vector, expressing the protein in *E. coli* strain BL21, and purifying the protein by affinity chromatography as described under “Experimental Procedures.” The aPanK protein was purified in a single step (Table I), and typically 10–12 mg was obtained from a 500-ml culture of cells (5 × 10^8/ml) in logarithmic growth. The major variable was the efficiency of the IPTG induction step before lysis of the cells. The purified aPanK preparation consisted of a single polypeptide species (Fig. 2B). The apparent subunit molecular mass of the purified protein, 46 kDa, was consistent with the molecular mass, 46.9 kDa, predicted from the cDNA sequence. The specific activity of the purified aPanK ranged from 1.5 to 2.5 μmol/min/mg among several preparations when assayed under the standard conditions described under “Experimental Procedures” using an ATP concentration of 2.5 mM. The in vitro kinase reaction was optimal over a broad pH range; and pH values between 6 and 8.5 supported ≥ 80% of the maximum activity (data not shown). Cofactors were not required for the reaction, and the addition of Zn^{2+} was inhibitory at ≥ 50 μM. Of the other cations tested, the addition of Ca^{2+}, Mg^{2+}, or Mn^{2+} did not affect activity in the standard assay mixture. Other nucleotides could replace ATP somewhat in the assay, with GTP supporting up to 72% of the reaction (Table II). Manganese could replace Mg^{2+} up to 48% (Table III).

The affinity constants of the purified aPanK for the two substrates were determined. The pattern of parallel lines observed in Fig. 4 is characteristic of ping-pong kinetic systems. However, there are examples of kinases that operate by ordered bi-bi systems where the lines seem parallel but really are not (32, 33). This effect arises when the kinase has a high affinity for ATP, but because of the relative values of the other rate constants the apparent K_m is much larger. The same thing is observed when both substrates are varied (Fig. 4). Although the lines appear parallel, the family of plots intersect far to the left of the (velocity) ^{-1} axis and far below the (substrate concentration) ^{-1} axis. The apparent K_m for pantothenate at saturating ATP concentrations was 60 μM (Fig. 4A), and the K_m for ATP at saturating pantothenate concentrations was 145 μM (Fig. 4B).

Regulation of aPanK by CoA and Its Thioesters—PanK activity from different sources has been reported to be inhibited by CoA or CoA thioesters (14, 21–23). We found that acetyl-CoA was the most potent regulator of the aPanK (see Fig. 6A), and we investigated the mode of inhibition of enzyme activity (Fig. 5, A and B). The double reciprocal plot of pantothenate concentration versus kinase activity indicated that acetyl-CoA inhibited the interaction between pantothenate and enzyme in a noncompetitive manner (Fig. 5A). In these experiments, acetyl-CoA at 80 μM reduced pantothenate kinase activity by 66% at 1 mM ATP. In contrast, the interaction of ATP with the enzyme was inhibited in a competitive manner by acetyl-CoA (Fig. 5B). Acetyl-CoA at 32 μM reduced activity by 51% at 50 μM pantothenate. The K_i for acetyl-CoA was calculated as 9 μM from the replot of the slopes from these data.

The kinetic mechanism of inhibition by a CoA species was the same in both the pantothenate kinases from *Aspergillus* and from *E. coli* (20, 21) in that the inhibitor was competitive with respect to the ATP substrate. However, the pantothenate kinases differed in their sensitivities to either free CoA or acetyl-CoA. We found previously that bPanK was inhibited potently by nonesterified CoA (21). Acetyl-CoA and succinyl-CoA inhibited the bacterial enzyme to a much lesser extent, and malonyl-CoA was without effect (21). Acetyl-CoA had been reported to inhibit the mammalian pantothenate kinase activity, whereas free CoA or other CoA thioesters had little or no effect (14, 22, 23). We investigated the regulation of the purified aPanK by CoA and its thioesters and directly compared it with regulation of the bPanK purified from *E. coli* (Fig. 6, A and B). Both enzymes were assayed at 1 mM ATP at CoA concentrations up to 128 μM. Inhibition of aPanK by nonesterified CoA was not evident, whereas acetyl-CoA was very effective (Fig. 6A). On the other hand, bPanK was inhibited most potently by nonesterified CoA and acetyl-CoA was considerably less effective, reducing activity by only 25% at the maximum concentration of inhibitor (Fig. 6B). Malonyl-CoA was not a potent inhibitor of either aPanK or bPanK at 1 mM ATP (Fig. 6, A and B). These data indicated that the ATP binding site of aPanK had a distinctly different structural context that interacted selectively with the acetyl moiety of acetyl-CoA as well as the adenine moiety of either ATP or CoA. The lack of similarity between the primary structures of aPanK and bPanK, particularly at the predicted nucleotide binding site (Fig. 3), was consistent with these results.

**DISCUSSION**

The identification of the protein sequence for a eukaryotic PanK will enable the identification and molecular character-
subcellular compartmentation and metabolism. The eukaryotic version of PanK characterized in this report has a primary structure that bears little resemblance to the previously described enzyme expressed in prokaryotic cells. The similarities between bPanK and aPanK/yPanK are so weak that a definitive identification of the eukaryotic PanK in the S. cerevisiae genome is not possible using standard search algorithms with bPanK as the query. Nonetheless, the eukaryotic aPanK carries out the same reaction as bPanK, and its ability to complement functionally the E. coli mutants expressing a temperature-sensitive bPanK permitted the isolation of the aPanK cDNA. Retrospectively, the bPanK and the aPanK/yPanK sequences do have some similarities revealed by the introduction of significant gaps in the bPanK sequence in its alignment with aPanK (Fig. 3). For example, the critical lysine residue involved in nucleotide binding in bPanK (Lys-101) (20) appears to be conserved in aPanK (Lys-141) and yPanK (Lys-85). However, experimental verification of this point will be essential because the sequences surrounding Lys-101 in bPanK differ significantly from the sequences surrounding the conserved lysine in aPanK/yPanK. Furthermore, the sequence, AVIDIGGS, between residues 69 and 75 of aPanK, is very similar to a sequence in hexokinase I which is postulated to form part of the ATP binding pocket (34). By analogy, this sequence motif may also be part of the ATP site in aPanK, but it is missing from bPanK. The significance of the other very short regions of identity between these two proteins remains obscure.

The dissimilarities in the primary sequence of bPanK and aPanK reflect the distinct differences in the regulatory properties of PanK from E. coli and A. nidulans. CoA and its thioesters inhibit bPanK activity by blocking competitively the lysine in the proposed ATP binding site in aPanK (Fig. 3). The selectivity of aPanK for acetyl-CoA suggests that the ATP binding site is distinctly different from that found in bPanK or other ATP-utilizing enzymes. The amino acids flanking the lysine in the proposed ATP binding site in aPanK (Fig. 3) do not resemble either a type A or a type B nucleotide binding site (40) and may represent a unique ATP binding fold. A comparison of the crystal structure of bPanK with that of aPanK may reveal the residues that determine the specificity for the acetyl moiety as well as enhance our understanding of the divergent structures which catalyze identical enzymatic reactions.

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Cloning and Characterization of a Eukaryotic Pantothenate Kinase Gene (panK) from Aspergillus nidulans

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