Biochemical Properties of Human Pantothenate Kinase 2 Isoforms and Mutations Linked to Pantothenate Kinase-associated Neurodegeneration*

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Yong-Mei Zhang, Charles O. Rock, and Suzanne Jackowski

From the Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105

The PANK2 gene encodes the human pantothenate kinase 2 protein isoforms, and PANK2 mutations are linked to pantothenate kinase-associated neurodegeneration. Two PanK2 protein forms are proteolytically processed to form a mitochondrially localized, mature PanK2. Another isoform arose from a proposed initiation at a leucine codon and was not processed further. The fifth isoform was postulated to arise from an alternative splicing event and was found to encode an inactive protein. Fourteen mutant PanK2 proteins with single amino acid substitutions, associated with either early or late onset disease, were evaluated for activity. The PanK2(G521R), the most frequent mutation in pantothenate kinase-associated neurodegeneration, was devoid of activity and did not fold properly. However, nine of the mutant proteins associated with disease possessed catalytic activities that were indistinguishable from wild type, including the frequently encountered PanK2(T528M) missense mutation. PanK2 was extremely sensitive to feedback inhibition by CoA thioesters (IC50 values between 250 and 500 nm), and the regulation of the active PanK2 mutants was comparable with that of the wild-type protein. Coexpression of the PanK2(G521R) and wild-type PanK2 did not interfere with wild-type enzyme activity, arguing against a dominant negative effect of the PanK2(G521R) mutation in heterozygous patients. These data described the unique biochemical features of the PanK2 isoforms and suggested that catalytic defects may not be the sole cause for the neurodegenerative phenotype.

The inherited PanK2-associated neurodegeneration (PKAN) is an autosomal recessive disease and is linked to mutations in the human PANK2 gene (1). PKAN patients include those with hypoprebetalipoproteinemia, acanthocytosis, retinitis pigmentosa, and pallidal degeneration (2) and constitute a subset of those diagnosed with neurodegeneration with brain iron accumulation, formerly known as Hallervorden-Spatz syndrome (3). PKAN patients have a pathological accumulation of iron in the basal ganglia and a combination of motor symptoms in the form of dystonia, dysarthria, intellectual impairment, and gait disturbance (3). Early onset patients have rapidly progressive disease and later onset patients have a slowly progressive, atypical disease, where parkinsonism is common (3, 4). A cohort of patients with either early or late onset disease was heterozygous with respect to the PANK2 gene, and it has been suggested that a frequent mutation is semidominant (4). A distinct subset of patients was symptomatically indistinguishable yet did not harbor PANK2 mutations (3). Two hypotheses have been proposed to explain the clinical sequence associated with an assumed loss of PanK2 activity: accumulation of cysteine-containing substrates because of the inhibition of CoA synthesis, which may undergo rapid auto-oxidation in the presence of iron, leading to free radical generation and cell damage (1), or mitochondrial CoA deficiency leading to increased oxidative stress from free radicals (5).

PanK catalyzes the first committed step and is the primary rate-controlling enzyme in CoA biosynthesis (6). PanK2, like the other PanK isoforms (7–9), is a dimer and is feedback-regulated by CoA (10). Human PanK2 localizes to mitochondria (5, 10, 11), distinguishing PanK2 from the cytosolic PanK proteins encoded by separate genes (6, 9). The PanK2 protein translated from the most 5′ start site is sequentially cleaved at two sites by the mitochondrial processing peptidase, generating a long-lived 48-kDa mature protein (10). Two shorter PanK2 isoforms have been described, the first one also localizes to mitochondria (5) and the second does not, because of lack of a targeting sequence (11). The PKAN mutations described thus far occur in the coding sequence of the shorter mitochondrial form (1, 4), and it is thought that early onset patients tend to have more severe mutations resulting in truncated PanK2 proteins, whereas the later onset patients would have mutations with amino acid changes that compromise activity (12). Cell lines expressing three of the PanK2 mutant proteins have decreased activity, although cell lines expressing three other mutants appear active (10), raising questions about the relationship between PanK2 activity and the PKAN phenotypes. Disruption of the PanK2 gene in mice does not produce a neurodegenerative phenotype (13), although it does result in retinal degeneration, often linked with the human disease, and azoosperma, which is also associated with reduced PanK activity in Drosophila melanogaster (14). The importance of understanding the phenotypic and genetic heterogeneity of this disorder prompted us to examine the biochemical and regulatory properties of the PanK2 isoforms and a comprehensive set of the most frequently encountered mutant proteins.

EXPERIMENTAL PROCEDURES

Cloning of the Human PanK2—The splice variant of PanK2 (spPanK2) was amplified by PCR from a human brain Marathon-Ready cDNA library (BD Biosciences). The forward primer (spPanK2-F) (Table 1) contained an NbI restriction site, and the reverse primer (PanK2-R) contained an EcoRI restriction site downstream of the stop codon. The PCR product was subcloned into pCR2.1 and sequenced. The NbI- and EcoRI-digested fragment was then ligated into pcDNA3.1(−), and the resultant plasmid was named pKM3 (Table 2). The full-length PanK2 (pPanK2) was assembled from two ESTs of the...
human cDNA obtained from ATCC, EST 603204447F1 (Image clone ID 5270218; GenBank™ accession number BI643406) and EST 603241690F1 (Image clone ID 5284174; GenBank™ accession number BI544308). The iPanK2 constructed for this work was based on the predicted cleavage site of pPanK2 at position 44 (11). After our work was completed, the actual cleavage site was determined to be at amino acid position 32, upstream of the predicted site (10). Thus, our iPanK2 constructs lack 12 amino acids at the N terminus that are present in the cellular intermediate (iPanK2). An Nhel restriction site was introduced upstream of the Kozak sequence with primers iPank2-F, iPank2-R, and sPank2-F to clone the pPank2, iPank2, and sPank2 between the Nhel and EcoRI sites of pcDNA3.1(−) to yield pKm4, pKm5, and pKm3L, respectively. iPank2 was also expressed with an N-terminal His tag from pKm7, which was constructed by ligation of the iPanK2 sequence into pcDNA3.1-HisA, possessing the mature sequence of PanK2 with an N-terminal His tag cloned into pcDNA3.1-HisA.

**Construction of the Plasmid for Coexpression of iPanK2 with pPanK2**—To test whether the PanK2 (G521R) mutation had a dominant negative effect on PanK2, we constructed a plasmid to coexpress the wild-type and G521R forms of iPanK2 in tandem from a bicistronic mRNA. iPanK2-PmeL and iPanK2-Blpl-2 primers were used to introduce a PmeL and a Bpl1 restriction site by PCR at the beginning and end of the wild-type iPanK2 coding sequence, respectively. The PCR product was cloned into pcR4Blunt-TOPO® (Invitrogen) and sequenced. Then the wild-type iPanK2 sequence was ligated into pcDNA3.1 vector (Novagen) within the PmeI and BlpI sites, and the resulting plasmid was named pPJ273. iPanK2-PmeL and iPanK2-Blpl-2 primers were used to introduce a PmeL and a Bpl1 restriction site into the pPanK2 preparation. The PCR product was cloned into pcR4Blunt-TOPO® and sequenced. The plasmid containing the correct mutant sequence was digested with PstI, treated with Klenow polymerase to generate blunt ends, and then digested with BstZ17I. The gel-purified mutant fragment was inserted into the pTandem-1 vector (Novagen) within the PmeL and Blp1 sites, and the resulting plasmid was named pPJ273. iPanK2-MstI-F and iPanK2-MstI-R primers were used to introduce a PstI and a BstZ17I restriction site into the pPanK2(G521R) and pPanK2(G521R)-MstI cloning sites, respectively. The PCR product was inserted into the pTandem-1 vector digested previously with PstI, treated with Klenow, and BstZ17I. The gel-purified mutant fragment was inserted into the pTandem-1 vector digested previously with PstI, treated with Klenow, and BstZ17I. The gel-purified mutant fragment was inserted into the pTandem-1 vector digested previously with PstI, treated with Klenow, and BstZ17I. The gel-purified mutant fragment was inserted into the pTandem-1 vector digested previously with PstI, treated with Klenow, and BstZ17I. The gel-purified mutant fragment was inserted into the pTandem-1 vector digested previously with PstI, treated with Klenow, and BstZ17I. The gel-purified mutant fragment was inserted into the pTandem-1 vector digested previously with PstI, treated with Klenow, and BstZ17I.
carrying the empty vector contained endogenous PanK activity at less that 0.2% of the level expressed by the wild-type protein. This same value was found with the iPanK2(K224A) mutant.

The activities of the mutants were compared with the wild-type protein as follows. The protein concentrations in the dialyzed extracts were determined by the Bradford method (17). Aliquots containing 2.5, 5, and 10 μg of protein were assayed for PanK activity as described above, and the apparent specific activity was calculated by regression analysis. These same three protein concentrations were also fractionated by SDS-gel electrophoresis, and the PanK2 proteins were detected by immunoblotting with the anti-PanK antibody as described above. The relative amount of each PanK mutant was determined by quantification of the fluorescent signal obtained from the Typhoon 9200. The ECF method was employed for quantification because there is a linear relationship between the fluorescent signal and the amount of protein over a 25-fold dilution range. These values were normalized to the wild-type protein, which was given a value of 1, and the normalization factor was multiplied by the specific activity to obtain the normalized activity reported in the tables and figures. In the case where multiple PanK2 species were detected by immunoblotting, the signals from each band were added together. A wild-type protein expression control was used in all transfection experiments, which were performed at least twice, and the data were combined.

**Determination of the Oligomerization State of PanK2**—The Stokes radius of PanK2 was evaluated by using gel filtration chromatography on a Superdex™ 200 10/300 GL column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM dithiothreitol. Protein molecular weight markers were purchased from Amersham Biosciences.

**In Vitro Transcription and Translation**—Because spPanK2 was not expressed in 293T cells, we used the TNT® Quick-coupled Transcription/Translation System (Promega) to express spPanK2 in vitro according to the manufacturer’s protocol. Briefly, the 50-μl reaction mix contained 1 μg of pKM3 (template plasmid for spPanK2), 40 μl of TNT® Quick Master Mix, and 2 μl of [35S]methionine (1,000 Ci/mmol at 16.7 mCi/ml; Amersham Biosciences). After 90 min of incubation at 30°C, 5 μl of the reaction mix was loaded onto a 12% SDS-polyacrylamide gel to determine the expression level of spPanK2. The dried gel was exposed to a PhosphorImager screen, and the autoradiograph was scanned using a Typhoon 9200 (Amersham Biosciences) and analyzed with ImageQuant software, version 5.2 (Amersham Biosciences). The activity of the spPanK2 expressed in vitro was tested using 15 μl of the reaction mix in the standard PanK assay as described above. Reactions using template plasmid for iPanK2 (pKM5), pcDNA3.1(−) vector and no template DNA were included as controls.

**RESULTS**

**Expression and Activity of Human PanK2 Isoforms**—Four homologous human PANK genes have been identified in silico (1), and multiple transcripts were thought to arise from the PANK2 gene (1, 10, 11). The longest cDNA encodes a precursor protein, termed pPanK2, consisting of 570 residues (10) (Fig. 1A). The validity of the larger pPanK2 isoform being the primary transcript is supported by 5′-rapid amplification of cDNA end results (11), PCR cloning from a Marathon cDNA library (10), and the identification of an EST clone corresponding to the 5′ end of the transcript we used to assemble the cDNA. This protein is sequentially processed by peptidase(s) to yield a transient 57.6-kDa intermediate, termed iPanK2, and a long lived 48-kDa mature form, mPanK2 (Fig. 1A). The first cDNA that was isolated encoded a smaller 50.6-kDa isoform, termed sPanK2, that initiates at Leu-111 (1), and subsequently a 288-amino acid splice variant, termed spPanK2, with a predicted size of 30.8 kDa was described that arises from a putative alternative initiation exon (11). We cloned and expressed by transient transfection all of these PanK2 isoforms in 293T cells, and we determined their apparent molecular sizes by gel electrophoresis and Western blotting with an antibody that recognizes a common element within the catalytic core domain (Fig. 1B). Both the pPanK2 and iPanK2 were expressed well and were partially converted to mPanK2. The sPanK2 expressed well but was not processed to the mPanK2 form (Fig. 1B). The expressed mPanK2 protein was not processed further (Fig. 1B).

All of the PanK2 isoforms that were expressed in 293T cells were catalytically active (Fig. 1C). This was not a surprising result, because the catalytic core identified in all mammalian PanKs encompasses residues 211–570 (7, 8), and all of the isoforms, except spPanK2, possess this identical central sequence. Furthermore, normalization of the expression levels using Western blotting and activity determinations over a range of protein concentrations showed that the overall activities of the different isoforms were nearly identical (Fig. 1C). The only exception was the sPanK2 species, which had a slightly lower specific activity in these experiments. Pantothenate kinase activity above base line was never detected in the spPanK2-transfected cell lysates, and neither were we able to detect the presence of immunoreactive protein (not shown). Therefore, we expressed the spPanK2 cDNA by using an in vitro coupled transcription/translation system. In this expression system, the amount of spPanK2 protein expressed was equal to or higher than the level of iPanK2 (Fig. 1D). Thus, we concluded that the reason the spPanK2 was not detected when expressed in 293T cells was because of rapid protein degradation. The transcription/translation products were then assayed for pantothenate kinase activity (Fig. 1E). Although the iPanK2 extract possessed pantothenate kinase activity, the spPanK2 extract did not. We concluded that spPanK2 cDNA does not encode a functional pantothenate kinase. This finding is consistent with the spPanK2 protein lacking the highly conserved DIXGT(S)XXK motif present in all eukaryotic PanK catalytic domains (Fig. 1A). The mutagenesis experiments described below demonstrate that this motif is essential for pantothenate kinase activity.
by CoA and its thioesters. The mPanK2 was potently inhibited by all four CoA species tested with IC50 values between 250 and 500 nM (Fig. 2A). These findings were in sharp contrast to the significantly weaker inhibition of the highly related PanK1/H9252 isoform by acetyl-CoA (IC50 = 11005 M) and the lack of inhibition of the PanK1/H9252 isoform by CoA and palmitoyl-CoA (9). Another related isoform is PanK3, which exhibits differential sensitivity to the panel of CoA thioesters and is most potently inhibited by acetyl-CoA (IC50 = 1125 M) and the least inhibited by CoA (IC50 = 125 µM) (9). The protein domain of PanK3 responsible for the higher affinity CoA thioester binding was identified as residues 56–104 (9), and the catalytic core region of PanK2 is related to residues 56–104 of PanK3 and was clearly distinct from the same region of PanK1. This stringent regulation of PanK2 explains why we were unable to detect PanK2 activity in 293T cell lysates unless they were first dia-
lyzed to remove low molecular weight components, presumably CoA and its derivatives. This step was not necessary to analyze the activity of the PanK1 proteins (7, 8).

The kinetic constants for the two mPanK2 substrates were in the range typically found in the PanK proteins. The Km for ATP was 63.6 ± 10.3 (Fig. 2B), and the Km for pantothenate was 25.4 ± 3.9 (Fig. 2C). We also examined the kinetic constants for the iPanK2 isoform and found similar results (ATP Km = 68.2 ± 15.3; pantothenate Km = 9.4 ± 2.4). A bioinformatics analysis of the mammalian PanK protein group showed that these proteins belong to the actin/hexokinase/Hsc70 superfamily of ATP-binding proteins. Several x-ray structures of family members were available allowing the assignment of specific substrate binding functions to the highly conserved primary sequence motifs that define this family (18, 19). The DIGGT(S)XXK motif in the N terminus of mPanK2 is a conserved sequence known to be a phosphate-binding loop. We generated the PanK2(K224A) mutant in the wild-type iPanK2
to test the essential nature of this conserved residue for activity and to provide a base-line activity profile for an inactive mutant. Expression of the iPanK2(K224A) mutant in 293T cells did not yield pantothenate kinase activity above the background value obtained with an empty vector (Fig. 3). Based on these data, the spPanK2 variant should not be functional as a pantothenate kinase because it lacks the DIGGT(S)XXK.

Evaluation of the Human PanK2 Mutations—An important goal of this work was to determine the effect of mutations associated with PKAN neurodegeneration on the biochemical activity of PanK2 to test the hypothesis proposed by others (1) that the disease phenotype is because of the inactivation or alteration of the biochemical properties of the enzyme. The range of activities in the assay was established by comparing the activity of the expressed wild-type iPanK2 protein (100%) to the inactive iPanK2(K224A) mutant or the empty vector (0.2%) (Fig. 3). We numbered the affected residues in the PanK2 coding sequence that represent the spectrum of mutations identified in patients with PKAN (4) in relation to the initiating methionine in the longest isoform, pPanK2 (Fig. 1A). Several mutations result in a frameshift or premature stop codon early in the PanK2 coding sequence (Fig. 3), and these proteins are clearly predicted to be biochemically inactive because of the loss of the core catalytic domain sequence, including another conserved region, Val-Val-Phe-Val-Gly<sup>521</sup>-Asn-Phe-Leu-Arg (Fig. 3). These mutant proteins are listed in the table as inactive but were not constructed or assayed in this study.

Most of the wild-type overexpressed iPanK2 protein was unprocessed and accessible to the cytosolic compartment, although about 10% was processed to the mature form as determined by Western blotting (Fig. 1B). This allowed us to evaluate potential processing defects as well as catalytic activity of the mutants. The mature PanK2 predominates in human brain (10). We introduced the most frequent single amino acid mutants associated with both early and late onset disease with a focus on PanK2(G521R), which has the highest incidence in PKAN patients (4). Similar processing of many of the overexpressed iPanK2 mutants, including N404I, R286C, and T528M, from the intermediate to the mature form was evident in our Western blots as reported previously (10). Both forms, when present, were included in the quantification of protein. These three mutant proteins were catalytically active when expressed as the intermediate isoform (Fig. 3), and their respective mature forms were expressed and evaluated both for catalytic activity and feedback regulation (Fig. 5). In contrast, expression of the iPanK2(G521R) protein did not result in increased pantothenate kinase activity in the cell lysate (Fig. 3) nor was it processed to the mature form (Fig. 6A). Also, the iPanK2(R451Stop) mutant was not active, consistent with loss of a major portion of conserved downstream residues in the catalytic core domain. Thus, any mutations that truncated the protein upstream of Arg-451 were also predicted to be inactive (Fig. 3). Only two additional single amino acid missense mutations associated with the PKAN disease inactivated PanK2, iPanK2(G219V), and iPanK2(N500I), and these have been associated with either early or late onset disease, respectively (Fig. 3). The activities of 10 additional mutants were significant, and 8 of these had activities that were 78% of wild-type or higher (Fig. 3). These values indicate that many of the mutations did not inhibit the catalytic activity of the protein, which is proposed as a possible cause of the disease (1).

**FIGURE 2. Regulation and kinetics of mPanK2.** A, feedback inhibition of mPanK2 by CoA and its thioesters. Pantothenate kinase assays were performed in the presence of the indicated concentrations of CoA (○), acetyl-CoA (●), malonyl-CoA (□), or palmitoyl-CoA (▲). The ATP Km for mPanK2 was 63.6 ± 10.3 μM with a V<sub>max</sub> of 90.2 ± 5.4. C, the pantothenate Km for mPanK2 was 25.4 ± 3.9 μM, and V<sub>max</sub> of 92 ± 6.5. The insets in panels B and C show the Lineweaver-Burk plots of mPanK2 kinetics. Details of these experiments are described under “Experimental Procedures.”
The defect in the PanK2(G521R) mutant was investigated further by gel filtration chromatography (Fig. 4). All characterized pantothenate kinases from bacteria to humans are organized as homodimers (9, 10, 20, 21). The oligomeric state of the mature wild-type mPanK2 was verified by gel filtration chromatography, and the elution position of mPanK2 was consistent with it existing as a dimer in solution (Fig. 4A). The location of the activity corresponded to the location of the mPanK2 protein determined by immunoblotting (not shown). The intermediate and precursor forms of the protein were also dimeric (not shown), and these data agree with those of Kotzbauer et al. (10). The expressed iPanK2(G521R) mutant eluted in the void volume of the column (Fig. 4B). In this case, the protein was catalytically inactive and was located by Western blot analysis. There was a small immunoreactive peak eluting at 12.5 ml. This molecular size is consistent with the conclusion that iPanK2(G521R) did not fold properly and was either in a denatured conformation or highly aggregated state that prevented processing and interfered with catalytic activity.

### Table 1: DNA Mutations and Protein Activity

| DNA Mutation | Protein Change | Onset | Activity |
|--------------|---------------|-------|----------|
|              |               | Early | Late     | % Activity |
| WT           |               |       |          | 100 ± 9.6  |
| 1561G→A      | G521R         | 24    | 7        | <0.2      |
| 1583C→T      | T528M         | 3     | 7        | 146 ± 15  |
| 1351C→T      | R451stop      | 4     | 1        | 2.9 ± 0.1 |
| 448C→T      | Q150stop      | 3     | 0        | —         |
| 1432G→A      | S471N         | 3     | 0        | 16 ± 0.8  |
| 1211A→T      | N404I         | 0     | 3        | 83 ± 8.4  |
| 856C→T      | R286C         | 2     | 2        | 176 ± 22  |
| 545insA      | Frame shift (181/228) | 2 | 0 | — |
| 569insA      | Frame shift (193/203) | 2 | 0 | — |
| 927-933del   | Frame shift (310/325) | 2 | 0 | — |
| 980C→T      | T327I         | 2     | 0        | 91 ± 3.5  |
| 1176-1177del | V394stop      | 2     | 0        | —         |
| 1323-1326del | Frame shift (447/447) | 2 | 0 | — |
| 1499A→T      | N501I         | 2     | 0        | 3.9 ± 0.6 |
| 1501-1504dup | Frame shift (501/511) | 2 | 0 | — |
| 1526C→T      | A509V         | 2     | 0        | 105 ± 3.0 |
| 1594C→T      | R632W         | 2     | 0        | 95 ± 3.5  |
| 700A→G      | T234A         | 0     | 2        | 112 ± 4.7 |
| 1051T→C      | S351P         | 0     | 2        | 78 ± 4.4  |
| 656G→T      | G219V         | 0     | 1        | 0.4 ± 0.1 |

* Predicted to lack activity.
PanK2 is very sensitive to inhibition by CoA and CoA thioesters (Fig. 2A), and the active mutant enzymes were tested for defects in CoA regulation that might contribute to the PKAN disease phenotypes. Also, we wished to verify that the lack of catalytic defect exhibited by the expressed intermediate forms was true for the mature forms. We addressed these points by selecting three active mutants, mPanK2(T528M), mPanK2(N404I), and mPanK2(S471N), and we examined their inhibition by acetyl-CoA (Fig. 5). The mPanK2(T528M) mutation occurs with high frequency in both early and late onset diseases, whereas mPanK2(N404I) is associated with late onset, atypical disease, and mPanK2(S471N) is linked with early onset, rapidly progressive severe disease (4). There was no significant difference between the response of the mutant proteins compared with the wild-type mPanK2 (Fig. 5). None of these mutations were in the region of mPanK2 corresponding to the domain in mammalian pantothenate kinases associated with CoA regulation (9). Our PanK2 preparations were as sensitive to palmitoyl-CoA regulation as reported by Kotzbauer et al. (10); however, they were considerably more sensitive to CoA regulation. This may be due to the absence of a reducing agent in the prior assays and the presence of variable amounts of oxidized CoA in commercial preparations. These data do not support the idea that defective mPanK2 regulation by CoA thioesters is a factor leading to metabolic imbalance and development of the PKAN disorder.

Some PanK2 patients were heterozygous for the PanK2(G521R) mutation, and it was suggested that the mutation could be semidominant (4). We explored this idea by expressing the iPanK2(G521R) mutant in tandem with the wild-type iPanK2 in a single expression vector to determine whether there was a dominant negative effect on pantothenate kinase activity (Fig. 6). The expression of iPanK2(G521R) did not block iPanK2 processing to the 48-kDa mature form and did not inhibit its activity (Fig. 6). These data demonstrated that iPanK2(G521R) did not have a dominant negative effect on iPanK2 pantothenate kinase activity when coexpressed in the same cell (Fig. 6).

DISCUSSION

Our data suggest that the inactivation of PanK2 catalytic activity may not be the sole underlying cause of the PKAN neurodegenerative disease. Some PanK2 mutations, such as the most common PanK2(G521R) which accounts for about 30% of the cases, may not fold properly or may lack critical protein domains and as a result are catalytically inactive. However, the mPanK2(T528M) mutation is the second most common allele associated with PKAN, and there is no discernible defect in the catalytic or regulatory properties of this enzyme or nine other mutant proteins. One would predict that the mPanK2(T528M) mutant would be fully active because methionine is found in place of threonine at this position in mammalian PanK1. PanK3, on the other hand, has threonine (8, 9). The lack of catalytic activity is not always associated with early onset disease (Fig. 3). A subset of PanK2 patients are heterozygous, having both a wild-type and a PanK2(G521R) allele, and it has been suggested that mPanK2(G521R) may have a dominant negative effect on wild-type PanK2 to promote the disease. Our data do not support this idea because PanK2 activity is not blocked by the expression of PanK2(G521R) in the same cell. Recently, a PanK2 knock-out mouse was developed as a model for PKAN (13). Although these mice have a distinct phenotype, including retinal degeneration and azoospermia,
the knock-out animals do not develop the brain iron accumulation or motor disorders associated with PKAN in humans. Defects in PanK2 processing do not account for the disease in those patients expressing catalytically active PanK proteins, such as PanK2(T528M), PanK2(T234A), PanK2(E134G), and PanK2(S471N), which are targeted to mitochondria as assessed by colocalization of immunofluorescent staining with mitotracker red and do not exhibit defects in processing or stability as evaluated by quantitative pulse-chase experiments (10). Processing from the intermediate to the mature forms of these mutants was also evident in our experiments (data not shown). The PanK2(G521R) mutant, on the other hand, is targeted to the mitochondria (10) but is catalytically inactive and not processed (Fig. 6A), likely due to its misfolded conformation and/or oligomerization (Fig. 4B). Taken together, these data suggest that classical PKAN may not arise solely from abnormal PanK2 catalytic or regulatory functions. PanK2 may also have additional function(s) not yet appreciated. The data also raise the possibility that some of the missense mutations are polymorphisms in the PanK2 gene and unrelated to disease. There is a recent report of a patient with the classical PKAN eye-of-the-tiger pattern who does not carry a mutation in the PanK2 gene (22). A comparison of PanK2 gene sequences from a broad spectrum of unaffected individuals would be useful to address this issue, although sequencing of the PanK2 genes from a number of unrelated individuals was reported previously and did not indicate that the mutations analyzed in our work are polymorphic substitutions (4). Alternatively, evaluation of coding sequences upstream of Leu-111 or the PanK2 promoter regions in PKAN patients may reveal additional different mutations that may affect PanK2 function(s) or expression levels.

The localization with mitochondria distinguishes PanK2 from the other PanKs and suggests a unique function for this protein. Immunocytochemistry with an antibody that recognizes PanK1, PanK2, and PanK3 localizes the majority of the PanK protein in the cytoplasm (23). The localization of PanK2 to mitochondria raises the question of whether a CoA biosynthetic pathway exists within this organelle. The subcellular localization studies to date definitively show that PanK2 is associated with mitochondria (10), but they do not establish its location within the organelle. This is an important distinction because the CoA synthase, which catalyzes the last two steps in the pathway, is also processed and associated with the mitochondria but accesses the cytoplasmic surface of the organelle and not the matrix (24). Thus, no other components of the CoA biosynthetic pathway or the pantothenate substrate are known to exist within the mitochondria matrix, and the localization of PanK2 needs to be more closely investigated to interpret its role in CoA metabolism. This issue is directly relevant to understanding the significance of the regulatory properties of PanK2. The highest concentrations of CoA in the cell are found in the mitochondrial matrix, and measurements range from 2.2 to 5.0 mM (25, 26). Because PanK2 is potently inhibited by submicromolar concentrations of CoA and all its thioesters in the presence of 2.5 mM ATP, the concentrations of mitochondrial CoA are clearly sufficient to completely shut off enzymatic activity, thus raising the question of how this protein could function as a pantothenate kinase within the mitochondrial matrix. On the other hand, two enzymes that utilize long chain acyl-CoAs, carnitine palmitoyltransferase (27) and the glycerol-phosphate acetyltransferase (28, 29), are located on the outer membrane of mitochondria and compete for acyl-CoAs for $\beta$-oxidation and lipid synthesis, respectively. The possible localization of PanK2 with these proteins places it in a position to sense the concentration of acyl-CoA, malonyl-CoA, and CoA at the precise cellular location of this critical branch point in metabolism. Understanding the physiological role(s) of the wild-type PanK2, other than its catalytic activity, requires considerably more investigation in order to relate it to the PKAN human disease or the phenotype associated with the PanK2 knock-out mouse model.

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