Diversity of the Pyruvate Dehydrogenase Kinase Gene Family in Humans*

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Recent evidence from this laboratory indicates that at least two isoenzymic forms of pyruvate dehydrogenase kinase (PDK1 and PDK2) may be involved in the regulation of enzymatic activity of mammalian pyruvate dehydrogenase complex by phosphorylation (Popov, K. M., Kedishvili, N. Y., Zhao, Y., Gudi, R., and Harris, R. A. (1994) J. Biol. Chem. 269, 29720-29724). The present study was undertaken to further explore the diversity of the pyruvate dehydrogenase kinase gene family. Here we report the deduced amino acid sequences of three isoenzymic forms of PDK found in humans. In terms of their primary structures, two isoenzymes identified in humans correspond to rat PDK1 and PDK2, whereas a third gene (PDK3) encodes for a new isoenzyme that shares 68% and 67% of amino acid identities with PDK1 and PDK2, respectively. PDK3 cDNA expressed in Escherichia coli directs the synthesis of a polypeptide with a molecular mass of approximately 45,000 Da that possesses catalytic activity toward kinase-depleted pyruvate dehydrogenase. PDK3 appears to have the highest specific activity among the three isoenzymes tested as recombinant proteins.

Tissue distribution of all three isoenzymes of human PDK was characterized by Northern blot analysis. The highest amount of PDK2 mRNA was found in heart and skeletal muscle, the lowest amount in placenta and lung. Brain, kidney, pancreas, and liver expressed an intermediate amount of PDK2 (brain > kidney = pancreas > liver). The tissue distribution of PDK1 mRNA differs markedly from PDK2. The message for PDK1 was expressed predominantly in heart with only modest levels of expression in other tissues (skeletal muscle > liver > pancreas > brain > placenta > lung > kidney). In contrast to PDK1 and PDK2, which are expressed in all tissues tested, the message for PDK3 was found almost exclusively in heart and skeletal muscle, indicating that PDK3 may serve specialized functions characteristic of muscle tissues. In all tissues tested thus far, the level of expression of PDK2 mRNA was essentially higher than that of PDK1 and PDK3, consistent with the idea that PDK2 is a major isoenzyme responsible for regulation of pyruvate dehydrogenase in human tissues.

Mitochondrial multienzyme complex, pyruvate dehydrogenase (PDH),1 catalyzes the oxidative decarboxylation of pyruvate: pyruvate + CoA + NAD+
\(-\rightarrow\) acetyl-CoA + NADH + H+ + CO2 and is one of the major enzymes responsible for the regulation of homoeostasis of carbohydrate fuels in mammals (for review, see Ref. 1). The enzymatic activity of PDH is controlled by a phosphorylation/dephosphorylation cycle (2–5). The phosphorylation of PDH that leads to the complete inactivation of its enzymatic activity is catalyzed by a highly specific pyruvate dehydrogenase kinase (PDK) (6, 7). The dephosphorylation with concomitant reactivation is catalyzed by pyruvate dehydrogenase phosphatase (8, 9). It is generally believed that the phosphorylation state of PDH in mitochondria is determined by the activity of its intrinsic kinase, which itself is regulated by the products and substrates of the dehydrogenase reaction (10–12). The products stimulate the kinase activity, whereas the substrates are inhibitory. The kinase activity is also inhibited by ATP (competitive with ATP) that acts synergistically with pyruvate (13). Therefore, in mitochondria, the steady state activity of the kinase should depend on intramitochondrial ratios of CoA/acetyl-CoA, NAD+/NADH, and ADP/ATP, as well as on the intramitochondrial concentration of pyruvate.

Recent evidence from this laboratory indicates that, at least in rodents, there are two isoenzymic forms of PDK sharing up to 70% of amino acid identity (14). These isoenzymes of PDK have been designated as PDK1 and PDK2. Both isoenzymes, obtained as the recombinant proteins, were found able to catalyze the phosphorylation and inactivation of PDH (14, 15). The tissue distribution of isoenzymes appeared to be quite different. By Northern blot analysis, the isoenzyme PDK1 was found to be expressed predominantly in cardiac muscle. The isoenzyme PDK2, in contrast, is highly expressed in all tissues tested thus far, therefore suggesting that PDK2 may be responsible for regulation of the enzymatic activity of PDH complex in most of the tissues (14). Consistent with this idea, some recent immunological evidence indicates that PDK2 corresponds to one of the subunits of PDK from bovine kidney mitochondria.

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1 The abbreviations used are: PDH, pyruvate dehydrogenase; PDK1, PDK2, and PDK3, isoenzymes 1, 2, and 3 of the pyruvate dehydrogenase kinase; E1, pyruvate decarboxylase component of PDH; E2, dihydrolipoamide transacylase component of PDH; E3, dihydrolipoamide dehydrogenase component of PDH; bp, base pair(s); kb, kilobase(s); PCR, polymerase chain reaction; RACE, rapid amplification of the cDNA ends; HisTag, stretch of six consecutive histidine residues added to the recombinant protein in order to facilitate purification.
Isoenzymes of Pyruvate Dehydrogenase Kinase

(16) and also corresponds to the free catalytic subunit of PDK identified by Randell's laboratory in rat liver mitochondria (14, 17).

The physiological significance for existence of isoenzymes of PDK has not been established. Moreover, to date, it has not been shown whether isoenzymes of PDK exist in other mammalian species besides rodents. In the present study, we further explored the diversity of the PDK gene family. Here we report the data on the primary structures of three isoenzymic forms of PDK found in humans, one of which is a new isoenzyme of PDK, along with data on their tissue distribution and catalytic activities.

EXPERIMENTAL PROCEDURES

Polymerase Chain Reaction—The gene-specific oligonucleotide primers (AA(A/G) AA(C/T) GCN ATG CGN (or AG(A/G)) GCN AC) and (GGN CGN GTN GAA(G) (or CT(A/G)) TAC AT(A/G) TA) for PCR were designed according to the amino acid sequences corresponding to the conserved subdomains II (-KNAMRAT-) and IV (-YMYSTAP-) of mitochondrial protein kinases. These primers were used to amplify human liver QUICK-Clone cDNA obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Each reaction mixture contained 50 pmol of each gene-specific primer, 1 ng of double-stranded cDNA along with deoxyribonucleoside 5’-triphosphates, buffer, and 0.5 unit of Taq polymerase added according to the manufacturer’s instructions (Perkin-Elmer). Forty cycles of PCR were performed using 1 min at 94°C for denaturation, 1 min at 52°C for annealing, and 1 min at 72°C for extension. The PCR products of approximately 180 bp were purified and subcloned in M13mp18 for sequencing.

5’-Stretch tag10 Human Liver cDNA Library Screening—Human liver cDNA library obtained from Clontech was screened with random-primer 32P-labeled cDNAs of rat PDK1, PDK2, or PDK3, with partial cDNA of human PDK3 corresponding to PCR product obtained during amplification. Approximately 0.3–1.0 × 108 plaque-forming units of cDNA library were screened essentially as described by Sambrook et al. (18). Hybridization conditions were as follows: 5 × SSPE (1 × SSPE = 180 mM NaCl, 10 mM NaH2PO4, 1 mM Na2EDTA, pH 7.1), 5 × Denhardt’s solution (0.1% [w/v] bovine serum albumin, 0.1% [w/v] polyvinylpyrrolidone, 0.1% [w/v] Ficoll 400), 50% (v/v) formamide, 1% (w/v) sodium dodecyl sulfate, 0.1 mg/ml denatured salmon sperm DNA, and the radiolabeled probe (2 × 106 cpm/ml) at 37°C for 17 h. The filters were washed 6 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.5) containing 0.1% SDS four times at room temperature, two times with 2 × SSC, 0.1% (w/v) SDS at room temperature, and two times with 0.2 × SSC, 0.1% (w/v) SDS at 42°C for 5 min. Positive plaques were purified through four more cycles of plating and screening. Respective cDNAs were cut out of plasmid EcoRl and religated in EcoRl-digested M13mp18 for sequencing.

Rapid Amplification of 5’-End of PDK3 cDNA (5’-RACE)—5’-RACE protocol was used in order to do the 5’-end of human PDK3 cDNA. Human 5’-RACE-ready cDNA for this purpose was obtained from Clontech. The first round of amplification was performed following the manufacturer’s instructions with gene-specific primer 1 (CTT AAT GGA TAA GTC TTC ACC TCT GAA GGT TCC AGA ATC GAT AG) obtained from Clontech. Gene-specific primer 2 corresponding to the positions 794–821 of PDK3 cDNA (CTC TTT TTC TCT TTC ATA GAG TTC) was used for the second round of amplification. PCR product of 821 bp was purified and subcloned in M13mp18 for sequencing. The nucleotide sequences of PCR products obtained from three independent amplifications were analyzed.

Expression and Purification of PDK1, PDK2, and PDK3 cDNAs in E. coli—PDK1, PDK2, and PDK3 cDNAs were expressed as described elsewhere.2 Briefly, SacI and Xhol restriction sites flanking the coding regions of rat PDK1 and PDK2 cDNAs, as well as the cDNA for human PDK3, were constructed by PCR. Resulting SacI/Xhol fragments were ligated with PET-28a expression vector (Novagen, Madison, WI), cut with SacI and Xhol. In order to facilitate the purification of the recombinant isoenzymes of PDK, the stretches of six consecutive histidine residues (His6Tag) were constructed at the amino termini of the respective proteins. The resulting plasmids pPD1, pPD2, and pPD3 were expressed in Escherichia coli HMS 174 (DE3) (Novagen) as described (14).

Purification of recombinant proteins was achieved by chromatography on HisBind resin (Novagen) (19). PDK Activity Assay—ATP-dependent inactivation of the kinase-depleted PDH complex in the presence of recombinant PDK1, PDK2, or PDK3 was assayed essentially as described previously (7).

DNA Sequencing—Single-stranded M13 DNA was prepared and sequenced by the dideoxy chain termination method (20) with Sequenase version 2.0 (U. S. Biochemical Corp.).

Northern Blot Analysis—Human multiple tissue Northern blots were obtained from Clontech. Blots were probed with random-primer 32P-labeled cDNAs of PDK1, PDK2, and PDK3, respectively. Hybridization conditions were as follows: 5 × SSPE, 5 × Denhardt’s solution, 1.0% (w/v) SDS, 50% (v/v) formaldehyde, 0.1 mg/ml denatured salmon sperm DNA, and radiolabeled probe (1.0–1.5 × 106 cpm/ml) at 42°C for 12 h. Blots were washed four times in 2 × SSC with 0.1% (w/v) SDS at room temperature and two times in 0.1 × SSC with 0.1% (w/v) SDS at 55°C for 5 min and then exposed to x-ray film overnight at ~70°C.

RESULTS AND DISCUSSION

Molecular Cloning of Human PDK1, PDK2, and PDK3 cDNAs—Amplification of the human liver cDNA with primers designed according to the sequences of conserved subdomains II and IV of mitochondrial protein kinases gave rise to a PCR product of the expected size of approximately 180 bp. Sequence analysis revealed that the obtained PCR product is a mixture of three cDNAs (data not shown). Two of them appeared to be almost identical with the cDNAs of PDK1 and PDK2 previously identified in rat tissues. The third cDNA, in spite of the high resemblance to cDNAs for PDK1 and PDK2, encoded for a different protein, indicating that multiple isoenzymes of PDK found previously in rodents (14, 15) do exist in humans as well. Moreover, these experiments yielded a new isoenzyme of PDK that has not been identified in rat tissues.

In order to obtain the full-length cDNAs encoding for human isoenzymes homologous to rat PDK1 and PDK2, human liver cDNA library was screened with corresponding rat cDNAs. Two positive clones were obtained during screening with PDK1 cDNA. One of the clones contained a cDNA of 1,593 bp that encodes for the full-length protein product with a molecular mass of 49,244 Da corresponding to the human PDK1. Screening of human liver cDNA library with cDNA of rat PDK2 yielded eight positive clones. Six of them appeared to be partial clones and each was two contained cDNAs of 1,330 and 1,422 bp that encode a full-length protein with a molecular mass of 46,181 Da homologous to the rat PDK2.

In order to clone a full-length cDNA of PDK3, human liver cDNA library was screened with the unique partial cDNA obtained by PCR as discussed above. Only one positive clone was found after screening of approximately 1.0 × 108 plaque-forming units, indicating that this cDNA is under-represented in the liver library. The analysis of the nucleotide sequence of the respective cDNA (914 bp long) revealed that it encodes for the carboxyl terminus of PDK3. The 5’-end of PDK3 cDNA was obtained by using a 5’-RACE protocol. Amplification of human heart templates with primers described under “Experimental Procedures” gave rise to an 821-bp-long PCR product that encodes for the complete 5’-coding region of PDK3 cDNA, as well as for the 5’-noncoding region. Further attempts to amplify the 5’-noncoding region of PDK3 cDNA with primers located near the 5’-end failed to produce products longer than expected from the sequence of 821 bp cDNA, suggesting that it covers almost the entire 5’-noncoding region. The resulting 1599 bp-long composite cDNA for PDK3 was constructed by aligning the 821-bp 5’-RACE product with the 914-bp cDNA obtained during library screening. It encodes for a protein with molecular mass of 46,938 Da.

Primary Structures of Human PDK1, PDK2, and PDK3—Analysis of the deduced amino acid sequences of human PDK1 and PDK2 revealed an extremely high resemblance to their rat...
counterparts. The identity between human and rat PDK 2 is as great as 96% (Fig. 1). For human isoenzyme PDK 1 the percent identity with the homologous rat isoenzyme was found to be somewhat lower, approximately 93%, due to the differences within the sequences of leader peptide. PDK 3 shares 68% and 67% identity with PDK 1 and PDK 2, respectively. The same range of similarity was found previously between rat PDK 1 and PDK 2, clearly indicating that PDK 3 is a new isoenzyme of pyruvate dehydrogenase kinase.

Five regions of an extremely high conservation that presumably define the putative catalytic domain of mitochondrial protein kinases were identified in previous studies (14–15, 21): subdomain I defined by an invariant histidine residue (His155, numbering here and below is in accord with the mature sequence of rat PDK 2); subdomain II defined by an invariant asparagine residue (Asn247); subdomain III corresponding to the first glycine rich loop Asp282-X-Gly284-X-Gly286; subdomain IV defined by aromatic residue (Tyr298); and subdomain V corresponding to the second glycine rich loop Gly317-X-Gly319-X-Gly321-Lys322-Pro323. As expected from these observations, the sequences corresponding to subdomains I, IV, and V are perfectly conserved among all isoenzymes of human PDK (Fig. 1). The minor differences were found within the sequences of subdomains II and III. It appeared that the alanine of subdomain II (-KNAMRAT-), as well as the arginine of subdomain III (-DRGGG-) of PDK 3, are substituted to serine (-KNSMRAT-) and leucine (-DLGGG-), respectively. The lower level of conservation was observed only in rather short stretches of spacers connecting subdomains II and III, as well as subdomains IV and V (positions 263–276 and 315–321 of human PDK 2, respectively). Surprisingly, an extremely high degree of conservation (up to 81%) was found within the amino termini of human isoenzymes of PDK (positions 26–73 of human PDK 2), in spite of the fact that the overall level of conservation within this region of mitochondrial protein kinases is fairly low (15). Presumably, it indicates that the amino terminus serves some specialized function characteristic of PDK, such as docking of the kinase to the complex. The greatest degree of divergency between the isoenzymes was found within the sequences immediately flanking subdomain I (positions 74–119 of PDK 2). Taking into account that the invariant histidine residue of subdomain I (His123 of PDK 2) may serve as a catalyst of the phosphotransfer reaction (21), it seems reasonable to suggest that the differences within sequences flanking subdomain I

![Fig. 1. Comparison of the predicted amino acid sequences of mitochondrial protein kinases.](image)
may be directly responsible for the unique catalytic properties of the isoenzymes of PDK.

The structural motifs of mitochondrial protein kinases were identified based on the analysis of the amino acid sequences deduced from the nucleotide sequences of five genes available at that time: rat PDK1 and PDK2, rat branched chain α-keto acid dehydrogenase kinase, hypothetical phosphoprotein 3 (HP3) from Trypanosoma brucei and ZK370.5 from Caenorhabditis elegans (22, 23). However, with four more sequences available now for comparison (human isoenzymes of PDK and ORF of hypothetical protein Y1L042C from yeast, GenBank accessions number Z47047), additional structural motifs of the mitochondrial protein kinases became apparent (Fig. 1). The first motif defined by consensus sequence Ala−Gly−(Val/Lys)−X−Glu is localized immediately downstream from the first domain I. The second motif, corresponding to consensus sequence Arg−(Ile/Met)−(Arg/Lys)−(Met/Lys)−(Arg/Lys)−Leu, is positioned in the middle of the spacer connecting the subdomains I and II. Two other consensus sequences Tyr−(Ala/Leu)−X−(Tyr/Leu)−(Phe/Leu)−X−Gly−Gly and Gly−Gly−X−Gly−Thr−Asp, are situated within the carboxyl terminus of the kinase molecule and flank the catalytic domain on its carboxyl terminus. It is generally believed that regions of high conservation are important for catalytic function, either directly as components of the active site or indirectly by contributing structurally to the formation of the active site. The functional significance of subdomains of mitochondrial protein kinases identified through the comparison of their primary structures will be determined during future structure-functional analysis.

The Catalytic Activity of the Recombinant Isoenzymes of PDK—Most of the studies on the regulation of PDH activity have been performed on purified preparations of the PDH complex or on the mitochondria prepared from different tissues with unspecified isoenzymic composition of PDK. Thus, the question about the contribution of individual isoenzymes of PDK to the observed regulatory properties of the kinase has never been addressed. Since the cDNAs of individual isoenzymes of PDK are now available, it is possible to determine the contribution of each isoenzyme to the regulation of the PDH complex by characterizing the respective recombinant enzymes. In the present study, the cDNAs of PDK1, PDK2, and the newly discovered PDK3 were cloned in E. coli. Purified recombinant kinases, and characterized their activities toward the kinase-depleted PDH complex.

To facilitate the purification of recombinant PDK1, PDK2, and PDK3, stretches of six consecutive histidine residues (HisTag) were constructed at the amino terminus of the respective proteins. Corresponding HisTagged kinases were purified from E. coli extracts in one step by using metal chelate chromatography. The recombinant kinases, purified in this way, were found to be more than 90% pure as judged by SDS-polyacrylamide gel electrophoresis analysis (Fig. 2). The electrophoretic mobility of purified PDK1 was found to be somewhat slower than that of PDK2, whereas PDK3 had electrophoretic mobility comparable to that of PDK2 in good agreement with previous estimates of the molecular weights of the respective isoenzymes based on the deduced amino acid sequences (in this manuscript and in Refs. 14 and 15). Preparations of isoenzyme PDK1 appear to consist of several closely sized bands on SDS gel (Fig. 2). This microheterogeneity was found to be due to phosphorylation occurring during expression.

The recombinant isoenzymes of PDK expressed and purified in the present study appeared to have enzymatic activity toward the kinase-depleted PDH complex (Fig. 3). Based on an ATP-dependent inactivation assay that measures the rate of ATP-dependent inactivation of PDH activity due to phosphorylation, PDK3 was found to be the most active isoenzyme. The enzymatic activity of PDK1 was approximately 30–50% lower than that of PDK3 in three independent experiments. The second isofrom of PDK had the lowest basal activity of all three isoenzymes. However, it is generally believed that in mitochondria the rate of kinase activity depends upon the relative concentrations of products and substrates of the dehydrogenase reaction. The products, NADH and acetyl-CoA, activate PDK, whereas the substrates, pyruvate, NAD+ and CoA inhibit the kinase. Therefore, direct comparison of the basal activities of isoenzymes of PDK may be somewhat misleading. Accordingly, it was found that the enzymatic activity of isoenzyme PDK2, when it is tested in the presence of high ratios of NADH/NAD+ and acetyl-CoA/CoA, increases almost five times and approaches the activity of isoenzyme PDK1, thus indicating that isoenzymes of PDK are different not only in terms of their basal activities, but also in terms of regulation of their enzymatic activities by the compounds known to affect kinase activity in vivo.

Tissue Distribution of Human PDK1, PDK2, and PDK3—Tissue distribution of all three isoforms of human PDK was characterized by Northern blot analysis (Fig. 4). The mRNA of PDK2 corresponded to a single hybridizing species of approximately 2.4 kb in all tissues tested. The highest amount of message was found in heart and skeletal muscle, the lowest amount in placenta and lung. Brain, kidney, pancreas, and liver expressed an intermediate amount of PDK2 (brain > kidney = pancreas > liver). Tissue distribution of PDK1 mRNA differed markedly from PDK2 (Fig. 4). The message for PDK1 (4.5 kb long) was abundantly expressed in heart with only modest levels of expression in other tissues (skeletal muscle > liver > pancreas > brain > placenta > lung > kidney). This is in contrast to PDK1 and PDK2 which are expressed in all tissues tested, the message for PDK3 (4.0 kb) was found almost exclusively in heart and skeletal muscle (Fig. 4), indicating that PDK3 may serve some specialized functions characteristic for muscle tissue. In all tissues tested thus far, the level of expression of PDK2 mRNA was higher than that of PDK1 and PDK3, suggesting that PDK2 may be a major isoform responsible for the regulation of enzymatic activity of the PDH complex in humans. However, whether the difference in the levels of mRNA directly translates in different levels of corresponding

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3 Y. Zhao and K. M. Popov, unpublished observations.
Accordingly, in heart muscle, liver is different (see Fig. 4 of this manuscript, as well as Ref. For example, the isoenzymic composition of PDK in heart and liver enzymes of PDK expressed in a particular tissue being studied. The reportedinpreviousstudiesmayreflecttherepertoireofisoen-
zymes of pyruvate dehydrogenase kinase. 40 milliliters of kinase-depleted PDH was reconstituted with 0.5 µg of the recombinant PDK1 ( ), PDK2 ( ), or PDK3 ( ), respectively, in 570 µl of the phosphorylation mixture containing 20 mM TrisHCl (pH 7.4), 5 mM MgCl₂, 50 mM KCl, 2 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin. Phosphorylation reactions were initiated with ATP (final concentration 100 µM) after a 5-min preincubation at room temperature. Aliquots of the phosphorylation mixture were withdrawn at indicated times for determination of the PDH residual activity. Each point represents an average from three independent expression experiments. Under comparable conditions of incubation, the kinase-depleted PDH complex itself did not show any inactivation.

The existence of multiple isoenzymes of PDK in mammalian tissues suggests that the differences in the properties of kinase reported in previous studies may reflect the repertoire of isoenzymes of PDK expressed in a particular tissue being studied. For example, the isoenzymic composition of PDK in heart and liver is different (see Fig. 4 of this manuscript, as well as Ref. 14). Accordingly, in heart muscle, β-oxidation of fatty acids is necessary for the effect of starvation on the level of the enzymatic activity of PDH complex and may be reversed easily by tetradecylglycidate, an inhibitor of β-oxidation (24). In contrast, in liver, tetradecylglycidate does not reverse the effect of starvation on the percent of active PDH complex (24). These observations are suggestive that the different isoenzymes ex-

protein remains to be established.

The Relationships within the Family of Mitochondrial Protein Kinases—The analysis of the phylogenetic tree of mitochondrial protein kinases indicates that isoenzymes of PDK along with protein ZK370.5 from C. elegans form one big cluster (Fig. 5). On the other hand, branched chain α-keto acid dehydrogenase kinase, HPP3 from T. brucei and YIL042C from yeast map on the tree quite distant from the main cluster (Fig. 5). Based on this analysis, mitochondrial protein kinases fall into four classes: isoenzymes of PDK and ZK370.5 from C. elegans in class I, branched chain α-keto acid dehydrogenase kinase in class II, HPP3 from T. brucei in class III, and YIL042C from yeast in class IV. Class I and II contain the best characterized enzymes, whereas the proteins of class III and IV along with class I protein ZK370.5 have not been characterized yet. However, some inferences may be made based on the analysis of the topology of phylogenetic tree. It seems reasonable to assume that members of a cluster are more similar to each other than to non-cluster members in terms of three-dimensional structure, mechanism of substrate recognition, and mechanism of phosphotransfer reaction. If this assumption is correct, gene ZK370.5 most likely encodes for a PDK for C. elegans. On the other hand, the proteins from class III and IV, even though they have the structural features characteristic of mitochondrial protein kinases, are less related to the other members of the family. They may have protein substrates other than mitochondrial multienzyme complexes.

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