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Short communication

Isolation and Characterization of cDNA Encoding Pyridoxal Kinase from Ovine Liver

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cDNA fragments of ovine liver pyridoxal kinase were amplified by PCR using degenerate oligonucleotide primers derived from partial amino acids sequences of the enzyme. Using PCR products as probes, several overlapping cDNA clones were isolated independently from an ovine liver and a human brain cDNA library. The largest cDNA clone for each was selected for sequence analysis. The ovine liver cDNA encodes a polypeptide of 297 amino acid residues with M_r of 32,925, whereas the human clone is comprised of an open reading frame encoding 312 amino acid residues with M_r of 35,102. The deduced sequence of the human brain enzyme is completely identical to that of human testes cDNA recently reported (Hanna *et al.*, 1997). The ovine enzymes have approximately 77% sequence identity with the human enzyme although the two sequences are completely different in the N-terminus comprising 32 residues. This result suggests that pyridoxal kinase is highly homologous in mammalian species.

Introduction

Vitamin B_6 generally refers to all 3-hydroxy-2-methyl pyridine derivatives. These compounds have been recently demonstrated to be useful molecular probes in investigating structural features of macromolecules (Kwon *et al.*, 1994; Kwon, 1996). Pyridoxal-5′-phosphate (PLP) is a biologically active form of this vitamin, which serves as a cofactor required by numerous enzymes that catalyze transamination and carboxylation reactions (McCormic *et al.*, 1961; Snell, 1990). The formation of PLP from ATP, pyridoxal, and a divalent cation (Zn^{2+}) is catalyzed by pyridoxal kinase (EC 2.7.1.35), which has been detected in all mammalian species (Kwok and Churchich, 1979; Cash *et al.*, 1980; Tagaya *et al.*, 1989; Hirakawa-Sakurai *et al.*, 1993) as well as in many microorganisms (White and Dempsey, 1970). The enzyme isolated from sheep brain has been identified to be a dimer with a molecular weight of 80 kDa. Limited chymotrypsin digestion of the enzyme yields two fragments of 24 and 16 kDa with concomitant loss of catalytic activity (Dominici *et al.*, 1989; Cho *et al.*, 1997).

The physiological significance of pyridoxal kinase is not fully understood. However, this enzyme is of particular interest because of the intimate relationship of vitamin B_6 metabolism to brain disorders. Several lines of evidence indicate that convulsive seizures occur during vitamin B_6 deficiency. Moreover, vitamin B_6 metabolism is known to be altered in Down's syndrome (McCoy *et al.*, 1969). Interestingly, the chromosome 21 trisomy displays increased pyridoxal kinase activity (Coburn *et al.*, 1991). Although there is still no obvious connection between them at present, pyridoxal kinase is clearly a candidate target for these disorders. Further progress in the physiological and functional studies of pyridoxal kinase depends upon detailed information of the structure of this target enzyme.

In order to further our understanding of the molecular structure and mechanism of pyridoxal kinase, we isolated and sequenced full-length cDNAs encoding the ovine liver and human brain enzymes. Recently, the genes from various species have been cloned in several laboratories (Hanna *et al.*, 1997; Gao *et al.*, 1998; Yang *et al.*, 1998). These advances in accumulation of structural information should pave the way for further studies on the relationship between structure and catalytic function of this enzyme.

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Materials and Methods

Materials An ovine liver λgt11 cDNA library and a human brain λZAP II cDNA library (frontal cortex) were purchased from Clontech (Palo Alto, USA) and Stratagene, Inc (La Jolla, USA), respectively. Enzymes used for the cloning procedures were purchased from Stratagene. Nucleic acid transfer membrane (Hybond-N^+), [α-^{32}P] dCTP, and [α-^{35}S] dATP were obtained from Amersham Corporation (Arlington Heights, USA). DNA sequencing reagents were from United States Biochemical (Cleveland, USA).

PCR screening of cDNA library cDNA fragments of pyridoxal kinase were amplified from a sheep liver λgt11 cDNA library using polymerase chain reaction (PCR) techniques. Two degenerate oligonucleotide primers, 5′-CA(TC) GT(AGTC) AA(CT) CA(AG) TA(CT) GA(CT) TA-3′ (forward primer) and 5′-GC(AG) TC(AGTC) AC(CT) TT(AG) TGC AT(CT) TCC AT-3′ (reverse primer) were used in the PCR amplification. These primers correspond to the sequence of two peptides from ovine pyridoxal kinase (Maras *et al.*, 1999), His-Val-Asn-Gln-Tyr-Asp-Tyr and Met-Glu-Met-His-Lys-Val-Asp-Ala, respectively.

Amplifications were carried out using a GeneAmp PCR system 2400 according to a procedure described elsewhere (Friedman *et al.*, 1988; Innis *et al.*, 1990). In a final volume of 100 μl of PCR reaction mixture was added 5 μl of cDNA library aliquots and 100 pmol of each primer in a PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl) containing 2 mM $MgCl_2$ and 200 μM each of dNTPs. The reaction mixture was heated for 5 min and cooled to the annealing temperature of 55°C. Next, 0.5 μl of AmpliTaq DNA polymerase (2.5 units/100 μl) was added to the mixture, followed by 40 cycles of extension at 72°C for 1 min, denaturation at 94°C for 1 min, and annealing at 55°C for 1.5 min.

PCR products were analyzed by agarose gel electrophoresis. The amplified fragment was then subcloned into a TA cloning vector (Invitrogen, San Diego, USA), and transformation was performed using an F′ one shot kit (Invitrogen, San Diego, USA). The procedures were performed according to the manufacturer's instructions, and the PCR product identity was analyzed by nucleotide sequencing.

Isolation of pyridoxal kinase clones The amplified gene fragments of ovine pyridoxal kinase were used as probes to isolate full-length clones from λgt11 ovine liver and λZAP human brain cDNA libraries. Probes were prepared by the nick translation method (Sambrook *et al.*, 1989), and approximately 5×10^5 bacteriophages were plated out for each screening. Duplicate filter replicas of the plaques were lifted onto nylon membranes, and plaque hybridization was carried out at 42°C overnight as described elsewhere (Sambrook *et al.*, 1989; Sohng and Yoo, 1996). Washing was performed twice at room temperature for 30 min in 2 × SSC and twice at 60°C for 30 min in 1 × SSC.

Several clones were picked after the tertiary screening. For ovine clones, the isolated phage DNAs were cleaved with *Eco*RI. The inserts were subcloned into pBluescript II SK(−) vector and amplified in *Escherichia coli* DH5αF′ cells. Human clones excised from the λZAP vector were subcloned into pBluescript SK(−) plasmid using the ExAssist/SOLR system, as described in the manufacturer's protocol (Stratagene).

DNA sequencing and computer-assisted analysis The largest clone was used for sequence analysis. Double-stranded sequencing reactions were carried out by the dideoxy chain-termination method (Sanger *et al.*, 1977) using a Sequenase version 2.0 DNA sequencing kit. Sequencing products were resolved in 6% polyacrylamide/7M urea gels. The primer walking method was followed using various synthetic oligonucleotide primers corresponding to the known sequences. The nucleotide sequence and protein structure were analyzed with the aid of the PC GENE computer program. For DNA and protein homology searches, the GenBank database was used.

Result and Discussion

We isolated full-length pyridoxal kinase clones independently from ovine liver and human brain cDNA libraries. Using two degenerate oligonucleotide primers corresponding to the amino acids sequence of the ovine brain enzyme, PCR amplifications were performed to screen an ovine liver λgt11 cDNA library. PCR products were analyzed by agarose gel electrophoresis, and only a single DNA band of approximately 0.5 kbp was detected. Further results from DNA sequencing analysis revealed that the amplified fragment consists of 465 bp encoding 155 amino acid residues, and that the deduced amino acids sequence coincides with the peptide sequence previously determined by Edman degradation (Maras *et al.*, 1999).

Cloned cDNA fragments from PCR were used for screening of the λgt11 ovine liver cDNA library to obtain a full-length clone. Using a nick-translated probe, several clones were isolated from the library. Sequence analysis was carried out on the largest clone of 2.1 kb, and the resulting nucleotide and deduced amino acids sequences of an open reading frame region are shown in Fig. 1. A termination codon (TGA) is adjacent to the triplet coding for the carboxyl terminus leucine. Instead of a polyadenylation consensus sequence, an AAAAAT sequence procedes a 12-nucleotide polyadenylate tail in the 3′-untranslated region. The cDNA encoding for the ovine liver enzyme consists of 295 amino acid residues with a molecular mass of 32,925 Da. A computer calculation revealed that the isoelectric point for the protein is 5.75. The hydropathy profile of pyridoxal kinase indicated a rather even distribution of hydrophobic and hydrophilic regions throughout the molecule (data not shown). Predictions of secondary structure indicated the presence of α-helices (38%) and β-pleated sheets (16%). The region of PCR product used for the probe is underlined in the figure.

A human brain cDNA library constructed in λZAPII (Stratagene) was also screened. After the third screening, five clones were isolated and sequence analyses were carried out. One of the clones consists of 1116 bp encoding a pyridoxal kinase of 312 amino acids with a molecular weight of 35,102. During the completion of this work, the sequence of a human testes cDNA encoding pyridoxal

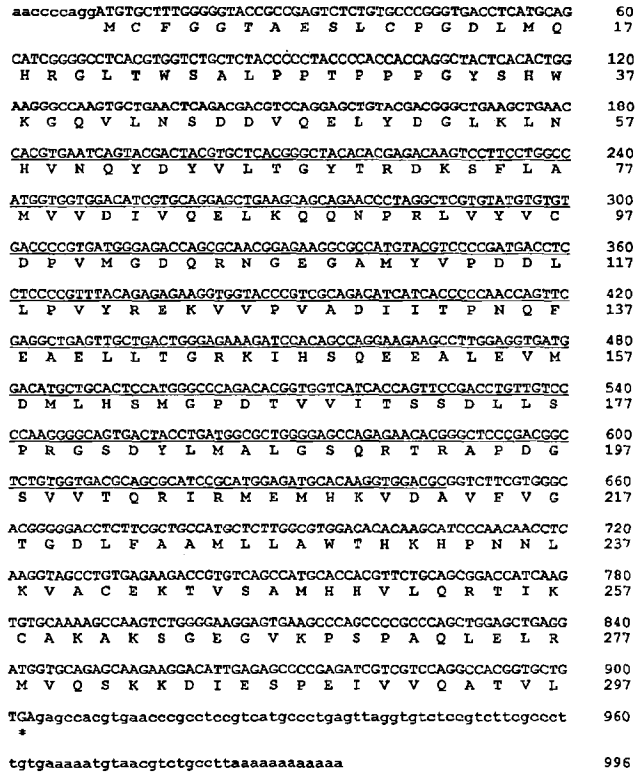

Fig. 1. Nucleotide and deduced amino acids sequences of ovine liver pyridoxal kinase (GenBank accession No.AF125374). The 5′ and 3′ flanking regions are indicated as lowercase and the open reading frame as uppercase letters. The predicted amino acids sequence (single-letter abbreviation) is shown below the nucleotide sequence. The termination codon is represented by an asterisk (*), and the region of PCR amplification is underlined.

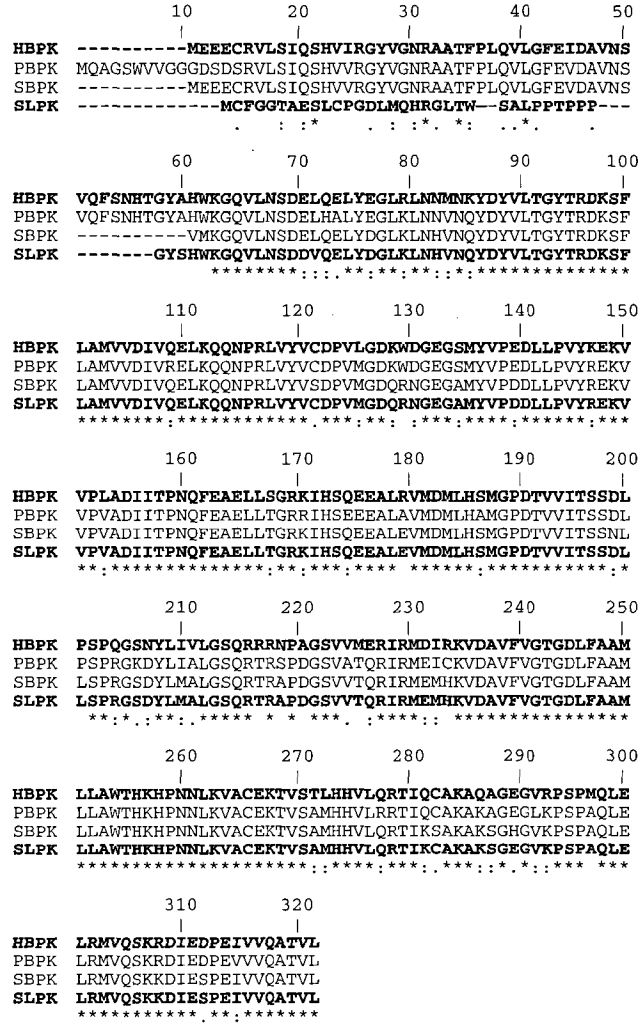

Fig. 2. Comparison of the amino acids sequence of mammalian pyridoxal kinases. The sequence for human brain pyridoxal kinase (HBPK) is from the present study and GenBank accession No.U89606, porcine brain enzyme (PBPK) from GenBank accession No. AF041255, sheep brain enzyme (SBPK) from direct protein sequencing, and sheep liver enzyme (SLPK) from the present study. Dashes indicate the gaps introduced to maximize alignment. Asterisks represent identical amino acids, double dots and single dots are high similarity and low similarity, respectively, and blank spaces are no homology.

kinase was reported by Hanna *et al.* (1997). It was found that the two sequences are completely identical.

Comparison of the amino acids sequence of ovine pyridoxal kinase with other mammalian enzymes, including the human pyridoxal kinase, is shown in Fig. 2. The amino acids sequence of ovine liver pyridoxal kinase bears remarkable homology with the human enzyme, and the overall sequence identity is approximately 77%. The C-terminal region, proposed to be indispensable for catalysis, is well conserved. In contrast, the N-terminus sequence of 32 amino acid residues is considerably different from those of the human enzyme. This result suggests that N-teminus sequences of pyridoxal kinase vary more than C-terminus sequences. It is interesting to note that the porcine brain enzyme (PBPK) also appears to have low similarity of the N-terminus due to additional residues in the region (Gao *et al.,* 1998).

The complete primary structure of the ovine brain enzyme was determined very recently by the Edman degradation method (Maras *et al.,* 1999). The sequence showed a high degree of overall similarity (86% identity) with the human enzyme. However, comparison of the sequence deduced from the ovine liver cDNA (SLPK) with the sequence obtained from ovine brain enzyme by direct protein sequencing (SBPK) showed some differences. As shown in Fig. 2, most are minor. At positions 71 and 72, cDNA shows aspartate and valine, whereas the peptide analysis indicates glutamate and leucine, respectively, and at position 199, aspartate is indicated instead of asparagine. These differences appear to be simply a trivial error arising from the two different approaches. However, a major difference between the two structures occurs at the N-terminus. Again, the N-terminal sequence of the liver

enzyme shows essentially no identity with that of the brain enzyme. Careful reconsideration with another full-length cDNA clone isolated independently from the ovine liver library confirmed that the sequence presented in Fig. 1 was correct. Although the possibility of a chimeric clone resulting from a cDNA construction error cannot be ruled out, the N-terminal discrepancy between the two structures could reflect the use of a genetic heterogeneity or the existence of an organ- specific isozyme. Further studies such as the isolation of a genomic clone and Northern blot analysis using the isolated clone should help to resolve this question. The availability of recombinant pyridoxal kinase will allow future studies using mutagenesis to further define the relationship between structure and catalytic mechanism of this enzyme.

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