cDNA Cloning and Expression of Pig Cytosolic Aspartate Aminotransferase in *Escherichia coli*: Amino-Terminal Heterogeneity of Expressed Products and Lack of Its Correlation with Enzyme Function†

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ABSTRACT: A full-length cDNA encoding the pig cytosolic aspartate aminotransferase (EC 2.6.1.1) (cAspAT) was constructed from two overlapping cDNA clones. One clone (Lm pcAAT-8) isolated from a λ gt10 pig heart cDNA library contained a 3′ untranslated sequence, a poly(A) segment, and a part of the coding region for amino acid positions 127–412. Another clone (Lm pcAAT-107) isolated from a λ gt10 primer extension library contained the coding region for amino acid positions 1–148 and a 5′ untranslated sequence. Rejoining of the cDNA inserts of the two clones and recloning into pUC18 gave rise to a cDNA covering an entire coding sequence for pig cAspAT mRNA. Insertion into pKK223-3 yielded an expression plasmid, ppcAAT200. *Escherichia coli* JM105 cells transfected with ppcAAT200 overproduced pig cAspAT to an extent of about 3% of the total cellular soluble proteins. The expressed product was indistinguishable from the α subform of cAspAT isolated from pig heart in terms of specific activity, absorption spectra, molecular size, crystalline form, and immunological reactivity with anti pig cAspAT antibody. Compared with the amino-terminal sequence (Ala-Pro-Pro-) reported for pig heart cAspAT, the recombinant pig cAspAT showed heterogeneity in the amino-terminal sequence: Ala1 (26%), Pro2 (54%), and Pro3 (19%). Construction of a mutant cAspAT with deletion of residues 1–3 and its comparison with the wild-type enzyme revealed that loss of the three amino-terminal residues does not affect the catalytic activity and structural integrity of the enzyme.

Cytosolic and mitochondrial isoenzymes of aspartate aminotransferase (EC 2.6.1.1) (AspAT)[1] have been extensively studied from both structural and functional aspects. The three-dimensional structure has been elucidated, particularly for the pig cytosolic isoenzyme (Arnone et al., 1985a) and chicken mitochondrial isoenzyme (Jansonius et al., 1985). Possible catalytic roles for several amino acid residues within the active site have been proposed on the basis of abundant information derived from the enzymological and crystallographic studies (Arnone et al., 1985b). Recent progress in recombinant DNA techniques has made an enzyme amenable to site-specific amino acid substitution studies that will aid in gaining an insight into the functions of catalytically and structurally critical amino acid residues. Prerequisites to this goal are, first, the cloning of cDNA for the enzyme into an expression vector and, second, systematic amino acid substitution by oligonucleotide-directed, site-specific mutagenesis. For an accurate interpretation of the consequences of alteration of target residues, it is imperative to distinguish between its primary and secondary effects on the catalytic events. This can only be performed by the crystallographic analysis of the mutated enzyme and comparison with that of the nonmutated enzyme. As a first step toward this ultimate goal, we attempted to isolate the cDNA clone for pig cytosolic AspAT (cAspAT) and establish its expression system in *Escherichia coli*.

Experimental Procedures

Materials. Restriction enzymes, DNA polymerase I (Klenow fragment), T4 polynucleotide kinase, DNA ligation kit, DNA sequence kit, and M13 reverse sequencing primer RV were obtained from Takara Shuzo Co. Ltd. (Kyoto). Calf intestinal alkaline phosphatase was obtained from Boehringer Mannhein. [γ-^{32}P]ATP (6000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from New England Nuclear and Amersham Corp., respectively. Peroxidase-conjugated sheep anti rabbit IgG antibody was obtained from Organon Teknika Corp-Cappel Products. Protease 401 from *Streptomyces violaceochromogenes* (Murao et al., 1984) was a gift of Dr. S. Murao.

Bacterial Strains, Plasmid, and Media. *E. coli* strain HB101 was used for screening the Okayama-Berg cDNA library. *E. coli* strains L87 and MN514[hfl$^+$] were used for λ gt10 phage library screening. *E. coli* JM105 was used for expression of pig cAspAT. pUC18 (Ap^r) and pKK223-3 (Ap^r; expression vector containing the *tac* promoter and *rrn*B ribosome RNA terminator) were obtained from Pharmacia. A

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[1] Abbreviations: AspAT, aspartate aminotransferase; cAspAT, cytosolic aspartate aminotransferase; mAspAT, mitochondrial aspartate aminotransferase; SD, Shine–Dalgarno; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SSC, standard saline citrate; ppcAAT, plasmid carrying pig cAspAT cDNA; Lm pcAAT, λ gt10 phage carrying cAspAT cDNA; kb, kilobase pairs; bp, base pairs; IPTG, isopropyl β-D-thiogalactoside; A_{550}, absorbance at 550 nm; CCB, Coomassie brilliant blue; PMSF, phenylmethanesulfonyl fluoride; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; EDTA, ethylenediaminetetraacetate.

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                140                 145
                Trp Glu Asn His Asn Gly
        mRNA 5'-UGG GAA AAU CAU AAU GG -3'
                    G   C   C   C
a,   Probe 3'-ACC CTT TTA GTA TTA CC -5'
                    C   G   G   G

                140                             148
                Trp Glu Asn His Asn Gly Val Phe Thr
        cDNA 5'-TGG GAA AAT CAC AAT GGA GTC TTC AC -3'
b,  Primer 3'-ACC CTT TTA GTG TTA CCT CAG AAG TG -5'

                127                     133
                Asn Asn Lys Asp Thr Pro Val
        cDNA 5'-AAC AAC AAA GAC ACG CCT GT -3'
c,   Probe 3'-TTG TTG TTT CTG TGC GGA CA -5'
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FIGURE 1: Synthetic oligodeoxyribonucleotides used for screening and priming cDNA libraries. Each oligodeoxyribonucleotide was synthesized according to the amino acid sequence of pig cAspAT as noted above the nucleotide sequence. (a) Probe for screening an Okayama-Berg cDNA library from pig liver; (b) primer used for construction of a primer extension library from pig heart; (c) probe for screening the primer extension library.

cDNA synthesis/cloning system λ gt10 was purchased from Amersham. PYG broth (Miki et al., 1987) was used for the expression of cAspAT.

Preparation of RNA. Extraction of total RNA from pig heart was carried out by using guanidine thiocyanate procedures (Chirgwin et al., 1979). Poly(A)+ RNA was prepared by oligo(dT)–cellulose (P-L Biochemicals) chromatography, and the yield was about 5% of the total RNA.

Oligodeoxyribonucleotide Synthesis. Oligodeoxyribonucleotides were synthesized by the β-cyanoethylphosphoramidite method on a Beckman System 1 Plus DNA synthesizer. After treatment with ammonia, 5′-(dimethoxytrityl)oligodeoxyribonucleotides were purified on a Sep-Pack C18 column (Millipore Co. Ltd.) as described by Lo et al. (1984).

A mixture of the 16 oligodeoxyribonucleotides (17 base long) was synthesized against the known pig cAspAT amino acid sequence (Ovchinnikov et al., 1973) from positions 140 to 145 (Figure 1a). A 26-mer oligodeoxyribonucleotide, corresponding to the protein sequence from positions 140 to 148, was synthesized as a primer for construction of a primer extension library (Figure 1b), and a 20-mer oligodeoxyribonucleotide, corresponding to the protein sequence from positions 127 to 132, was synthesized as a screening probe (Figure 1c).

Nucleic Acid Labeling. The *Xho*I/*Ava*II and *Pst*I/*Sca*I fragments used as hybridization probes were excised from the recombinant plasmids containing mouse cAspAT cDNA and pig cAspAT cDNA (Obaru et al., 1986), respectively. Nick translation was performed according to the method of Rigby et al. (1977). An oligodeoxyribonucleotide probe was labeled with ^{32}P by using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Construction of Pig Heart cDNA Library. A pig heart cDNA library was constructed by using cDNA synthesis/cloning system λ gt10, according to the manufacturer's manuals. About 1 μg of cDNAs was obtained from 5 μg of pig heart poly(A)+ RNA as a template. One-tenth of cDNAs thus obtained was inserted into λ gt10 arms and transfected to NM514.

Construction of Primer Extension Library. The use of 5 μg of poly(A)+ RNA as a template and 0.5 μg of a 26-mer oligodeoxyribonucleotide (corresponding to the protein sequence from 140 to 148) (see Figure 1b) as a specific primer yielded about 0.5 μg of cDNAs. About 0.1 μg of cDNAs was inserted into λ gt10 and was transfected to NM514. The manipulations were the same as described above.

Screening for Clones Containing cAspAT cDNA. The pig liver cDNA library used here had been constructed in our laboratory (Obaru et al., 1986) according to the method of Okayama and Berg (1982). The transformed clones were screened by the in situ colony hybridization method (Grunstein & Hogness, 1975) using the ^{32}P-labeled mixed 17-mer oligodeoxyribonucleotide probe (Figure 1a).

The pig heart cDNA library in λ gt10 phage was screened by the in situ plaque hybridization method described by Maniatis et al. (1982). The filters were hybridized at 42 °C with a nick-translated *Xho*I/*Ava*II fragment (350 bp) derived from mouse cAspAT cDNA (Obaru et al., 1986).

The pig heart primer extension library was screened by the in situ plaque hybridization method. The filters were hybridized at 37 °C with the ^{32}P-labeled synthetic oligodeoxyribonucleotide probe (Figure 1c).

Characterization of cDNA Clones and Sequence Analysis. cDNA inserts in phage libraries were subclones into pUC18. Recombinant plasmids from selected colonies were purified and digested with various restriction enzymes and electrophoresed in agarose gels to determine the size. DNA sequence was determined according to the method of Maxam and Gilbert (1980) for ppcAAT-1 and by the dideoxy chain termination method using denatured plasmid template as described by Hattori and Sakaki (1986), after subcloning into a plasmid pUC18, for ppcAAT-1, Lm pcAAT-17, Lm pcAAT-8, and Lm pcAAT-107 (see Results).

Cell Growth and Preparation of Extracts. JM105 cells harboring the plasmid ppcAAT200 (see Results) were grown in PYG broth. The culture was diluted 20 times with the same medium and incubated at 37 °C on a rotary shaker. At a cell density A_{550} (absorbance at 550 nm) of 0.8 (1-cm light path), isopropyl β-D-thiogalactoside (IPTG) was added to a concentration of 1.0 mM, and incubation was continued for 22 h. Cells grown in 100 mL of culture media were collected by centrifugation and suspended in 2 mL of a solution containing 50 mM Tris-HCl buffer (pH 7.0), 1 mM EDTA, 0.05 mM pyridoxal 5′-phosphate, 1 mM *o*-phenanthroline, and 1 mM PMSF. Suspensions were mixed with glass beads (0.25–0.45 mm in diameter) for 5 min under stirring. Cell debris and glass beads were removed by centrifugation. The supernatant solutions were examined for enzyme activity and subjected to SDS–polyacrylamide electrophoresis (SDS–PAGE).

Enzyme Assay. The reaction mixture for the enzyme assay contained in a total volume of 1 mL 20 mM L-aspartate, 10 mM 2-oxoglutarate, and 0.1 M Tris-HCl buffer (pH 8.0). The reaction was started by adding an enzyme sample and carried out at 25 °C. The increase in absorbance at 280 nm ($\epsilon_{oxalacetate}$ = 570 M^{-1} cm^{-1}) was recorded on a Hitachi 200 spectrophotometer. One enzyme unit was defined as the amount of enzyme producing 1 μmol of oxalacetate/min at 25 °C. Specific activity was expressed as the number of units per milligram of protein. The maximal velocity (V_{max}) and Michaelis constants (K_m) for substrates were determined by double-reciprocal plots of initial velocities versus substrate concentrations.

Purification of Pig cAspAT Expressed in E. coli. Ten-liter cultures of *E. coli* JM105 cells harboring plasmid ppcAAT200 grown as described above yielded 40 g of cells (wet weight). Cells were disrupted in an X-press Model X-25 (BIOX, Stockholm) and extracted with the buffer solution, as described above. The specific activity of the enzyme in extracts was 5.8 units/mg of protein. Ammonium sulfate fractionation and DEAE-Sephadex column chromatography were performed as described for the purification of cAspAT from pig heart

(Morino et al., 1977). Subsequent chromatographies on a butyl-Toyopearl and a Sephacryl S200 column yielded 59.5 mg (77% yield) of pure enzyme with a specific activity of 280 units/mg of protein (see Table I).

Purification of the α Subform of cAspAT from Pig Heart. The α subform of cAspAT was purified from pig heart muscle as described by Martinez-Carrion et al. (1967) and Morino et al. (1977).

SDS–PAGE. SDS–PAGE was performed by using 12.5% acrylamide in slab gels and was stained with CBB R-250. In some cases, the bands were transferred to a Millipore membrane, GVHP, by using a Zartorius semidry blotter and detected immunologically.

Immunochemical Analysis. Anti-cAspAT antibody was prepared as described (Teranishi et al., 1978). Immunoblotting was performed by a modification of the procedures described by Kyhse-Andersen (1984). Double-immunodiffusion analysis on agar gel was performed as described (Ouchterlony, 1949).

Crystallization. The recombinant pig cAspAT was crystallized by vapor diffusion of solutions containing about 30 mg/mL of enzyme sample in 4% polyethylene glycol (Carbowax PEG 6000) (McPherson, 1976) as described by Metzler et al. (1978) for pig heart cAspAT.

Site-Directed Mutagenesis. A 1.4-kb *Eco*RI/*Eco*RI fragment covering the entire coding region for pig cAspAT was excised from ppcAAT200 and inserted into phage M13mp9. Synthetic oligonucleotide used to generate a mutant cAspAT with deletion at positions 1–3 was AATTCATGTCAGCTCTTT, in which the initiation codon ATG is followed by the codons for Ser, Val, and Phe at positions 4, 5, and 6, respectively, of pig cAspAT. The mutant was constructed by the gappled duplex method (Kramer et al., 1983).

Amino-Terminal Sequence Determination. This was performed by an automated gas-phase protein sequencer (Applied Biosystems Model 470A) equipped with an online HPLC, Model 120A.

pH Titration of Internal Aldimine Schiff Base. The pyridoxal form of purified cAspAT (wild-type or mutant enzyme) (20 mg/mL) was dialyzed against 1 L of 10 mM PIPES buffer (pH 6.5) containing 0.01 mM dithiothreitol and 0.01 mM EDTA. Dialyzed enzymes were diluted to 0.6 mg/mL by 50 mM good buffers containing 50 mM KCl. Good buffers used were MES (pH 4.6–6.2), PIPES (pH 6.2–7.5), and TAPS (pH 7.5–8.5).

Reaction with Protease 401. The reaction mixture contained 0.2 mg of the pyridoxal form of wild-type or mutant cAspAT and 2 μg of protease 401 in 0.2 mL of 50 mM Tris-HCl buffer (pH 8.5) and was incubated at 25 °C. At various times, aliquots of 20 μL were withdrawn and assayed for transamination activity. A pseudo-first-order rate constant was obtained on the basis of the relation $k = 0.693/t_{1/2}$, where $t_{1/2}$ is the half-time for inactivation.

Thermal Stability. The reaction mixture contained 0.6 mg of the pyridoxal form of wild-type or mutant cAspAT and 50 mM NaCl in 1 mL of 50 mM MES buffer (pH 6.0) and was kept at 76 °C. At various times, aliquots of 20 μL were withdrawn and assayed for the enzyme activity. A pseudo-first-order rate constant for inactivation was obtained as described above.

All of the cloning procedures were conducted in accord with the guidelines for research involving recombinant DNA molecules issued by the Ministry of Education, Science and Culture of Japan.

Results

Isolation and Characterization of Pig cAspAT cDNA Clones. The cAspAT cDNA clone isolated from a pig liver cDNA library (see Experimental Procedures), designated ppcAAT-1, carried a cDNA insert of about 1.7 kb in length. This clone lacked the 5′ part of the coding sequence that corresponded to the 136 amino-terminal amino acid residues of cAspAT, as shown by the later sequence analysis.

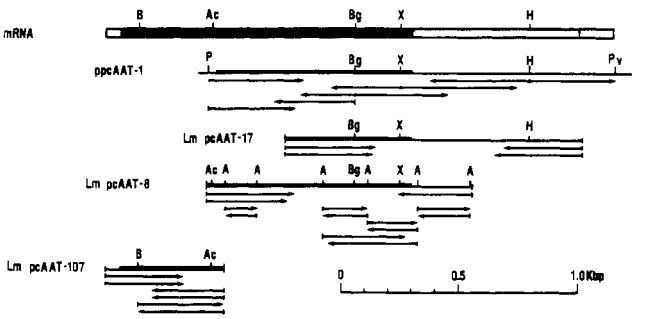

FIGURE 2: Restriction maps and sequence strategy for cAspAT cDNA clones. The sequence of ppcAAT-1 was determined according to the method of Maxam and Gilbert (1980), except for the *Pst*I/*Bgl*II fragment. Other sequences were determined by the dideoxy chain termination method using denatured plasmid template. Some parts (65%) of the sequence were determined on both strands of cDNA, and other parts of the sequence were confirmed by duplicated sequence reactions. Sequencing strategy is summarized beneath the maps with solid dots (for the Maxam–Gilbert method) or short vertical bars (for the dideoxy method) indicating positions of 5′-end labeling and horizontal arrows indicating direction and extent of sequencing from a given labeled end. The structure at the top represents a full-length pig cAspAT cDNA constructed by appropriate combination of cDNA inserts of Lm pcAAT-8, Lm pcAAT-17, and Lm pcAAT-107. The thick region represents the coding region; the hatched region, the 5′ and 3′ untranslated regions; and the open region, poly(A) tract. Plasmid ppcAAT-1 isolated from a pig liver cDNA library comprised the region encoding a partial amino acid sequence from positions 138 to 413. The coding region contained in Lm pcAAT-8 insert corresponded to the amino acid sequence of cAspAT from positions 127 to 413. Lm pcAAT-107 isolated from a primer extension library constructed from pig heart mRNAs contained the coding region for the amino acid sequence from positions 1 to 148. Thick bars denote the coding regions; thin bars, 5′ and 3′ untranslated regions. (B) *Bam*HI; (Ac) *Acc*I; (Bg) *Bgl*II; (H) *Hin*dIII; (P) *Pst*I; (Pv) *Pvu*II; (A) *Alu*I. The scale at the bottom is for the nucleotide position.

In an attempt to obtain a clone carrying a full-length cAspAT cDNA, a λ gt10 pig heart cDNA library was screened by using the 350-bp-long *Xho*I/*Ava*II fragment of mouse cAspAT cDNA (Obaru et al., 1986). Several positive clones were isolated by screening 3×10^5 plaques. One clone, Lm pcAAT-8, contained a cDNA insert of about 1.1 kb in length. This clone was composed of a 3′ untranslated sequence (269 bp), a poly(A) segment, and the coding sequence that had a sequence extending to the codon for the amino acid residue at position 127 of cAspAT (Figures 2 and 3). Another clone, Lm pcAAT-17, with a cDNA insert of 1.2 kb, covered a 3′ untranslated sequence (709 bp), a poly(A) segment, and a part of the protein-coding sequence that corresponded to the 166 carboxy-terminal amino acid residues (Figures 2 and 3).

To obtain cDNA clones carrying the 5′ untranslated region, a λ gt10 primer extension library was constructed from pig heart poly(A)+ RNA. Four positive clones were isolated by screening 5×10^4 clones. The insert of the longest cDNA clone, designated Lm pcAAT-107, was subcloned into a plasmid vector, pUC18. This clone possessed a cDNA insert that covered the coding region missing in Lm pcAAT-8 and a further extended 5′ untranslated sequence (53 bp) of the cAspAT mRNA (Figure 2).

Nucleotide Sequence and Predicted Amino Acid Sequence. The restriction maps of cAspAT cDNAs and sequence analysis strategy are shown in Figure 2. The nucleotide and predicted amino acid sequences are shown in Figure 3. All clones except

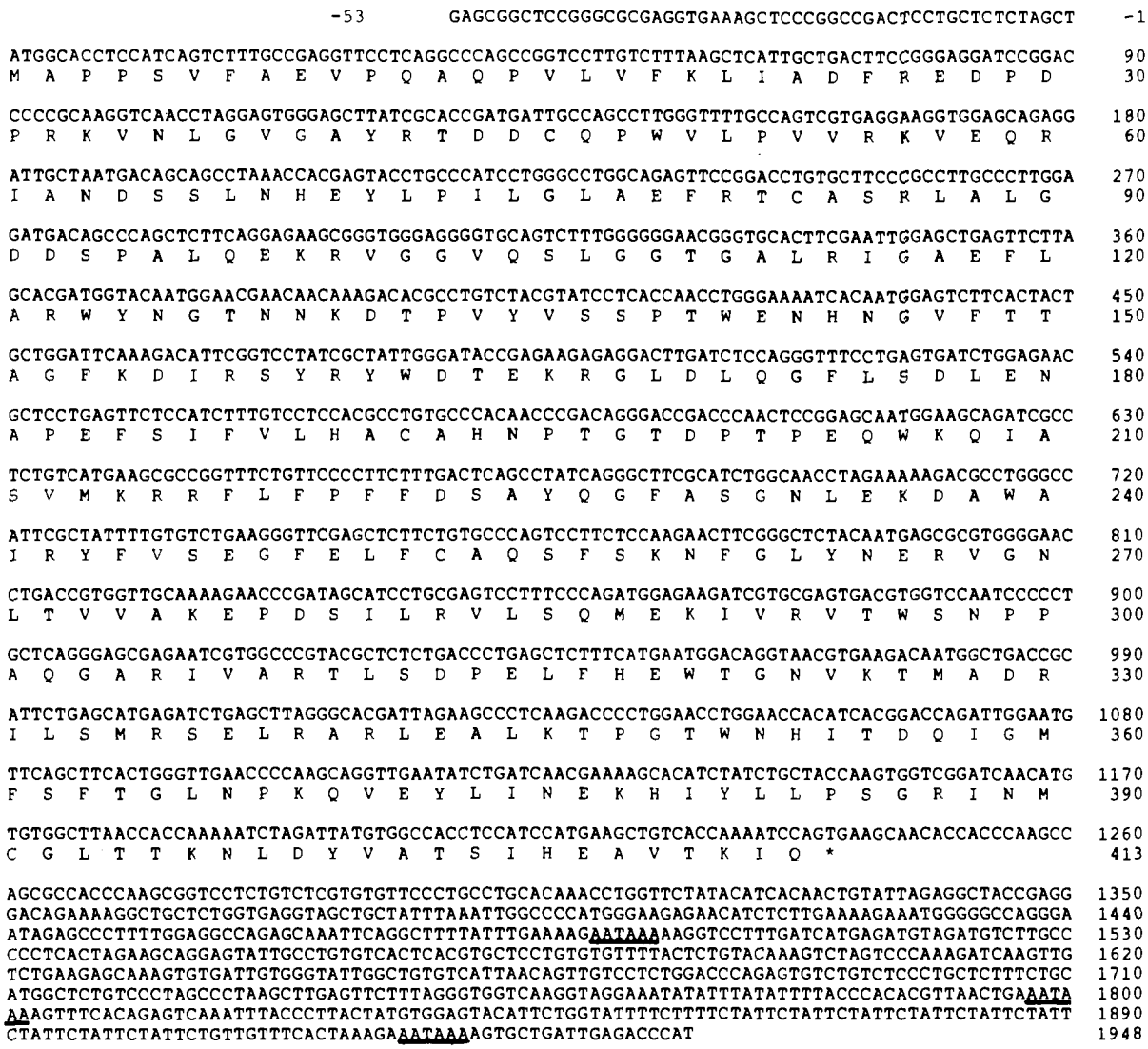

FIGURE 3: Nucleotide and deduced amino acid sequences for pig cAspAT cDNA. Amino acid residues are shown by one-letter notation. For both the nucleotide and amino acid sequences, positive numbers start at the initiation methionine residue/codon. The underlined sequences in the 3′ untranslated region are potential poly(A) addition signals.

Lm pcAAT-107 contained in their 3′ untranslated regions one poly(A) tract and either single or triple AATAAA sequences. This indicated that all of the three cDNAs covered an entire 3′ untranslated region of the pig cAspAT mRNA. Although the 3′ untranslated sequence of Lm pcAAT-8 was shorter than that of other two clones, this clone also possessed an AATAAA sequence and a poly(A) tract. In contrast, ppcAAT-1 and Lm pcAAT-17 contained three AATAAA sequences (Figure 3). The initiation site for translation was assigned to the methionine codon ATG at nucleotide positions 1–3 (Figure 3). The amino acid sequence deduced from the nucleotide sequence agree completely with the sequence determined from peptides (Ovchinnikov et al., 1973) except for the initiation methionine (Figure 3). The cDNA contained the 5′ untranslated sequence (53 bp), the protein coding sequence (1239 bp), and the 3′ untranslated sequence (709 bp). In the 5′ untranslated region, 5-bp direct repeats were found at nucleotide positions −49 and −28. Three putative polyadenylation signals, AATAAA, were present at positions 1490, 1797, and 1924. The first hexanucleotide presumably functions as a polyadenylation signal in the generation of mRNA cloned in Lm pcAAT-8, while the third one functions for those cloned in ppcAAT-1 and Lm pcAAT-17. At nucleotide position 1859, an unusual block of $(TTCTA)_9$ was observed. There were TATTT sequences at nucleotide positions 1479, 1768, 1774, and 1850 (Figure 3). The significance of this repeat was not determined.

Construction of Expression Plasmid for Pig cAspAT in E. coli. Prior to construction of an expression plasmid, a full-length cDNA for pig cAspAT was constructed by combining the two cDNAs excised from Lm pcAAT-8 and Lm pcAAT-107. For this purpose, these two cDNAs were subcloned separately into pUC18 and were designated ppcAAT-8 and ppcAAT-107, respectively. The 444-bp *Eco*RI/*Acc*I fragment of the ppcAAT-107 (nucleotide sequence from −53 to 390, Figure 3) was inserted into *Eco*RI/*Acc*I double-digested pUC18. The resulting clone, ppcAAT-10, was then digested by *Acc*I and *Hin*dIII and was ligated with the 1117-bp *Acc*I/*Hin*dIII fragment (nucleotide positions 391–1508, see Figure 3) of ppcAAT-8 to yield ppcAAT-100 (Figure 4). The ppcAAT-100 contained the entire coding region for pig cAspAT and unique restriction enzyme sites for *Ban*I and *Nco*I. The *Ban*I site was located between the first methionine and the second alanine codons. The *Nco*I site was at the 3′ untranslated region, 155 bp downstream from the termination codon. The initiation codon ATG triplet was incorporated into the *Ban*I/*Nco*I fragment as follows. The *Ban*I/*Nco*I fragment was treated with the Klenow fragment and cloned into ppcAAT100, previously cleaved by *Nco*I, dephosphorylated, and then repaired with the Klenow fragment. The resulting ppcAAT101 was digested by *Nco*I to yield the *Nco*I/*Nco*I

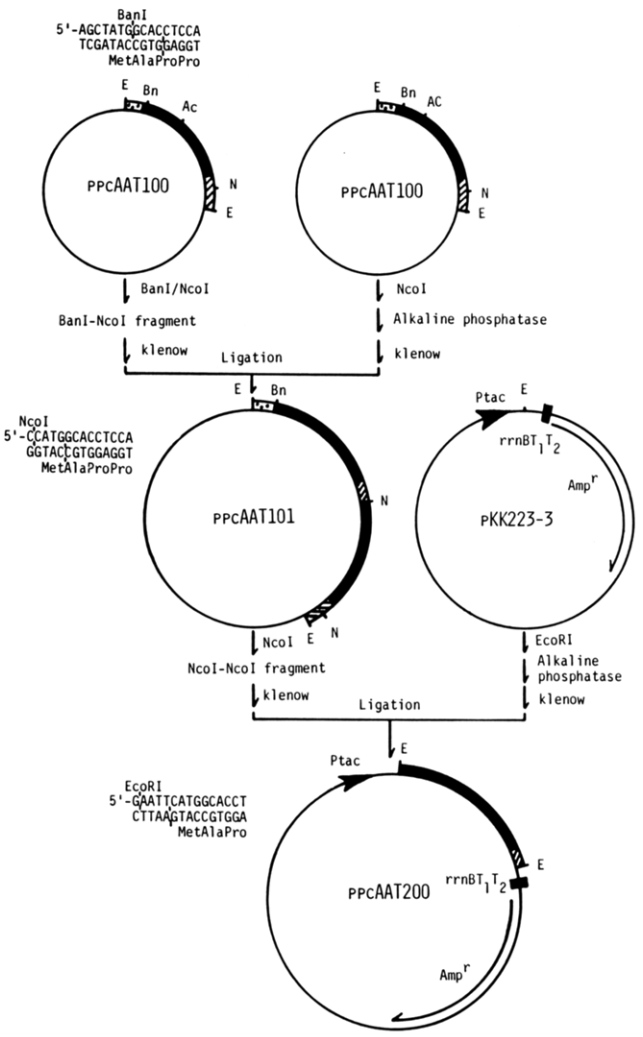

FIGURE 4: Construction of a plasmid expressing pig cAspAT in *E. coli*. The *Ban*I/*Nco*I fragment excised from ppcAAT100 contains an entire coding region for pig cAspAT, except for the initiator methionine codon. Insertion of this fragment into a *Nco*I-digested ppcAAT100 introduced an *Nco*I site as well as an ATG codon just before the alanine codon to yield ppcAAT101. A *Nco*I/*Nco*I fragment from ppcAAT101 was then inserted into an *Eco*RI site in the multicloning site of pKK223-3. Plasmid pKK223-2 comprises a *tac* promoter (P_{tac}), a Shine–Dalgarno (SD) sequence, a multicloning site, an *rrn*B terminator, and a segment from pBR322 containing an origin of replication and ampicillin resistance gene. Resulting recombinant plasmids were screened, and a clone, ppcAAT200, with the correct insertion was selected on the basis of restriction mapping data. Partial nucleotide sequence analysis revealed that the distance between the SD sequence and the ATG codon in plasmid ppcAAT200 is 10 bp long (*AGGA*AACAGAATTC*ATG*).

fragment, which contained the initiation codon. The *Nco*I/*Nco*I fragment was then treated with the Klenow fragment and inserted into the repaired (Klenow fragment) *Eco*RI site of pKK223-3. The resulting recombinant plasmids were screened. One clone, ppcAAT200 (Figure 4), was selected on the basis of the results of restriction mapping, which were consistent with the correct insertion of the initiation methionine codon followed by the entire coding sequence just downstream of the *tac* promoter and the SD sequence of pKK223-3. *E. coli* cells harboring this plasmid were found to overproduce cytosolic AspAT, as shown below.

Production of Pig cAspAT in E. coli JM105. JM105 cells harboring ppcAAT200 were grown overnight at 37 °C in PYG broth. The culture was diluted 20-fold and incubated at 37 °C. At a cell density A_{550} of 0.8, the culture was divided into two portions. To one portion was added IPTG at a concentration of 1 mM, and the culture was incubated for an additional 22 h at 37 °C. The other portion of the culture without the inducer was similarly incubated. Extracts were prepared from harvested cells and analyzed on SDS–PAGE. The protein band corresponding in mobility to cAspAT was hardly detectable upon staining with CBB. However, immunoblotting clearly revealed an immunoreactive protein band that comigrated with cAspAT purified from pig heart (data not shown). The enzyme assay revealed that the extract from the IPTG-induced cells had an activity of 9.35 units/mg of protein. The extract prepared from noninduced cells showed an activity of only 0.39 unit/mg. The latter value was the same as that for nontransfected cells. The amount of pig cAspAT expressed in the IPTG-induced cells was estimated to be about 3% of the total soluble *E. coli* proteins.

Table I: Purification of Pig cAspAT Expressed in *E. coli*[a]

step	vol (mL)	act. (units/mL)	proteins (mg/mL)	spec act. (units/mg)	yield (%)
crude extracts	340	63.1	10.9	5.8	100
$(NH_4)_2SO_4$ fractionation[b]	38	554	49	11.3	98
DEAE-Sephadex[c]	145	126	1.54	82.8	85
butyl-Toyopearl[d]	30	615	3.15	195	86
Sephacryl-200[e]	26	640	2.29	279[f]	77

[a] Crude extracts were obtained from 40 g (wet weight) of cells from 10-L cultures. [b] Precipitates at 45–75% saturation. [c] DEAE-Sephadex column (4 × 17 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4) containing 10 mM potassium succinate. Elution with the same buffer. [d] Butyl-Toyopearl column (1.6 × 21.5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 1.5 M $(NH_2)_2SO_4$. Elution with a gradient from 1.5 to 0 M $(NH_4)_2SO_4$. [e] Sephacryl-200 column (2.1 × 38 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 M KCl. Elution with the same buffer. [f] Based on A_{280nm} = 1.25 for a 1.0 mg/mL solution of the pure enzyme.

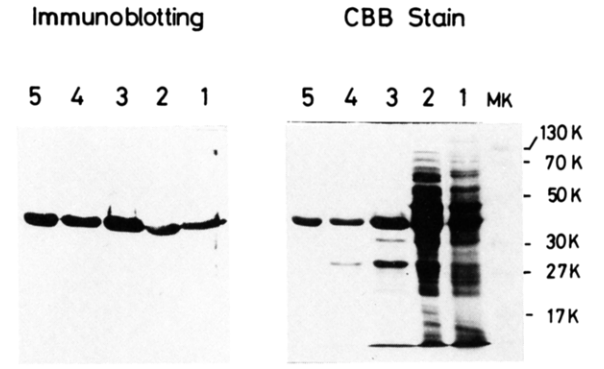

FIGURE 5: Electrophoretic (right) and immunological (left) characterization of proteins during purification of the recombinant pig cAspAT. SDS–PAGE was performed as described under Experimental Procedures. (Right, CCB stain) (lane 1) crude extracts from *E. coli* JM105 cells transformed with ppcAAT200; (lane 2) ammonium sulfate fraction (40–65% saturation); (lane 3) eluate from a DEAE-Sephadex column; (lane 4) eluate from a butyl-Toyopearl column; (lane 5) fraction from a Sephacryl 200 column. Lane MK shows the patterns for the proteins used as molecular mass standards. (Left, immunoblotting) numbering corresponds to those for the CBB stain patterns.

Expression Product. Purification of pig cAspAT overproduced in *E. coli* is summarized in Table I. Figure 5 shows SDS–PAGE patterns of proteins at various stages of purification of the recombinant cAspAT. Only the 44K band was reactive with the anti pig cAspAT antiserum. The mobility conforms to the molecular weight (46 344) for the subunit of pig cAspAT. Purified preparations of pig cAspAT overproduced in *E. coli* showed a specific activity of 280 units/mg of protein, which corresponds to the value for the α subform

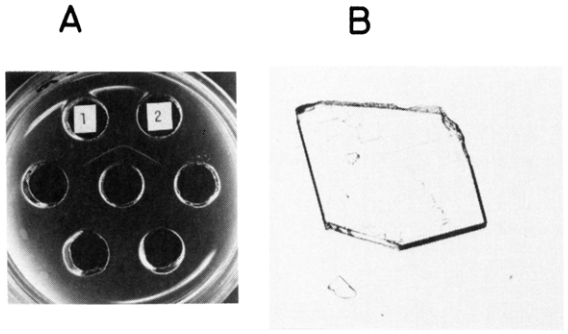

FIGURE 6: Structural comparison of pig cAspAT produced in *E. coli* with cAspAT purified from pig heart. (A) Double-diffusion analysis on agar gel. (Center well) Anti pig cAspAT antiserum; (well 1) recombinant pig cAspAT; (well 2) cAspAT purified from pig heart. (B) Crystal of recombinant cAspAT.

of cAspAT purified from pig heart. The pyridoxal form of the enzyme showed an absorbance ratio A_{430nm}/A_{340nm} of 4.8 in 0.1 M sodium acetate buffer (pH 5.0), a value that represents the typical spectral property of the α subform (Martinez-Carrion et al., 1967). Upon double-immunodiffusion analysis, the recombinant pig cAspAT formed a single precipitation line that fused with that for cAspAT purified from pig heart (Figure 6A). The recombinant cAspAT was readily crystallized from polyethylene glycol (Figure 6B) and was identical in crystalline form with that purified from the pig heart (Arnone et al., 1977). The amino-terminal sequence reported for the pig heart cAspAT is Ala-Pro-Pro- (Ovchinnikov et al., 1973). In contrast, amino acid sequence analysis of a purified sample of the recombinant cAspAT showed heterogeneity in the amino-terminal structure; amino-terminal residues were Ala1 (26%), Pro2 (54%), and Pro3 (19%). Since this enzyme sample shows a specific activity of 280 units/mg of enzyme, a value identical with that of the α subform purified from pig heart, it appears that loss of the amino-terminal Ala1 and Pro2 may not affect the enzyme activity.

Mutant cAspAT with Deletion at Positions 1–3. To examine whether the deletion of the amino-terminal region as observed with the expression products would affect the enzyme activity or not, a mutant cAspAT with deletion from positions 1 to 3 was designed by using the technique of site-directed mutagenesis. *E. coli* cells harboring the mutant plasmids produced the mutant enzyme at a level similar to that of the wild-type enzyme. The mutant enzyme was thus purified by the same procedure as that used for the purification of the wild-type enzyme. Amino-terminal sequence analysis of the mutant enzyme revealed a single sequence, Ser-Val-Phe-Ala-Glu-Val-Pro-, consistent with the designed deletion of residues 1–3. No trace of methionine was detected. Comparison between the purified preparations of the wild-type and mutant enzymes was made in the following kinetic properties: specific activity for the overall transamination reaction between aspartate and 2-oxoglutarate [wild type and mutant, 280 μmol min^{-1} (mg of protein)$^{-1}$ at 25 °C] and K_m values for substrates (wild type, 1.85 mM for aspartate and 0.08 mM for 2-oxoglutarate; mutant, 1.86 mM for aspartate and 0.07 mM for 2-oxoglutarate at pH 8.0). The pyridoxal form of pig cAspAT shows two absorption bands at 362 and 430 nm that are attributed to the unprotonated and protonated forms of the internal aldimine Shiff base. pH titration of both the wild-type cAspAT and the deletion mutant enzyme showed an identical pK_a value of 6.3, which is also identical with the value reported for pig heart cAspAT (Jenkins et al., 1959).

It has been well documented that protease 401 inactivates pig cAspAT by a strictly specific limited proteolysis at Leu20 (Nagashima et al., 1986) but does not act on the mitochondrial isoenzyme (mAspAT), the amino-terminal segment of which bears a low sequence homology with that of cAspAT. The strict specificity of this protease was utilized to probe into a possible conformational difference that might be induced by loss of residues 1–3 in the amino-terminal segment. The wild-type cAspAT was inactivated in a pseudo-first-order fashion with a rate of 0.063 min^{-1} at a ratio (in weight) of the protease to cAspAT of 1:100 at pH 8.5 and 25 °C. The corresponding value for the mutant enzyme was 0.059 min^{-1} under identical conditions, indicating that loss of the amino-terminal residues 1–3 does not affect the susceptibility of the amino-terminal segment to the protease.

Pig cAspAT is fairly stable at high temperature (Jenkins et al., 1959). Inactivation of the wild-type and mutant cAspAT followed pseudo-first-order kinetics with rates of 0.0033 and 0.0034 min^{-1}, respectively, at 76 °C and pH 6.0, suggesting that deletion of amino-terminal residues 1–3 would not affect the thermal stability of this enzyme.

All of these results demonstrate that the deletion of three amino-terminal residues does not affect the enzyme function and verify that the heterogeneity produced in the amino-terminal structure of the expressed products would be silent in terms of the catalytic activity and the structural integrity of the enzyme.

Discussion

Structure of Pig cAspAT cDNA. We determined the entire coding sequence of pig cAspAT. The predicted amino acid sequence of cAspAT was identical with that determined for the pig heart cAspAT by direct amino acid sequence analysis (Ovchinnikov et al., 1973). The 3′ untranslated region of the cAspAT mRNA contains three putative polyadenylation signals (Figure 3). The presence of at least two types of mRNA for cAspAT is obvious, in terms of the length in the 3′ untranslated region of the cDNA clones isolated in the present study (Figure 2). One type is characterized by functioning of the first hexanucleotide AATAAA at nucleotide positions 1490–1495 and another type by functioning of the third hexanucleotide at positions 1924–1929. However, Northern blot analysis revealed only one radioactive band for cAspAT mRNA at a position corresponding to the size of 2.1 kb (data not shown). Thus, the third hexanucleotide at positions 1924–1929 may be preferentially used as a polyadenylation signal. Difference in size of cAspAT mRNA was not observed between liver and heart (data not shown).

Codon Usage. Codon usage for cAspAT mRNAs of pig, mouse, and rat together with that for the *E. coli* AspAT mRNA is summarized in Table II. Codon utilization for mammalian cAspATs is not random and exhibits a preference for codons ending with G or C. For instance, in the pig cAspAT, 24 codons for valine end in G or C, while 5 codons end in T or A; similarly, 18 codons for threonine end in G or C, while 7 codons end in A or T. The preferred codons are AAG (lysine), GAG (glutamic acid), TTC (phenylalanine), TGT (cysteine), and CAG (glutamine). Such nonrandom codon usage has been observed in genes from animals (Wain-Hobson et al., 1981). Exceptions were observed: 14 codons for alanine end in G or C, while 18 codons end in A or T; similarly, 11 codons for glycine end in G or C, while 17 codons end in A or T. Some codons differ in preference between mammalian cAspATs and *E. coli* AspAT mRNAs. For example, AAA (lysine) is preferred in the *E. coli* AspAT, but AAG is preferred in mammalian AspATs; similarly, GAA (glutamine) is preferred in *E. coli* AspAT, but GAG is preferred in mammalian cAspATs.

Table II: Comparison of Codon Usage in cDNAs for AspATs from Different Species[a]

		P	M	R	E			P	M	R	E			P	M	R	E			P	M	R	E
TTT	Phe	7	10	11	10	TCT	Ser	6	9	7	5	TAT	Tyr	8	5	6	5	TGT	Cys	4	3	3	1
TTC	Phe	16	13	14	10	TCC	Ser	9	7	9	2	TAC	Tyr	4	7	6	6	TGC	Cys	1	2	1	4
TTA	Leu	3	1	1	2	TCA	Ser	3	3	3	0	TAA	end	0	0	0	1	TGA	end	1	1	1	0
TTG	Leu	3	6	6	1	TCG	Ser	0	1	1	1	TAG	end	0	0	0	0	TGG	Trp	9	9	9	5
CTT	Leu	8	3	4	1	CCT	Pro	8	2	4	3	CAT	His	2	4	2	4	CGT	Arg	1	1	1	12
CTC	Leu	8	12	11	4	CCC	Pro	6	9	9	1	CAC	His	6	3	6	2	CGC	Arg	8	5	5	10
CTA	Leu	5	4	4	1	CCA	Pro	6	8	6	5	CAA	Gln	1	2	0	6	CGA	Arg	5	2	4	0
CTG	Leu	11	11	10	29	CCG	Pro	4	6	4	6	CAG	Gln	15	14	15	10	CGG	Arg	6	9	8	0
ATT	Ile	7	9	9	11	ACT	Thr	4	4	5	6	AAT	Asn	7	9	8	8	AGT	Ser	2	2	1	3
ATC	Ile	12	10	9	6	ACC	Thr	12	12	12	9	AAC	Asn	14	14	15	15	AGC	Ser	6	4	5	10
ATA	Ile	0	0	0	0	ACA	Thr	3	6	3	3	AAA	Lys	6	4	5	15	AGA	Arg	3	4	3	0
ATG	Met	7	7	8	8	ACG	Thr	6	0	2	4	AAG	Lys	13	16	16	3	AGG	Arg	3	3	3	0
GTT	Val	4	6	5	10	GCT	Ala	11	14	12	17	GAT	Asp	9	9	9	11	GGT	Gly	4	5	5	8
GTC	Val	11	12	12	3	GCC	Ala	13	11	12	11	GAC	Asp	12	12	12	9	GGC	Gly	4	7	7	17
GTA	Val	1	3	4	4	GCA	Ala	7	3	4	10	GAA	Glu	9	6	6	20	GGA	Gly	13	9	9	1
GTG	Val	13	9	10	9	GCG	Ala	1	6	4	8	GAG	Glu	16	18	19	7	GGG	Gly	7	8	9	4

[a] Numbers show the frequency for codons used. P, pig cAspAT; M, mouse cAspAT; R, rat cAspAT; E, *E. coli* AspAT.

Deletion of Residues 1–3 Does Not Affect the Enzyme Activity. The overall tertiary structure of pig heart cAspAT (Arnone et al., 1985) is extremely similar to chicken heart mAspAT (Jansonius et al., 1985). In both enzymes, each subunit is largely composed of a large domain (residues 76–300) that binds the coenzyme and a small domain (residues 15–47 and 359–410). Upon binding of substrate and its analogues such as 2-methyl aspartate, maleate, and 2-oxoglutarate, the small domain moves by as much as 4–5 Å toward the large domain. This movement results in the closure of the active-site cleft by some parts (residues 15–17 and 37–38) of the amino-terminal segment, which is believed to be prerequisite to catalysis. The end of the amino-terminal segment is anchored at the back of the coenzyme binding domain of the other subunit. Thus, this part of intersubunit interface contributes not only to the integrity of the dimeric structure of the enzyme but also to the substrate-induced movement of the small domain. The present finding that loss of the amino-terminal residues 1–3 did not affect the function of the enzyme indicates that this part of the amino-terminal region would not significantly contribute to the anchoring of the amino-terminal segment to the neighboring subunit. In fact, X-ray analysis shows that the side chains of Val5, Phe6, and Val7 are involved in strongly nonpolar interaction in the intersubunit interface.

Heterogeneity of Recombinant Pig cAspAT Produced in E. coli. Expressed products represent a mixture of at least three species generated by cleavage of the peptide bonds between the initiator methionine and Ala1 (26%), Ala1 and Pro2 (54%), and Pro2 and Pro3 (19%). Candidate exopeptidases responsible for the production of these amino-terminal deletions may by amino-terminal methionine aminopeptidase (Ben-Bassat et al., 1987) and aminopeptidase P (Yoshimoto et al., 1988). The initiator methionine residue would be removed by amino-terminal methionine aminopeptidase, while the cleavage of the bonds, Ala1–Pro2 and Pro2–Pro3, may be catalyzed by aminopeptidase P. Although these aminopeptidases are reported to be inhibitable by EDTA, inclusion of the chelating reagent in the extraction buffer did not completely protect the expression products from aminopeptidase action. It is likely that the cleavage occurs during intracellular synthesis of the protein. Thus, formation of products with heterogeneous amino-terminal deletions could not be avoided. However, this situation would not impose any serious problem in further use of the present expression system for structure–function studies by site-directed mutagenesis technique.

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Registry No. cAspAT, 9000-97-9; pig cAspAT cDNA, 118246-79-0; methionine-initiated pig cAspAT, 118246-82-5; pig cAspAT, 118246-81-4; Pro2 recombinant pig cAspAT, 118246-83-6; Pro3 recombinant pig cAspAT, 118246-84-7; mutant cAspAT cDNA, 118246-80-3; methionine-initiated mutant cAspAT, 118246-85-8; mutant cAspAT, 118246-86-9.

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Inducible (Class 3) Aldehyde Dehydrogenase from Rat Hepatocellular Carcinoma and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Treated Liver: Distant Relationship to the Class 1 and 2 Enzymes from Mammalian Liver Cytosol/Mitochondria†

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ABSTRACT: Peptides from rat liver aldehyde dehydrogenase (AlDH) induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treatment match the AlDH structure from HTC rat hepatoma cells (HTC-AlDH) at all positions examined, indicating induction of the same gene product by two independent routes. This 452 amino acid residue, class 3 AlDH structure differs substantially from the 500-residue AlDH structures isolated from normal liver cytosol (class 1) and mitochondria (class 2). Despite a 29.8% identity in 429 overlapping amino acids vs the human class 1 enzyme (27.7% vs class 2), neither the N- nor C-termini coincide, and gaps are introduced to optimize the alignment. Two residues placed in the active site of human liver AlDH by chemical modification, Cys-302 and Glu-268, are conserved in class 3 AlDH as Cys-243 and Glu-209. Cys-243/302 is the only cysteine residue conserved in *all* known AlDH structures. Gly-245 and Gly-250 of class 1/2 AlDHs, fitting the patterns of glycine residues in coenzyme binding fold of other dehydrogenases, are also conserved. Otherwise, Cys-49, Cys-162, and Glu-487, to which functional importance has also been ascribed, are not retained in the class 3 structure. Overall, a high conservation of Gly, Pro, and Trp and similar patterns of predicted secondary structure indicate general conservation of tertiary structure, as noted with other distantly related proteins. Three exon boundaries from the human liver mitochondria AlDH gene directly correspond to the N-terminus of the rat class 3 protein and to two of the gaps in the alignment. Three contiguous matches also occur within the 16-residue C-terminal "extension" of the class 3 structure vs the hypothetical protein sequence obtaining from the 3′ noncoding sequence of the human mitochondrial gene in the absence of the stop codon. Thus, exon addition, splice junction alterations, and stop codon "migration" appear to have occurred during evolution of the class 1 and 2 structures from an ancestor common to class 3.

Liver aldehyde dehydrogenases (AlDHs)[1] display a wide substrate specificity and notably participate in clearance of ethanol-derived acetaldehyde. In human, bovine, equine, and ovine liver, two NAD-dependent enzyme forms, one cytosolic and one mitochondrial, have been described (Eckfeldt & Yonetani, 1976; Greenfield & Pietruszko, 1977; Crow et al., 1974; Kitabatake et al., 1981). At present, the complete primary structures of the human (Hempel et al., 1984, 1985;

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[1] Abbreviations: HTC, hepatocellular carcinoma; AlDH, aldehyde dehydrogenase (EC 1.2.1.3); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HPLC, high-performance liquid chromatography; TLCK, N^{α}-tosyllysine chloromethyl ketone; DABITC, 4-(*N*,*N*-dimethylamino)azobenzene 4′-isothiocyanate.

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