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Cloning of a Gene for an Acyl-CoA Dehydrogenase from *Pisum sativum* L. and Purification and Characterization of Its Product as an Isovaleryl-CoA Dehydrogenase*

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Isovaleryl-CoA dehydrogenase (IVD, EC 1.3.99.10) catalyzes the third step in the catabolism of leucine in mammals. Deficiency of this enzyme leads to the clinical disorder isovaleric acidemia. IVD has been purified and characterized from human and rat liver, and the x-ray crystallographic structure of purified recombinant human IVD has been reported. Nothing is known about IVD activity in plants, although cDNA clones from *Arabidopsis thaliana* and partial sequences from *Gossypium hirsutum* and *Oryza sativa* have been identified as putative IVDs based on sequence homology and immuno cross-reactivity. In this report we describe the identification and characterization of an IVD from pea, purification of the enzyme using a novel and rapid auxin affinity chromatography matrix, and cloning of the corresponding gene. At the amino acid level, pea IVD is 60% similar to human and rat IVD. The specific activity and abundance of plant IVD was found to be significantly lower than for its human counterpart and exhibits developmental regulation. Substrate specificity of the plant enzyme is similar to the human IVD, and it cross-reacts to anti-human IVD antibodies. Molecular modeling of the pea enzyme based on the structure of human IVD indicates a high degree of structural similarity among these enzymes. Glu-244, shown to function as the catalytic base in human IVD along with most of the amino acids that make up the acyl CoA binding pocket, is conserved in pea IVD. The genomic structure of the plant IVD gene consists of 13 exons and 12 introns, spanning approximately 4 kilobases, and the predicted RNA splicing sites exhibit the extended consensus sequence described for other plant genes.

Isovaleryl-CoA dehydrogenase (IVD[1]; EC 1.3.99.10) is a 172-kDa intramitochondrial homotetrameric flavoenzyme that catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA in the leucine catabolism pathway, transferring the electrons from substrate to the electron-transferring flavoprotein (ETF) (1). IVD is encoded in the nuclear genome and is translated in the cytosol as a 45-kDa precursor that must subsequently be imported into mitochondria, cleaved to a 43-kDa subunit, then assembled into its final 172-kDa tetramer (2). IVD is a member of a family of at least seven homologous enzymes known as the acyl-CoA dehydrogenases. Four of these enzymes catalyze the first step in the β-oxidation of straight chain fatty acids, namely short chain (EC 1.3.99.2), medium chain (EC 1.3.99.3), long chain (EC 1.3.99.13), and the very long chain acyl-CoA dehydrogenase. The remaining members of acyl-CoA dehydrogenase gene family are active in the catabolism of amino acids. These include IVD, short/branched chain acyl-CoA dehydrogenase (which uses 2-methylbutyryl-CoA generated from isoleucine as substrate) and glutaryl-CoA dehydrogenase (active in lysine catabolism) (Ref. 3, and references therein). The substrate specificity of a novel human acyl-CoA dehydrogenase recently identified in a chromosome sequencing project has not yet been characterized (4). The human IVD gene has been completely sequenced and consists of 12 exons separated by 11 introns that span 15 kilobases.

IVD has previously been purified to homogeneity from rat and human liver (1, 5), and the structure of purified recombinant human IVD protein produced in *Escherichia coli* has been solved at a 2.6-Å resolution. In this structure, Glu-254 can be seen to be in position to act as the catalytic base that abstracts the α-proton of substrate, whereas Gly-374, located in the substrate binding pocket (which is a tyrosine in all other acyl-CoA dehydrogenases), appears to be important in conferring branched chain substrate specificity to IVD (6). A number of acyl-CoA dehydrogenases from plants have previously been described (7). In 1995 a partial cDNA sequence from rice (*Oryza sativa*) with homology to IVD was published but was not identified as an IVD (GenBank™ accession number D24729). Recently cDNA clones have been isolated from cotton (*Gossypium hirsutum*, GenBank™ accession AI731517, partial) and *Arabidopsis thaliana* (GenBank™ accession numbers AF160729 and Y12695) that show a similarity of about 60% to both human and rat IVD. No functional or physiological data have been provided for any of these sequences (8). In the current report we describe a new, fast, and highly efficient purification method for plant IVD relying on its auxin binding characteristics (9). Immunologic and enzymatic studies confirm the identity of this enzyme (10). We also describe the genomic structure of the pea IVD gene and its cDNA sequence, both submitted to EMBL (GenBank™ accession numbers AJ010945 (cDNA) and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ010945.

[1] The abbreviations used are: IVD, isovaleryl-CoA dehydrogenase; ETF, electron-transferring flavoprotein; MES, 4-morpholineethanesulfonic acid; 4-OH-PAA, 4-hydroxyphenylacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

AJ010946 (genomic sequence)). Molecular modeling of the three-dimensional structure of the pea enzyme based on the structure of human IVD indicates a high degree of structural similarity between these enzymes, including those residues thought to be important in determining substrate specificity for human IVD.

EXPERIMENTAL PROCEDURES

Plant Material— Seeds of *Pisum sativum* L. (cv. Solara) were sown in moist vermiculite and grown for 5–6 d at 20 °C in the dark. Shoot tips of the etiolated seedlings were harvested, frozen in liquid nitrogen, and stored at −20 °C.

Purification Method via Affinity Chromatography—Apices from etiolated pea seedlings were homogenized in 2 volumes (v/w) of extraction buffer (0.05 M Tris-HCl, pH 7.8, 4 mM diethyldithiocarbamic acid (sodium salt), 3% (w/v) polyvinylpyrrolidone, 1 mM phenylmethyl-sulfonyl fluoride, and 0.5 μg/ml leupeptin) in a Bühler homogenizer. The slurry was filtered through nylon nets (pore size 100 μm and 60 μm) and subjected to ultracentrifugation for 70 min at 4 °C, 150,000 × *g*. The supernatant was precipitated with 50% (w/v) ammonium sulfate and subjected to centrifugation at 20,000 × *g* for 15 min. The resulting pellet was resuspended in 50 mM MES buffer, pH 5.5, incubated 15 min on ice, and centrifuged again for 15 min at 4 °C and 20,000 × *g*. The supernatant was applied to chromatography through an affinity matrix prepared as follows. 4-OH-PAA was coupled to epoxy-activated Sepharose 6B (Amersham Pharmacia Biotech) according to the manufacturer's protocols. Coupling efficiency was determined by measuring UV absorption (250 nm) before and after coupling. 1.5–3 ml of the 4-OH-PAA-coupled beads were packed into a C-10 column (Amersham Pharmacia Biotech). Protein was loaded at 0.5 ml/min onto two affinity columns in parallel and recirculated three times. Columns were washed with 400 ml of 50 mM MES buffer, pH 5.5, overnight. IVD was eluted with 1 M Tris-HCl, pH 8.0. In some experiments, *e.g.* for microsequencing, 0.1 M NaCl was added to both the wash buffer and the elution buffer.

Enzyme Assays—Enzyme activity was determined using the anaerobic ETF fluorescence quenching assay with 50 μM isovaleryl-CoA (Sigma) as substrate as described previously (10). All solutions are prewarmed to 32 °C. The reaction mixture contained 50 mM Tris, pH 8.0, 0.5% glucose, and 50 μM acyl-CoA substrate calculated to give a final volume of 0.8 ml after subsequent additions. The mixture was deaerated by repeated vacuum and layering with oxygen-free argon (10 cycles of 60 s each) in a tightly sealed quartz, semimicro cuvette. Five microliters containing 40 units of glucose oxidase and 1 unit of catalase were added to remove any remaining dissolved oxygen, followed by the addition of purified pig ETF to give a final concentration of 1 μM. The reaction was initiated by adding the desired amount of enzyme sample, and activity was determined by exciting the ETF flavin at 342 nm and monitoring quenching of fluorescence at 496 nm using an LS 50 B luminescence spectrometer with a heated sample block (PerkinElmer Life Sciences). Sample concentration was adjusted to give a pseudo-linear reaction over a time course of 1 min. The slope of the line was calculated using linear regression analysis, and 1 unit was defined as the amount of enzyme needed to completely reduce 1 μmol of ETF/min at 32 °C. The regression line must have achieved a correlation coefficient of −0.95 or greater for the analysis to be accepted (11). For kinetic analyses, the isovaleryl-CoA concentration was varied from 1 to 50 μM; K_m and V_{max} were calculated on a Macintosh computer using the UltraFit software package from Biosoft (Ferguson, MO).

SDS-PAGE and Immunostaining—SDS-PAGE and Western blotting onto activated polyvinylidene difluoride membranes were carried out as described previously (12). For immunostaining, membranes were blocked in 1% (w/v) bovine serum albumin, 0.02% (w/v) NaN_3, and 1% (w/v) fetal calf serum in phosphate-buffered saline overnight. Antibodies were diluted in 0.05% (w/v) BSA, 0.025% (v/v) Tween 20, 0.01% (w/v) NaN_3, and 5% (w/v) lamb serum in phosphate-buffered saline. A polyclonal rabbit serum against human isovaleryl-CoA dehydrogenase (10) was used in a dilution of 1: 1500 as first antibody; goat-anti-rabbit Ig alkaline phosphatase (Sigma) was diluted 1:1000 and used as second antibody. Washing between each step was performed four times with phosphate-buffered saline, and final visualization was performed using the BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) system.

Microsequencing—After SDS-PAGE, proteins were transferred onto an activated polyvinylidene difluoride membrane in Dunn carbonate buffer (13). A derivatization of the cysteine residues was performed according to Fullmer (14). The membrane was washed thoroughly, stained with Coomassie Brilliant Blue R-250 (0.1% (w/v) in 40% (v/v) methanol, 7% (v/v) acetic acid), and the band corresponding to IVD was excised. The membrane slice was incubated for 5 min in a 0.2% (w/v) solution of polyvinylpyrrolidone (PVP 40, Sigma) in methanol, washed twice in water and once in 50 mM Tris-HCl (pH 8.5) and 1 mM EDTA, and incubated overnight at 37 °C in 100 ml of the same buffer containing 1 mg of endoproteinase LysC (Merck, sequencing grade). The digestion solution was transferred to a fresh tube, and the membrane slice was washed once with 50 ml of 70% formic acid and twice with 75 ml water. All solutions were combined, and the proteolytic fragments were separated by narrow-bore high performance liquid chromatography (130A, Applied Biosystems) on a C4 reverse-phase column (Vydac C4, 300Å pore size, 5-mm particle size, 2.1 × 250 mm). The peptides were eluted with a linear gradient (0–100% B in 50 min; solvent A: water, 0.1% trifluoroacetic acid; solvent B: 70% acetonitrile, 0.09% trifluoroacetic acid) at a flow rate of 200 μl/min. Peptide-containing fractions detected at 210 nm were collected manually into siliconized Eppendorf tubes and frozen immediately. A control digestion with a piece of the original blot containing no detectable traces of protein was run in parallel. Peptide sequences were determined by standard Edman degradation using an automatic protein sequencer (model 476A, PE Biosystems). The N-terminal sequence of the protein (21 amino acids) was determined at an initial yield of 18 pmol with an average repetitive yield of 93.5%. The sequence of an internal peptide of p44 obtained after LysC digestion was determined at an initial yield of 26 pmol with an average repetitive yield of 89% for the first 10 degradation steps.

Construction of cDNA—Isolation of total RNA from 100 mg of 3-day-old etiolated pea seedlings was carried out using the RNeasy plant kit (Qiagen). A cDNA library was constructed using the SMART™ cDNA library construction kit (CLONTECH); the Gigapack III kit from Stratagene was used for packaging into λ-phage. All procedures were carried out as indicated by the manufactures.

cDNA Screening and Analysis of the Genomic Structure—Pea cDNA

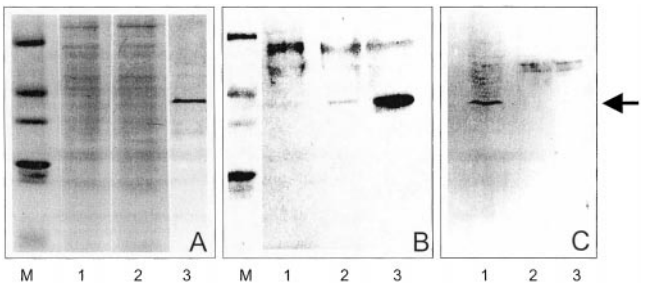

FIG. 1. *A*, purification of IVD from the apex of etiolated pea seedlings using 4-OH-PAA-affinity chromatography. Samples were trichloroacetic acid-precipitated, separated by electrophoresis on a 10% SDS-PAGE, and stained with Coomassie Blue dye. *Lane 1*, protein extracts before loading onto the 4-OH-PAA affinity column; *lane 2*, flow through from the 4-OH-PAA-affinity column; *lane 3*, 4-OH-PAA affinity column bound protein after elution; *lane M*, molecular mass marker (Sigma: 66, 45, 36, and 29 kDa). *B*, immunostaining using polyclonal rabbit antiserum raised against human IVD. *Lane 1*, flow through from the 4-OH-PAA-affinity column; *lane 2*, protein extracts before loading onto the 4-OH-PAA affinity column; *lane 3*, 4-OH-PAA affinity column bound protein after elution; *lane M*, molecular mass marker. Migration of purified human IVD is marked with an *arrow*. The faint bands at 64 kDa are unspecific cross-reactions of the antiserum. *C*, Immunostaining using polyclonal rabbit antiserum raised against human IVD revealed the presence of IVD in young apices (3 day) from pea (*lane 1*). In older apices (10 days, *lane 2*) or internodes (*lane 3*) only faint bands are visible, even though higher protein amounts were applied to *lanes 2* and *3*. This indicates a strong regulation of this protein in etiolated seedlings.

TABLE I
The amino acid sequence and degenerate primers derived from microsequence

Amino acids underlined were used for primer construction, amino acids labeled *X* could not be identified, and amino acids in brackets could not be determined unambiguously.

N-terminal sequence and *X*TSFL<u>FDDTQIQ</u>F*X*ESVASF(A/N)
Derived primer: 5′-TTY GAYGAYACNCARATHCART-3′
Internal sequence and AD(R/C)V<u>DGGYVLNG</u>N(K)
Derived, reverse primer:
3′-CTRCCNCCNATRCANRANTTRCC-5′

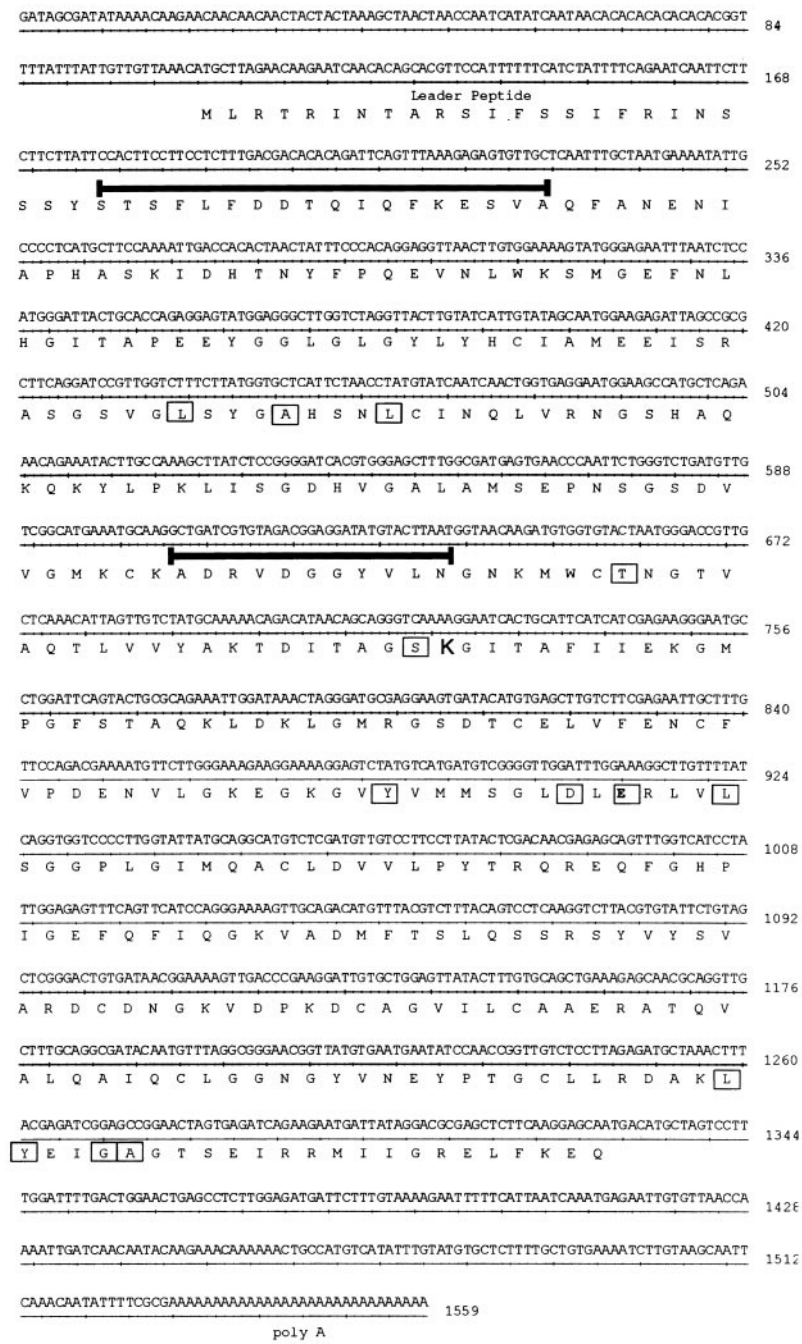

FIG. 2. **Nucleotide and deduced amino acid sequences of the IVD cDNA from pea.** The open reading frame starts with the leader sequence (first 24 amino acids). Amino acids that correspond to the two sequences obtained from microsequencing are *overlined*. Amino acids homologous to those in human IVD shown to be important in substrate binding are *boxed* (see text). The catalytic base is written in *bold*. A difference between pea and human IVD can be found at Arg-191, which is Lys (*bold*) in pea. The sequence has been submitted to EMBL data base under accession number AJ010945.

was used either directly or after subcloning as the template for PCR-based screening for IVD sequences. Degenerate oligonucleotide primers deduced from the protein microsequence were used in the initial PCR amplification. Conditions for PCR were as follows: 29 cycles (90 s 94 °C; 2 min, 60 °C; 2 min, 72 °C), the 10 min at 72 °C. The PCR product was subcloned into pGEM-T vector (Promega) and sequenced. Additional specific oligonucleotide primers were then constructed from this sequence. 5′- and 3′-RACE (rapid amplification of cDNA ends) was performed with sequence-specific primers combined with the NN-1 T30 primer and SMART™ primer, respectively. All cDNA sequences were analyzed from at least two different PCR reactions either directly or after subcloning. For the analysis of the genomic structure, DNA was extracted from pea seedlings according to Doyle and Doyle (15) and used as the template for PCR. The PCR product was subcloned and sequenced using at least two different clones.

DNA and Amino Acid Sequence Analysis—DNA sequencing was done by Seqlab (Göttingen). Analyses were performed using the program DNA Star (Lasergene). Amino acid alignment of IVD sequences from the various species was performed using the Clustal method with standard parameters. The three-dimensional structure of pea IVD was modeled based on that of human CoA persulfide-bound IVD crystals (Brookhaven Protein Data Bank, identification code 1IVH) (6) using the homology module of the Insight II 98.0 software package (Molecular Simulations, Inc., San Diego, CA) on a Silicon Graphics O_2 workstation (Mountain View, CA). The "Manual Rotomer" option was used to optimize the conformation of the side chains of specific amino acid residues.

RESULTS

An isovaleryl CoA dehydrogenase from etiolated pea seedlings was originally identified as auxin-binding protein (ABP_{44}) from pea (9). Because of the low amounts present in the plant tissue and the low yield of IVD, we developed a new and more efficient method for its purification based on its auxin binding characteristics. This allowed purification to near homogeneity as judged by SDS-PAGE (Fig. 1*A*). The purified plant IVD cross-reacts with a polyclonal rabbit antiserum raised against human IVD (Fig. 1*B*), confirming its identity with the human homologue. Only a faint immuno-reactive band was visible in

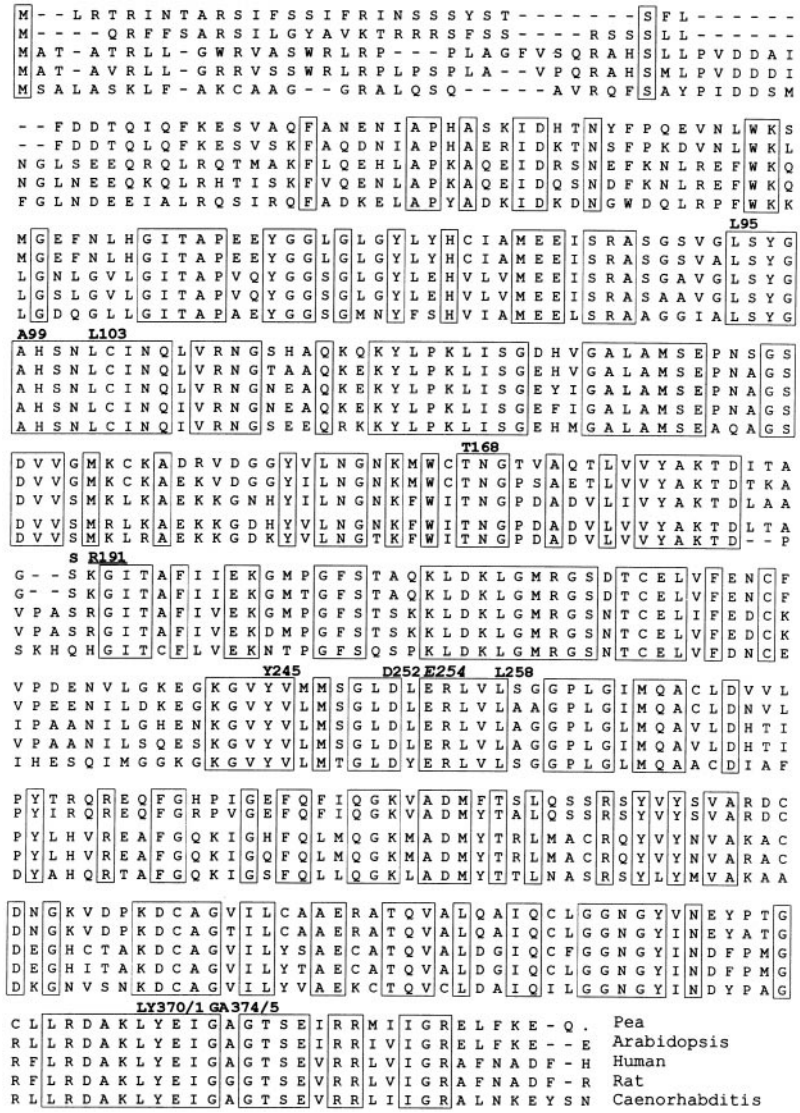

FIG. 3. **Amino acid alignment of pea IVD with homologs from *Arabidopsis* (accession number CAA73227), human (accession number P26440), rat (accession number P12007), and *C. elegans* (accession number AAD21088).** Amino acids identical among all sequences are *boxed*. Amino acids that are important in the human IVD functions listed in the text are labeled below the last line (numbering corresponds to the human protein). Glu-254, which is the catalytic base in human IVD, is *bold face*.

wash fractions of the column, indicating that the IVD in the crude extract was bound nearly to completion to the column. Unfortunately, use of the auxin binding column led to loss of the essential FAD coenzyme from the holoenzyme (data not shown). This led to loss of enzymatic activity, which could not always be restored by incubation with FAD. This did not, however, affect the auxin binding capability of the purified protein.

The plant IVD cannot be found in all parts of the plant. As described previously for auxin binding (9), IVD can be detected in young apices but not in internodes or older apices from etiolated seedlings in immunostaining (Fig. 1*C*).

To characterize the enzymatic properties of the plant IVD, gentler chromatographic conditions were employed that led to enrichment of IVD to approximately 30% of sample protein while still maintaining activity (9). This partially purified enzyme sample showed maximum activity with isovaleryl-CoA as substrate (300 milliunits/mg of protein). Measurable activity was also obtained with *n*-valeryl-CoA as substrate (30 milliunits/mg of protein) but not with any of the following: butyryl-CoA, isobutyryl-CoA, 3-methylcrotonyl-CoA, 2-methylbutyryl-CoA (racemic), octanoyl-CoA, hexanoyl-CoA, valproyl-CoA, 2-ethylhexanoyl-CoA, 2-methylpalmitoyl-CoA (2meC16), myristoyl-CoA (C14), myristoleoyl-CoA (C14:1), or glutaryl-CoA. Apparent K_m values for isovaleryl-CoA and *n*-valeryl-CoA were 6.0 μM ($\pm$ 0.49) and 74.8 μM ($\pm$ 20.7), respectively. In comparison, the K_m of the purified human IVD is 4.2 μM ($\pm$1.1), calculated with isovaleryl CoA as substrate (11, 16). The higher S.E. for valeryl-CoA simply reflects the low activity.

Microsequencing data from highly purified pea IVD (Fig. 1*A*, *lane 3*) was used to derive degenerate primers to the amino terminus and an internal fragment of pea IVD (Table I) for PCR-based screening of a cDNA library prepared from 3-day-old etiolated seedlings. Discrepancies between the sequences determined experimentally by Edman sequencing and the protein sequence derived from the cDNA were found only in positions where the Edman data did not allow an unambiguous identification of the amino acid. The amplified PCR products obtained using the degenerate primers were subcloned and sequenced, and a 400-base pair fragment was identified that contained regions corresponding to the amino acid sequences obtained by microsequencing. A modified 5′- and 3′-RACE (rapid amplification of cDNA ends) procedure was then employed to identify a full-length cDNA sequence of IVD from pea (Fig. 2; EMBL data base accession number AJ010945). The putative coding region is framed by a 130-base pair 5′-UTR and 198-base pair 3′-UTR, and a consensus mitochondrial targeting leader peptide of 24 amino acids can be identified. Pea IVD is 58% homologous to *Caenorhabditis elegans*, 60% homologous to the human and rat enzymes, and 83% homologous to *Arabi-*

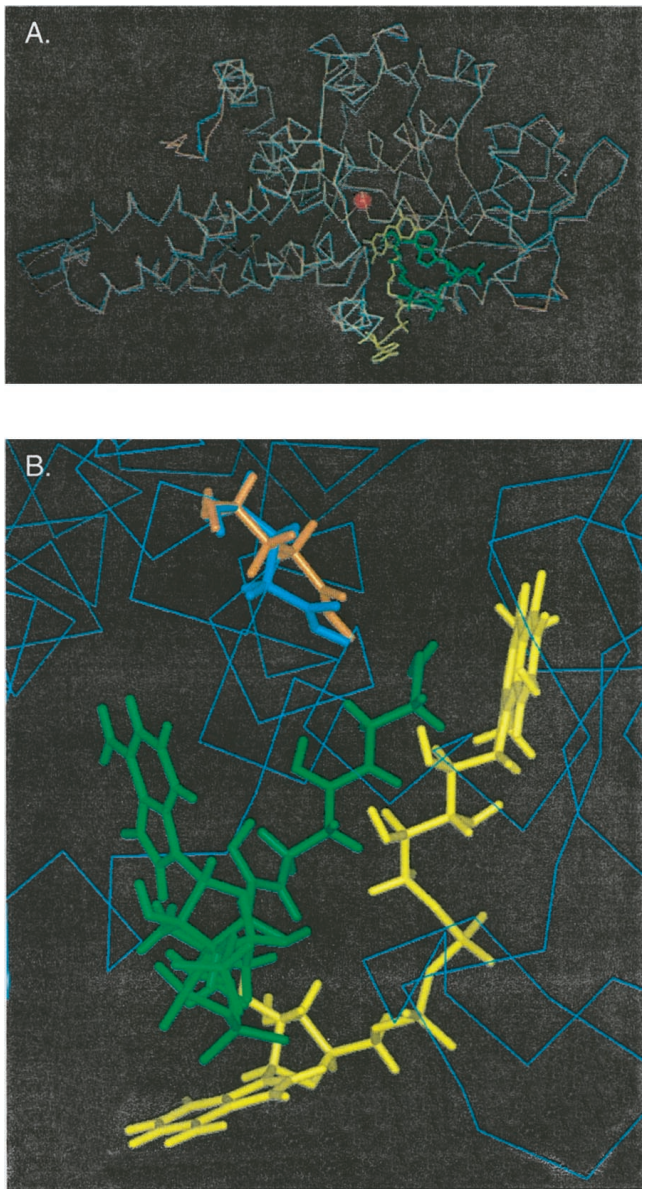

FIG. 4. **Computer-based molecular modeling of the structure of pea IVD.** *A*, the structure of human IVD (*orange*) is overlaid with the predicted structure of the pea enzyme (*blue*). The FAD coenzyme is rendered in *yellow*, and the bound CoA persulfide substrate analogue found in the purified recombinant human enzyme is rendered in *green*. Pea IVD residue Glu244 (the predicted catalytic base) is shown as a *red ball*. *B*, demonstration of the catalytic base in pea IVD. The predicted protein backbone of pea IVD (*blue*) is shown with the Glu-244 rendered *solid*. Glu-254, the catalytic base in human IVD is rendered in *orange* for comparison. The CoA analogue and FAD are as in *A*.

dopsis IVD (Fig. 3). The greatest sequence differences between the plant and animal IVDs are at the N terminus of the proteins. An x-ray crystal structure of human IVD has been published (8), and based on the high level of amino acid homology between the human and pea enzymes, we generated a predicted structure for pea IVD (Fig. 4). This model reveals a high conservation of protein backbone folding, with preservation of the position of Glu-244 in pea IVD relative to Glu-254 of the human enzyme, the catalytic base responsible for abstracting the α-proton of substrate. A number of other residues in the human enzyme that are important in facilitating substrate binding are also conserved in the pea enzyme. These include Leu-95 (human)/85 (pea), Ala-99/91, Leu-103/95, Thr-168/160, Leu-258/248, Leu-370/360, Tyr-371/361, Gly-374/364, and Ala-375/365, all of which contribute to fatty acyl-CoA binding, Asp252/242, which interacts with Asn-6 of the adenosine moiety of substrate, and Tyr-245/235 and Ser-190/180, which are involved in hydrogen bonding to the 3′ phosphate of substrate. Gly-374 in human IVD has been postulated to introduce a wider substrate binding pocket in this enzyme *versus* the other acyl-CoA dehydrogenases, thus allowing utilization of the branched substrate isovaleryl-CoA (6).This residue (Gly-364) is conserved in the pea IVD.

By comparing the genomic sequence obtained (accession number AJ010946) with the cDNA sequence (accession number AJ010945) we could analyze the genomic structure of the pea IVD gene. The important amino acids mentioned above are located in exons IV, VII, X, and XII. The pea IVD gene spans approximately 4 kilobases and consists of 13 exons and 12 introns, with exon sizes ranging from 41 to 205 base pairs. This is a similar genomic arrangement to that reported for human and the mouse IVD (17),[2] although the plant gene is smaller than its human (15 kilobases) and mouse (17 kilobases) counterparts. The DNA sequences at the exon-intron junctions of the pea IVD gene are summarized in Table II. Donor sites at the 3′ site of exons 3, 4, 7, 10, and 12 do not correspond well to the donor splice site consensus sequence from *Arabidopsis* as determined using the NetGen2 search engine (18), whereas all acceptor sites are in agreement with consensus sequence. With respect to the reading frame, all three types of splice junctions were found. Type 0, occurring between codons, was found at five junctions. Type I and type II, which interrupt codons at the first and second nucleotide of the codon, were found at three and four junctions, respectively.

DISCUSSION

In this paper we describe the enzymatic characterization and molecular cloning of an isovaleryl-CoA dehydrogenase from pea, originally identified as an auxin-binding protein (9, 12). Based on the affinity of IVD to auxin we were able to obtain enough highly purified IVD for microsequencing and subsequent cloning experiments, but the purification procedure led to loss of the essential FAD coenzyme and subsequent inactivity of the enzyme. In contrast to reports for IVD purified from rat liver, restoration of pea IVD activity after loss of FAD by the addition of exogenous FAD was not possible (2). Enzymatic assay of active, but less pure enzyme preparations, confirms the identity of the pea enzyme as an IVD, with reduced activity for *n*-valeryl CoA and no activity for any other substrates tested. A similarly narrow pattern of substrate specificity has also been described for the corresponding enzyme from animals (1, 19). Molecular modeling of the structure of pea IVD based on the known structure of human IVD provides significant insights into the likely functional motifs of the plant enzyme. Only slight differences are predicted in the protein backbone chain, whereas important functional amino acid residues such as the catalytic base and a number of amino acids important in interacting with substrate in the substrate binding pocket as well as FAD binding are conserved between the two enzymes. Pea IVD shows the highest homology to a putative IVD from *Arabidopsis* (8). The N terminus of the pea IVD, a region of low homology when comparing all acyl-CoA dehydrogenase sequences and/or structures, differs from the human protein, as shown by the structural model (Fig. 4) as well as by the homology alignment of the amino acid sequences (Fig. 3). The mitochondrial leader peptide sequence diverges significantly from human IVD but is consistent with a consensus mitochondrial targeting sequence. Besides pea, sequences homologous to IVD

[2] J. Willard and J. Vockley, unpublished information.

TABLE II
Intron-exon junctions in the pea IVD gene

Exon	Exon size	5′ Splice donor	Intron size	3′ Splice acceptor	Codon phase
1	108	ACACGCAGATTCA**Gg**tttcaccct	476	cctgttgca**gT**TTAAAGAGAG	0
2	84	TTTCCCACA**Gg**tatgtttca	110	ctaatttta**gG**AGGTTAACT	0
3	58	CACCA**Ga**ggagg	166	ttatttgca**gA**GGAGTATGG	I
4	124	AATCAAC**Ta**attga	86	tttgttttta**gG**TGAGGAATG	0
5	45	CTTGCCAAA**Gg**ttctagatt	549	aattttgaa**gC**TTATCTCCG	0
6	49	GAACCCAAT**Tg**taagatatg	83	tcttctgca**gC**TGGGTCTGA	I
7	187	ATGCCT**Gg**ataattt	112	ttgactaca**gA**TTCAGTACT	II
8	51	GAAGTGATA**Cg**tatgtatgc	95	Ttttctcggca**gA**TGTGAGCTT	II
9	65	GAAGGAAAA**Gg**tctgaaaat	86	Ttttgttcag GAGTCTATGT	I
10	155	TTCATCCA**Gt**ggcataatt	86	ttggttacag GGAAAAGTTG	0
11	41	AGTCCTCAA**Gg**tagaagatt	71	Tttattccag GTCTTACGTG	II
12	51	AGTTGACC**Ct**gcctcc	92	ctaatttca**gG**AAGGGATTG	II
13	206	CAATGA (STOP)			

are present in *Arabidopsis*, cotton (partial), and rice (partial), suggesting that IVD is present throughout the plant kingdom.

Auxin binding capability in cellular extracts is limited to the apices of etiolated, 3-day-old pea seedlings (9). This is also true of IVD protein as determined by immunoblot (Fig. 1*C*). Since no photosynthetic activity is present in growing etiolated pea seedlings, catabolism of stored proteins is utilized for energy. It is likely therefore that IVD functions mainly for the degradation of leucine for energy generation in this situation. This is supported by the identification of the branched chain keto acid dehydrogenase and 3-methylcrotonyl-CoA carboxylase, responsible for the catalytic steps preceding and following IVD in the leucine catabolic pathway in various plant species (20–22).

In summary, we have identified and characterized IVD in etiolated, 3-day-old pea seedlings and cloned cDNA and genomic sequences for this enzyme. The cDNA sequence of pea IVD contains a mitochondrial targeting leader peptide identifying the mitochondrial matrix as the likely subcellular localization for this enzyme. These findings confirm the presence of a leucine catabolic pathway in plants and suggest a role for this pathway in energy metabolism early in plant development. Further study of this enzyme will provide additional insights into important structure/function relationships within the IVD gene family as well as the importance of leucine catabolism in plants.

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