J. Biochem. 119, 1118-1123 (1996)

Identification and Characterization of the *acoD* Gene Encoding a Dihydrolipoamide Dehydrogenase of the *Klebsiella pneumoniae* Acetoin Dehydrogenase System[1]

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Received for publication, January 18, 1996

The *acoD* gene, which encodes a dihydrolipoamide dehydrogenase component of the acetoin dehydrogenase enzyme system of *Klebsiella pneumoniae* was isolated and the nucleotide sequence determined. The gene is capable of encoding a protein of 465 amino acid residues with conserved binding domains for NAD and FAD, and two redox-active cysteine residues. The *acoD* gene product exhibited a Michaelis constant of 170 μM for NAD, while NADP can not be used as a substrate. The purified enzyme appeared to be a dimer of the *acoD* gene product. It did not associate tightly with the E1 and E2 components of either acetoin dehydrogenase or 2-oxoglutarate dehydrogenase to form an active multi-enzyme complex.

Key words: acetoin dehydrogenase, *aco* operon, dihydrolipoamide dehydrogenase.

The enzyme dihydrolipoamide dehydrogenase belongs to the group of flavin-containing pyridine nucleotide disulfide oxidoreductases (*1, 2*), like thioredoxin reductase (*3*), glutathione reductase (*4*), and mercuric reductase (*5*). The enzyme is an integral component of several multienzyme complexes, such as pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH), branched-chain 2-oxoacid dehydrogenase (BCODH) (*6*), and glycine decarboxylase (*7*), in various organisms.

Multiple isoforms of dihydrolipoamide dehydrogenase (DHLDH) have been identified in *Escherichia coli* (*8, 9*) and in *Pseudomonas* spp (*10-13*). In *E. coli*, the first identified DHLDH, encoded by the *lpd* gene, is the E3 component for both PDH and OGDH enzyme complexes (*8*). The function of the later-discovered *E. coli* DHLDH (*9*), however, is not clear. There are three DHLDHs in *Pseudomonas* spp. The first DHLDH was identified as the E3 of the BCODH complex (*10, 11*). A second DHLDH, as in *E. coli*, is the E3 component of OGDH and PDH complexes (*10, 11*), and the L factor of the glycine oxidation system in *P. putida* (*12*). The third DHLDH detected in *P. putida* mutants, showing low amino acid identity with the other two, is more closely related to the eukaryotic DHLDH (*13*).

Acetoin is a major fermentation product of *Klebsiella pneumoniae* grown on a medium with excess carbohydrate. It is also an energy-storing metabolite which can be reutilized by *K. pneumoniae*, as well as several other bacterial species, through the acetoin dehydrogenase (AoDH) enzyme system. Since accumulation of acetaldehyde, a product of acetoin cleavage, is toxic to cells, the acetoin dissimilating activity must be tightly regulated at either the gene or the enzyme level. It is therefore of interest to identify the genes responsible for acetoin catabolism and to characterize the biochemical properties of their products.

The acetoin dehydrogenase enzyme systems (AoDH) which mediate the utilization of acetoin are composed of E1 (acetoin-dependent dichlorophenolindophenol oxidoreductase; Ao:DCPIP OR), E2 (dihydrolipoamide acetyltransferase; DHLAT), and E3 (DHLDH) components, like the PDH, OGDH, and BCODH enzyme complexes. In several bacteria, structural genes encoding the AoDH enzyme complex have been identified and were found to be clustered together as an operon, the *aco* operon (*14-17*). The primary sequences of the respective enzymes as deduced from their nucleotide sequences are conserved in relation to those of PDH, OGDH, and BCODH suggesting a common evolutionary origin of these enzyme complexes. In *Alcaligenes eutrophus*, the AoDH E3 encoding gene is not present in the operon and the participation of a DHLDH in the acetoin cleaving system remains obscure (*14*). In contrast, a DHLDH encoding gene has been identified in the *aco* operons of *Pelobacter carbinolicus* (*15*), *Clostridium magnum* (*16*), and *K. pneumoniae* (*17*). However, whether the particular DHLDH of AoDH could be shared with the PDH, OGDH, or BCODH enzyme systems is not clear. We have recently isolated and characterized the *K. pneumoniae aco* operon (*17*). Three structural genes in the order of *acoA, B*, and *C*, which encode for the α and β subunit of Ao:DCPIP OR and DHLAT, respectively, have been sequenced. We here report the nucleotide sequence of the fourth gene, *acoD*, located immediately downstream of

[1] This work was supported in part by grants from Chang Gung College of Medicine and Technology (CMRP457 to HLP and CMRP459 to HYC) and the National Science Council of the Republic of China (NSC 83-412-B182-059 to HLP and NSC 85-2331-B182-023 to HYC).

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Abbreviations: Ao DCPIP OR, acetoin-dependent dichlorophenolindophenol oxidoreductase; AoDH, acetoin dehydrogenase; BCODH, branched-chain 2-oxoacid dehydrogenase; bp, base pair(s); DHLDH, dihydrolipoamide dehydrogenase; DHLAT, dihydrolipoamide acetyltransferase; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase pairs; kDa, kilodaltons; LB, Luria-Bertani medium; OGDH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; [ ], denotes plasmid-carrying state.

acoC. The biochemical properties of the gene product are also described.

MATERIALS AND METHODS

Enzymes and Chemicals—All restriction endonucleases and DNA modifying enzymes were obtained from either Promega (Madison, WI) or New England BioLab (Beverly, MA) and used under the conditions recommended by the suppliers. *Pfu* DNA polymerase, which was used for amplification of the *acoD* coding region, was a product of Stratagene (LaJolla, CA). The Sequenase kit and [α-^{35}S]-dATP were purchased from Amersham (Buckinghamshire, UK). DEAE-cellulose was purchased from Whatman BioSystems (Kent, UK) and Sephacryl-300 HR was from Pharmacia Biotech (Uppsala, Sweden). Other chemicals, coenzymes, and buffers were obtained from Sigma Chemical (St. Louis, MO).

Bacterial Strains, Plasmids, and Growth Conditions—*E. coli* strain JRG1342 was a kind gift of Dr. John R. Guest, (Sheffield University, Sheffield, UK). The *ace-lpd* genes encoding the entire PDH were deleted in the strain (*18*). *E. coli* Novablue(DE3), a λDE3 lysogen with a T7 RNA polymerase gene in an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible form, was obtained from Novagen (Madison, WI) for heterologous expression of the recombinant protein in *E. coli*. The construction of pHP656 which contains the complete *K. pneumoniae aco* operon has been described previously (*17*). All bacteria were propagated at 37°C in Luria-Bertani broth (LB) except that the *E. coli* JRG1342 and its derivatives were grown in LB supplemented with 0.4% of glucose, and 4 mM each of acetate and succinate.

Recombinant DNA Techniques and DNA Sequencing—Plasmid DNA preparation and DNA manipulation were carried out essentially as described (*19*). DNA sequence determination was performed by the dideoxy chain-termination method (*20*) with the Sequenase kit. Both the universal *M13* primer and synthetic oligonucleotides for a primer-hopping strategy were used. The nucleotide and amino acid sequences were analyzed with the DNAstar program (DNASTAR, Madison, WI) on a Macintosh LC II computer.

Enzyme Assay and Kinetic Analysis—Since the Ao: DCPIP OR activity of the *K. pneumoniae* AoDH was too low to be accurately measured when synthesized in *E. coli* JRG1342, a more sensitive method that determines the exhaustion of acetoin was used (*17*). The assay mix included 100 mM potassium phosphate (pH 7.0), 0.08 mM thiamine pyrophosphate, 0.5 mM magnesium chloride, 0.034 mM acetoin, and the enzyme extract in a final volume of 1 ml. The reaction mixture was incubated at 37°C for 1 h. Creatine and α-naphthol, which react with acetoin to form a bright red color, were then added to determine the amount of acetoin depleted from the mixture. The DHLDH activity was monitored spectrophotometrically at 340 nm in a reaction mixture containing 100 mM Tris-HCl (pH 7.5), 5 mM EDTA, 2 mM dihydrolipoamide, 1 mM NAD, and the enzyme extract (*9*). One enzyme unit is defined as the amount of enzyme that catalyzes an initial rate of formation of 1 μmol of NADH per min. The dihydrolipoamide used was prepared by reducing lipoamide with sodium borohydride as described (*21*). For the determination of PDH and OGDH activity, the reaction mixture included 100 mM Tris-HCl (pH 7.5), 0.5 mM magnesium chloride, 1.2 mM NAD, 0.13 mM coenzyme A, 0.8 mM thiamine pyrophosphate, 6 mM dithiothreitol, 3 mM cysteine hydrochloride, and the protein sample to be tested. The reaction was started by adding either pyruvate or 2-oxoglutarate to a final concentration of 3 mM and the reduction of NAD was determined spectrophotometrically at 340 nm as described (*22*). Kinetic data were analyzed using the nonlinear regression computer program DNRP-EASY that has been described elsewhere (*23*).

Protein Purification—The *K. pneumoniae* DHLDH synthesized in *E. coli* JRG1342[pHP783] was purified by conventional column chromatography. All procedures were performed at 4°C unless otherwise indicated. Overnight-grown bacteria were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5). The cells were disrupted by sonication and the debris was removed by centrifugation. Ammonium sulfate precipitation was conducted and the proteins that salted out between 40 to 55% saturation were collected and dialyzed against 50 mM Tris-HCl (pH 7.5). The dialysate was passed through a DEAE-cellulose column and the enzyme was eluted with a linear gradient of 0-500 mM NaCl in 50 mM Tris-HCl (pH 7.5). The peak fractions of the DHLDH activity were pooled and concentrated by 70% ammonium sulfate precipitation, and then applied to a Sephacryl-300 gel filtration column. Eluted fractions with high enzyme activity were combined and stored either at 4°C or at −20°C in a freezer with glycerol added to a final concentration of 50% (w/v). The recombinant AcoD with an N-terminal S peptide tag was purified by affinity precipitation with the S protein resin (Novagen, Madison, WI) under the conditions recommended by the manufacturer.

Molecular Weight Determination—The molecular weight of the enzyme subunit was determined by SDS-polyacrylamide gel electrophoresis. Determination of the size of the native enzyme was conducted on a column of Sephacryl-300 HR (100×1.6 cm). The protein standards used were carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and blue dextran (2,000 kDa). The molecular weight of the purified protein was estimated by the standard method (*24*).

In Vivo Protein Synthesis—All bacteria for protein analysis were grown in LB at 37°C with vigorous shaking until $A_{600}=0.3$. IPTG was added to a final concentration of 1 mM and the incubation was continued for 90 min. The cells were collected, resuspended into 2×Laemmli sample

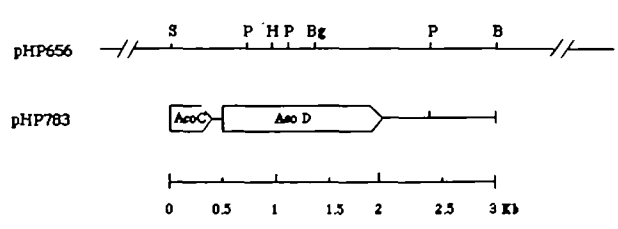

Fig. 1. **Restriction map of the *K. pneumoniae acoD*.** The relevant restriction endonuclease cutting sites are: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; S, *Stu*I. The direction of transcription is indicated.

buffer and the proteins were resolved on a 0.5% SDS-12.5% polyacrylamide gel (*25*). The protein profile was detected by staining with Coomassie Brilliant Blue R250.

RESULTS AND DISCUSSION

Isolation and Nucleotide Sequencing of the K. pneumoniae acoD Gene—We have previously shown that an open reading frame which is located immediately downstream of the *acoC* gene might encode a protein with the characteristics of DHLDH (*17*). In order to obtain the complete nucleotide sequence of the putative *acoD* gene, a DNA fragment which contains approximately 460 bp of the *acoC* coding sequence and a 2.5 kb region downstream of the *acoC* gene was excised from the cosmid clone pHP656 by *Stu*I and *Bam*HI double digestion. The DNA fragment was isolated and subcloned into pUC18. The restriction endonuclease cutting sites of the resulting plasmid, pHP783, were determined (Fig. 1) and overlapping DNA fragments were subcloned into *M13* for nucleotide sequencing. Analysis of the 3 kb sequence has revealed that the length of the partial open reading frame noted in our previous study was clearly extended and was capable of encoding a protein of 465 amino acid residues. The predicted size of the protein, 49,574 Da, is comparable with that of other DHLDHs (in the range of 49-55 kDa). At a distance of 7 bp, the start codon of *acoD* is preceded by a typical Shine-Dalgarno sequence (Fig. 2). The relatively close distance between *acoC* and *acoD* genes suggests that *acoD* is part of the *K. pneumoniae aco* operon.

Sequence Analysis of acoD Gene Product—The predicted primary sequence of the *acoD* gene product was used to search for homologous files using the BLAST network services of the National Center for Biotechnology Information. The *K. pneumoniae acoD* gene product displayed significant homologies with several bacterial DHLDHs (Fig. 3). The highest amino acid identity, 39.5% (183 out of 465 comparable amino acid residues), was noted between the gene products of *acoD* and *P. putida lpd3*. In addition, the sequence contains the characteristic motifs which are conserved within the pyridine nucleotide-disulfide oxidoreductase enzyme family (*26*). As shown in Fig. 3, a peptide which interacts with the adenine PP_i moiety of FAD (*27*) was noted at the N-terminal region of the *acoD* gene product. An additional conserved region which may also make contact with FAD (*27*) was found near the carboxyl-terminal portion of the molecule. The third region of sequence similarity among the representative members

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                                    acoC  TGATTGAAACCCCGACCTTGATGTTTATCCAGGAGACGAGCT   42
acoD
 M   H   D   K   Y   D   V   L   I   I   G   G   G   P   G   G   Y   V   A   A
ATG CAC GAT AAA TAC GAT GTG CTG ATC ATC GGC GGG GGT CCT GGG GGA TAC GTT GCC GCC  102
 I   R   A   G   Q   L   G   L   R   T   V   L   V   E   K   Q   H   L   G   G
ATC CGT GCC GGC CAG CTA GGG CTT CGT ACC GTA TTA GTG GAA AAA CAA CAT CTG GGC GGC  162
 I   C   L   N   W   G   C   I   P   T   K   A   L   L   H   G   A   E   V   A
ATC TGC CTG AAC TGG GGA TGC ATT CCA ACC AAG GCG CTG CTG CAT GGC GCT GAG GTT GCG  222
 H   T   I   T   H   A   S   Q   L   G   I   S   V   G   E   V   N   V   D   L
CAC ACT ATC ACC CAT GCC AGT CAG CTG GGC ATC AGC GTG GGC GAG GTG AAC GTC GAT CTG  282
 Q   K   L   V   Q   F   S   R   T   V   S   Q   Q   L   T   A   G   V   A   Y
CAG AAA CTG GTG CAG TTT AGC CGT ACC GTA TCG CAA CAG CTC ACC GCT GGG GTT GCG TAC  342
 L   L   K   K   N   G   V   R   V   I   D   G   T   A   R   L   R   G   K   G
CTG TTG AAG AAA AAT GGC GTG AGG GTG ATT GAT GGC ACC GCG CGG CTG CGC GGC AAG GGG  402
 Q   I   T   V   E   D   A   R   G   E   A   R   D   Y   R   A   D   H   V   I
CAA ATC ACG GTC GAG GAT GCC CGA GGG GAG GCG CGC GAT TAC CGG GCC GAT CAC GTG ATC  462
 L   A   T   G   A   R   P   R   A   L   P   G   I   A   P   D   G   E   H   I
CTG GCA ACC GGC GCC CGA CCA CGC GCA TTG CCA GGC ATA GCG CCA GAT GGC GAA CAT ATC  522
 W   T   Y   F   E   A   L   R   P   K   L   L   P   K   S   L   L   I   I   G
TGG ACC TAT TTC GAG GCG CTG CGG CCT AAG CTT TTA CCC AAG TCG CTC TTA ATC ATC GGT  582
 S   G   A   I   G   V   E   F   A   S   L   Y   N   D   L   G   C   K   V   T
TCA GGG GCG ATT GGC GTC GAG TTC GCC AGC CTT TAT AAC GAT CTG GGC TGT AAA GTG ACG  642
 L   V   E   L   A   S   Q   I   L   P   V   E   D   A   E   V   S   A   A   V
CTG GTC GAG CTA GCG TCG CAG ATT TTG CCA GTG GAA GAT GCC GAG GTG TCT GCA GCA GTG  702
 R   K   S   F   E   K   R   G   I   Q   I   H   T   Q   T   L   V   T   Q   V
CGT AAG TCA TTC GAA AAA CGC GGT ATT CAG ATC CAT ACC CAG ACC CTG GTG ACG CAA GTA  762
 Q   L   T   D   T   G   V   R   C   T   L   N   N   T   G   G   E   Y   S   Q
CAG CTC ACC GAT ACC GGG GTG CGC TGC ACA CTA AAC AAC ACC GGC GGC GAA TAT TCT CAG  822
 D   V   E   R   V   L   L   A   V   G   V   Q   P   N   I   E   D   L   G   L
GAT GTC GAA CGT GTG CTG CTG GCG GTC GGC GTA CAG CCG AAT ATT GAA GAT CTG GGG CTG  882
 E   T   L   G   V   E   L   D   R   G   F   I   K   T   D   A   A   C   R   T
GAA ACG CTG GGC GTT GAG TTA GAC CGC GGT TTT ATC AAG ACG GAC GCG GCT TGT CGC ACT  942
 N   V   F   G   L   Y   A   I   G   D   V   A   G   P   P   C   L   A   H   K
AAC GTA TTT GGG CTT TAT GCC ATC GGC GAT GTA GCC GGC CCG CCG TGT CTG GCG CAC AAG 1002
 A   S   H   E   G   V   L   C   V   E   T   L   A   G   V   E   G   A   H   P
GCC AGC CAC GAA GGC GTG CTC TGC GTT GAG ACC CTG GCC GGT GTC GAA GGC GCC CAC CCT 1062
 L   D   R   D   Y   V   P   G   C   T   Y   A   R   P   Q   V   A   S   L   G
CTC GAT CGC GAC TAT GTG CCT GGT TGC ACT TAT GCC CGG CCC CAG GTT GCC AGT CTG GGC 1122
 L   T   E   S   T   A   L   A   R   G   R   P   I   R   I   G   K   F   S   Y
CTG ACG GAA TCG ACG GCC CTG GCC AGG GGA CGG CCC ATC AGG ATC GGT AAG TTC TCT TAT 1182
 Q   S   N   G   K   A   L   V   S   G   E   T   E   G   F   V   K   T   I   F
CAG AGC AAT GGT AAG GCG CTG GTC AGC GGC GAG ACA GAG GGT TTT GTG AAG ACG ATT TTC 1242
 D   A   E   T   G   E   L   L   G   A   H   M   V   G   A   Q   V   T   E   Q
GAT GCT GAA ACC GGC GAG TTG CTG GGC GCG CAT ATG GTT GGC GCA CAG GTC ACG GAA CAG 1302
 I   Q   G   F   G   I   A   R   H   L   E   A   T   D   E   S   L   L   S   M
ATC CAG GGC TTT GGC ATC GCC CGT CAC CTG GAG GCC ACA GAC GAA AGC CTC CTG TCG ATG 1362
 I   F   A   H   P   T   L   S   E   A   M   H   E   S   I   L   A   A   C   D
ATC TTT GCG CAT CCG ACA CTT TCC GAA GCG ATG CAT GAG TCA ATC CTC GCG GCC TGC GAC 1422
 Q   P   L   H   Q   .
CAA CCG TTG CAT CAA TAA AAAATAAGGGAGGCGATAGCTGTGCGATACGGAGTAAGTTCCCGTTATCCCTCCC 1495
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Fig. 2. **Nucleotide sequence and translation of *K. pneumoniae acoD* gene.** Amino acids deduced from the nucleotide sequence are specified by one-letter abbreviations. The putative ribosome-binding site is underlined. The nucleotide sequence has been submitted to the GenBank under the accession number U30887.

given in Fig. 3 is a catalytic segment containing the redox-active cysteine residues. Unlike the thioredoxin reductases which contain a shorter redox-active peptide with two amino acids separating the functional cysteines (*26*), the two redox-active cysteines are four residues apart in the *acoD* gene product and in the related flavoproteins shown in Fig. 3. The consensus sequence involved in the binding of NAD(H), spanning residues 172 to 193 of the AcoD, is also conserved among the proteins. Finally, a region which has been proposed as the interface for dimerization (*27*) was also present near the C-terminus of the *acoD* gene product. These sequence comparisons provide compelling evidence that the *acoD* gene product is a DHLDH.

Overexpression of the acoD Gene in E. coli—The entire coding region of the *acoD* gene was PCR amplified using a high-fidelity DNA polymerase and inserted into the expression vector pET29c. The construct places the *acoD* coding region under the control of a strong T7 promoter in an IPTG-inducible manner. The plasmid, designated pHPA75, was transformed into *E. coli* Novablue(DE3) and the transformant was propagated in LB. Total cellular proteins of the IPTG-induced cells were then analyzed on SDS-polyacrylamide gel. As shown in Fig. 4, an approximately 54-kDa protein which is not present in *E. coli* Novablue (DE3) or Novablue(DE3) [pET29c] was detected in Novablue(DE3) [pHPA75] upon IPTG induction. The size of the protein is consistent with that deduced from the *acoD*

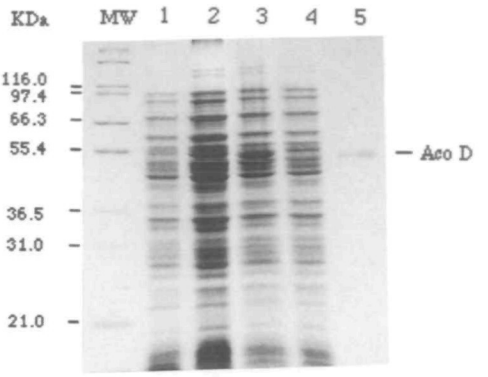

Fig 4 **Overexpression of *K. pneumoniae acoD* in *E. coli*.** Whole cell protein profiles were analyzed by SDS-polyacrylamide gel electrophoresis Lanes 1 and 3 contain total proteins isolated from IPTG-induced cells carrying pET29c and pHPA75, respectively The whole cell proteins in lanes 2 and 4 were obtained from the cells without IPTG induction Lanes 1 and 2, *E. coli* Novablue(DE3) [pET29c], 3 and 4, Novablue(DE3) [pHPA75], 5, AcoD purified through S-protein resin The sizes of the molecular weight markers are shown on the left The S-tag fusion AcoD protein is indicated on the right

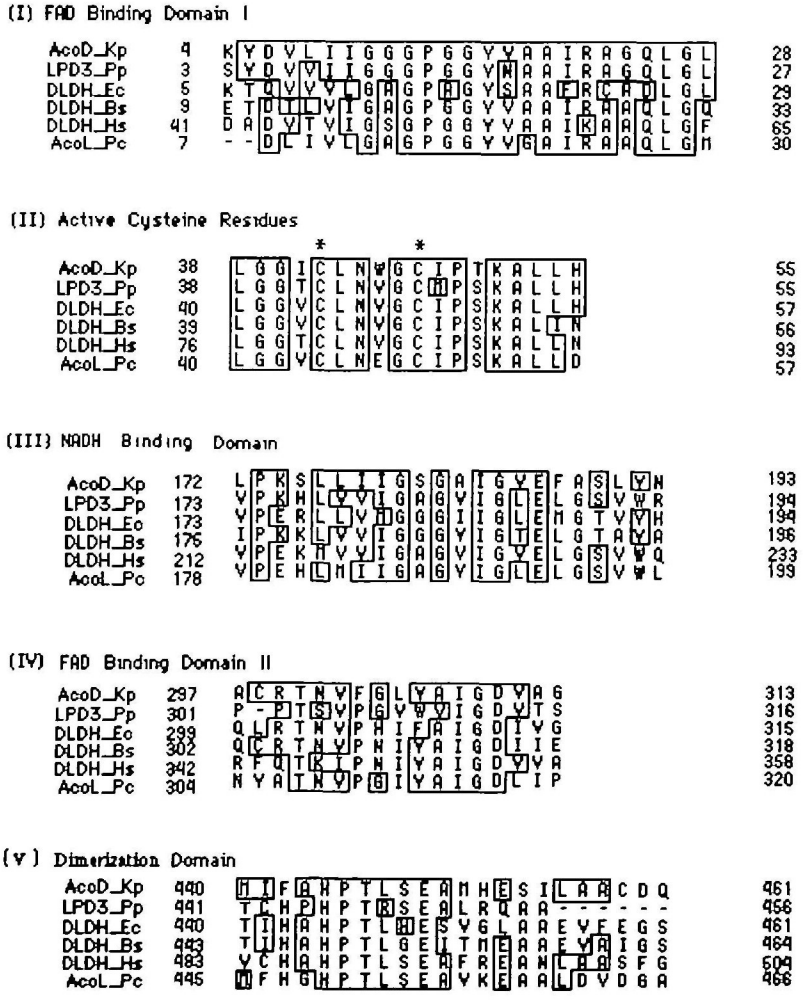

Fig 3 **Sequence comparison of the conserved domains of AcoD with related enzymes.** The sequences listed are: AcoD—Kp, *K pneumoniae* AcoD; LPD3—Pp, *P putida* Lpd3 (*13*); DLDH—Ec, *E coli* Lpd (*8*); DLDH—Bs, *Bacillus subtilis* E3 (*29*), DLDHHs, *Homo sapiens* E3 (*30*), AcoL—Pc, *P. carbinolycus* AcoL (*15*). (I) and (IV), FAD binding domains; (II), disulfide bridge active site, (III), NAD binding domain, (V), interface domain for dimeric form. The position of the first amino acid in the polypeptide is indicated to the left of the selected part of the chains; the position of the last amino acid is indicated to the right

gene (50 kDa) plus the N-terminal fusion S-Tag peptide (4 kDa). The protein, which was purified through S-protein resin, exhibited a specific DHLDH activity of approximately 16.05 U/mg protein.

Purification and Characterization of the DHLDH—To eliminate possible interference by the endogenous *E. coli* DHLDH activity in analysis of the biochemical properties of *acoD* gene product, an *ace-lpd* mutant strain JRG1342 was used as the recombinant DNA host for overexpression of the gene. Analysis of *E. coli* JRG1342[pHP783] lysate has demonstrated that the DHLDH activity is greatly increased in the strain. Approximately 0.19 U/mg protein of enzyme activity was observed in the lysate, while the activity was undetectable in the parental strain. *E. coli* JRG1342[pHP783] was therefore used as the source for purification of the *K. pneumoniae* DHLDH. The enzyme was purified through conventional methods and the final preparation was approximately 90% pure as judged by SDS-polyacrylamide gel electrophoresis. The enzyme activity obtained from the purified AcoD is approximately 13.84 U/mg protein (Table I), which is slightly less than that of the enzyme purified through the S-protein purification system. When stored at −20°C in the presence of 50% glycerol, approximately 50% of its activity was lost in 24 h. Like DHLDH of the *A. eutrophus* PDH enzyme complex (*22*), the *acoD* gene product utilized NAD but not NADP as a substrate. The Michaelis constant of the enzyme for NAD is 170 μM. The enzyme activity was eluted at a position of 110 kDa from the Sephacryl-300 gel permeation column, suggesting that like other DHLDHs, it is a homodimer of the *acoD*-encoded protein.

Elution Profile on Gel Filtration—Although *E. coli* JRG1342 is capable of synthesizing the E1 and E2 components of OGDH, a functional OGDH multienzyme complex could not be formed due to the deletion of the *ace-lpd* region (*18*). Thus, acetate and succinate must be supplied in LB to sustain the growth of the bacterium. Since the primary structure of the *acoD* gene product significantly resembles that of *lpd*, it is of interest to see whether the *K. pneumoniae acoD* gene product can complement the *lpd* mutation of *E. coli* JRG1342. Plasmid pHP783 was therefore introduced into *E. coli* JRG1342 and the transformants were tested for their requirement for acetate and succinate. However, we found that these transformants were still dependent on supply of acetate and succinate for growth, which suggested that the *acoD* gene product could not replace the function of the PDH E3. The chromatographic profile of the *E. coli* JRG1342[pHP783] extract eluted from the Sephacryl-300 column revealed only one DHLDH peak (Fig. 5A), approximately 110 kDa in size, confirming that most, if not all, of the *acoD* gene product did not form a multienzyme complex with the E1 and E2 components of OGDH.

To address the question of whether the AcoD is organized into a multienzyme structure with the E1 and E2 components of AoDH, the capability of NAD reduction in the extract of *E. coli* JRG1342[pHP656], which contains the three enzyme components of AoDH, was tested. Although the acetoin consumption could be detected with the cell extract, no NAD reduction at the expense of acetoin was noted. Furthermore, the chromatographic profile of *E. coli* JRG1342[pHP656] revealed one major DHLDH peak corresponding to that noted previously for *E. coli* JRG1342 [pHP783]. The data suggested that the AcoD was not able

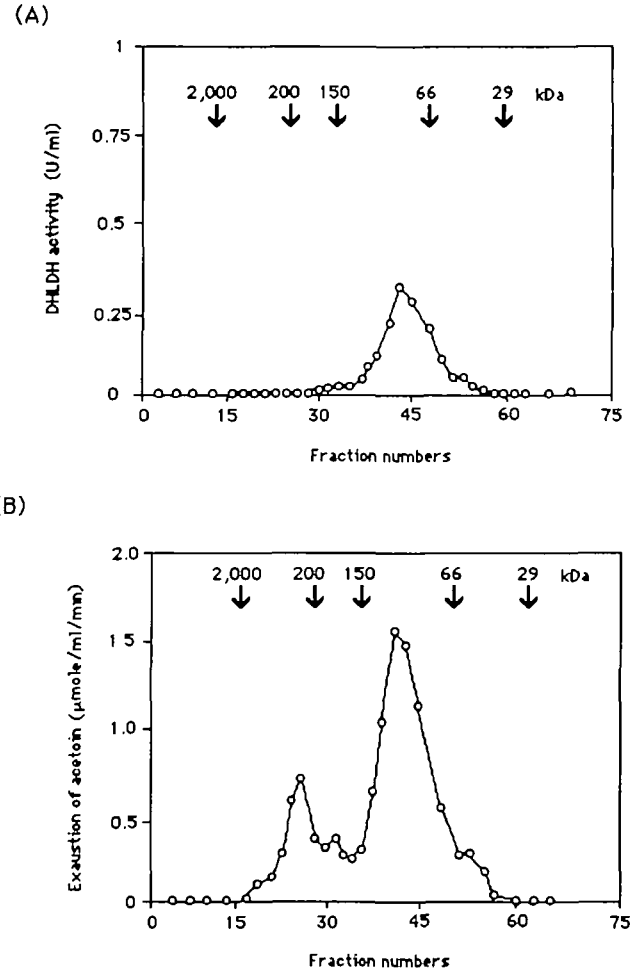

Fig. 5. **Gel filtration profiles of (A) DHLDH activity in *E. coli* JRG1342[pHP783] and (B) acetoin depleting activity in *E. coli* JRG1342[pHP656].** The collected overnight-grown cells were disrupted by sonication and the cell debris was removed by centrifugation at 100,000×*g* for 1 h. The ammonium sulfate precipitate between 40 and 50% saturation, which contained most of the DHLDH activity, was collected and then applied to a Sephacryl-300 gel filtration column.

TABLE I. **Purification of the AcoD from *E. coli* JRG1342[pHP783].**

Step	Vol. (ml)	Protein conc. (mg/ml)	Total protein (mg)	Total activity (U)	Sp. act. (U/mg)	Purification (fold)	Recovery (%)
Crude extract	60.0	2.37	142.2	27.6	0.19	1.0	100
Ammonium sulfate	3.5	5.37	18.8	24.3	1.29	6.8	88
DEAE column	66.0	0.03	1.9	15.2	7.95	42.0	55
Gel filtration	32.0	0.012	0.37	5.12	13.84	72.8	19

to organize with the other components of the AoDH into a high-molecular-weight enzyme complex. This is not certain, however, because of the relatively low DHLDH activity synthesized in *E. coli* JRG1342[pHP656]. In order to obtain a more conclusive result, acetoin depletion, which represents E1 activity, was also measured in these fractions. As shown in Fig. 5B, two peaks of acetoin-depleting activity were observed. The size estimated for the higher level of the activity was approximately 120 kDa, which corresponds to the size of a heterotetrameric form of the E1 of AoDH. The other peak was much smaller, and accounted for approximately one-fifth of the total acetoin depleting activity obtained from the extract of *E. coli* JRG1342 [pHP656]; this enzyme activity was found in fractions eluted soon after the void volume, indicating that its molecular mass was rather large. Taken together, these data suggest that although a small quantity of high-molecular-weight enzyme structure may be organized, most of the E1 and E3 components of *K. pneumoniae* AoDH did not associate to form a multienzyme complex.

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