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Overproduction of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* and site-directed substitutions in the E1p and E2p subunits

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The *aceEF-lpd* operon of *Escherichia coli* encodes the pyruvate dehydrogenase (E1p), dihydrolipoamide acetyltransferase (E2p) and dihydrolipoamide dehydrogenase (E3) subunits of the pyruvate dehydrogenase multienzyme complex (PDH complex). An isopropyl β-D-thiogalactopyranoside-inducible expression system was developed for amplifying fully lipoylated wild-type and mutant PDH complexes to over 30% of soluble protein. The extent of lipoylation was related to the degree of aeration during amplification. The specific activities of the isolated PDH complexes and the E1p component were 50–75% of the values normally observed for the unamplified complex. This could be due to altered stoichiometries of the overproduced complexes (higher E3 and lower E1p contents) or inactivation of E1p. The chaperonin, GroEL, was identified as a contaminant which copurifies with the complex. Site-directed substitutions of an invariant glycine residue (G231A, G231S and G231M) in the putative thiamine pyrophosphate-binding fold of the E1p component had no effect on the production of high-molecular-mass PDH complexes but their E1p and PDH complex activities were very low or undetectable, indicating that G231 is essential for the structural or catalytic integrity of E1p. A minor correction to the nucleotide sequence, which leads to the insertion of an isoleucine residue immediately after residue 273, was made. Substitution of the conserved histidine and arginine residues (H602 and R603) in the putative active-site motif of the E2p subunit confirmed that H602 of the *E. coli* E2p is essential, whereas R603 could be replaced without inactivating E2p. Deletions affecting putative secondary structural elements at the boundary of the E2p catalytic domain inhibited catalytic activity without affecting the assembly of the E2p core or its ability to bind E1p, indicating that the latter functions are determined elsewhere in the domain. The results further consolidate the view that chloramphenicol acetyltransferase serves as a useful structural and functional model for the catalytic domain of the lipoate acyltransferases.

INTRODUCTION

The pyruvate dehydrogenase (PDH) multienzyme complex of *Escherichia coli* is the best characterized of the 2-oxoacid dehydrogenase complexes. It has been extensively studied using molecular-genetic, enzymological and both ^{1}H n.m.r. and mass-spectrometric techniques (Guest *et al.*, 1989, 1990; Perham, 1991). The complex catalyses the conversion of pyruvate to acetyl-CoA by three integrated reactions. Pyruvate is oxidatively decarboxylated by the thiamin pyrophosphate (TPP)-dependent pyruvate dehydrogenase component (E1p, EC 1.2.4.1) with the concomitant reductive acetylation of the covalently bound lipoyl cofactors on the lipoate acetyltransferase subunits (E2p, EC 2.3.1.12). The acetyl groups are transferred to CoA by E2p and the reduced E2p lipoyl groups are reoxidized by the NAD-dependent lipoamide dehydrogenase (E3, EC 1.8.1.4). The complex is assembled on a cubic core of 24 E2p subunits, multiple copies of the dimeric E1p and E3 components being bound to the respective edges and faces with an approximate stoichiometry of 1.0:1.0:0.5 (E1p/E2p/E3). The cloning and sequence analysis of the *aceE-aceF-lpd* operon (Fig. 1) encoding the E1p, E2p and E3 components of the PDH complex have been described previously (Guest *et al.*, 1989; Stephens *et al.*, 1983*a*,*b*,*c*).

Research on the structure–function relationships of the PDH complex has concentrated on the organization of the outer domains of the E2p subunit and the covalently bound lipoyl cofactors (Guest *et al.*, 1989, 1990; Perham, 1991). The DNA-derived amino acid sequence of the *E. coli* E2p showed that the E2p subunit has a segmented structure with three homologous *N*-terminal domains of about 80 residues, each containing a lipoylatable lysine residue, although two of these domains appear redundant (Stephens *et al.*, 1983*b*; Guest *et al.*, 1985: Packman *et al.*, 1991). The lipoyl domains are separated from each other, and from the rest of the E2p chain, by short sequences which are rich in alanine, proline and charged residues. These linker sequences are highly sensitive to proteolysis (Packman & Perham, 1987) and are conformationally mobile (Miles *et al.*, 1988; Texter *et al.*, 1988). Internal to the lipoyl domains is a 50-residue segment which is required for binding E3 subunits (Packman & Perham, 1986) and is also flanked by linker sequences. The *C*-terminal domain contains the E1p and E2p binding sites and the lipoate acetyltransferase activity. Here a remote but significant similarity with chloramphenicol acetyltransferase (CAT) was detected at both the primary and predicted secondary structural levels, and it was suggested that the lipoate acyltransferases use a general-base-catalysed mechanism for acyl transfer similar to that proposed for CAT (Guest, 1987; Guest *et al.*, 1989). Multiple sequence alignments for 16 lipoate acyltransferases (Hemila *et al.*, 1990; Repetto & Tzagoloff, 1990; Borges *et al.*, 1990; Allen & Perham, 1991; Russell & Guest, 1991*a*,*b*) further revealed some highly conserved residues in the acyltransferase domains. These include a DHRXXDG motif which resembles the HHXXXDG motif containing the active-site histidine residue in CAT, and a serine (or threonine) residue corresponding to the

Abbreviations used: PDH complex, pyruvate dehydrogenase multienzyme complex; ODH complex, 2-oxoglutarate dehydrogenase multienzyme complex; CAT, chloramphenicol acetyltransferase; TPP, thiamin pyrophosphate; IPTG, isopropyl β-D-thiogalactopyranoside.

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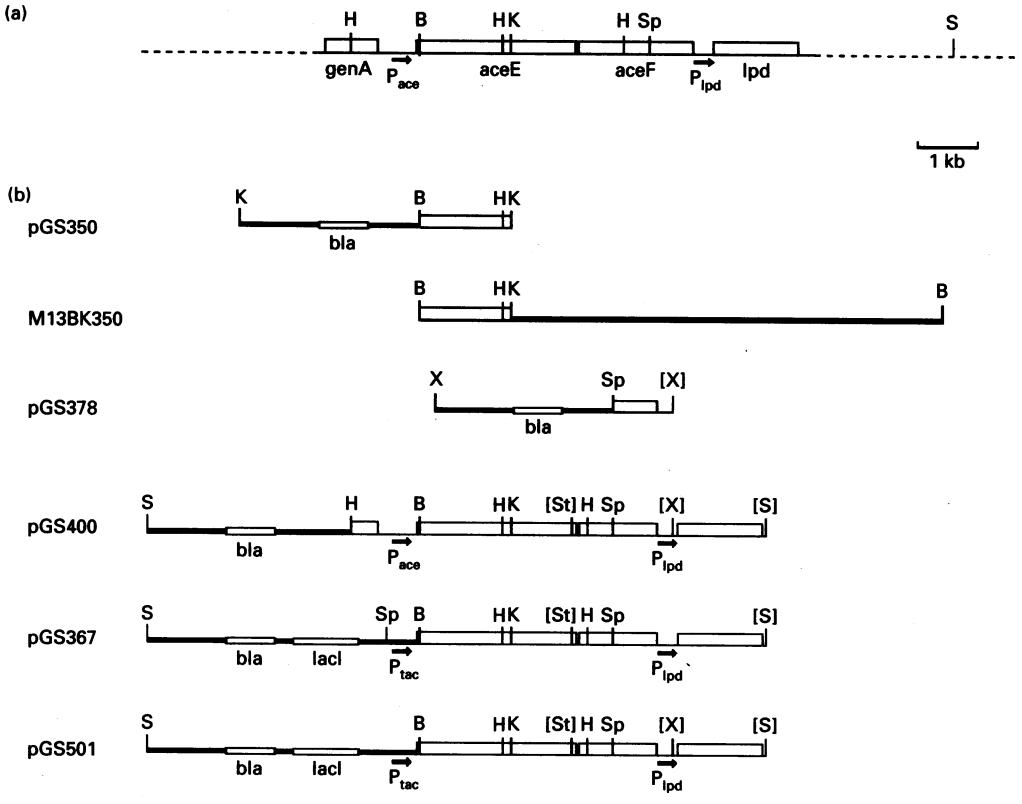

Fig. 1. Structure of the *aceEF-lpd* operon and derivative plasmids

(*a*) The *aceEF-lpd* operon. (*b*) Plasmids, phages and phagemids containing the *aceEF-lpd* operon or specific segments: pGS350 and M13BK350 (*aceE* cassette); pGS378 (*aceF* cassette). Genes (open boxes), promoters (arrows) and relevant restriction sites [B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sp, *Sph*I; St, *Sst*I; X, *Xho*I (engineered restriction sites are enclosed by square brackets)] are shown. Vector DNA (thick lines) and vector genes, β-lactamase (*bla*) and *lac* repressor (*lacI*), are indicated. The plasmids pGS367, pGS400 and pGS501 encode PDH complexes with one lipoyl domain per E2p chain.

transition-state-stabilizing serine residue of CAT (Leslie *et al.*, 1988; Leslie, 1990; Lewendon *et al.*, 1990; Russell & Guest, 1991*b*). The structural and functional relationship between the lipoate and CATs was seemingly confirmed by the inactivating effects of site-directed substitutions of the putative active-site histidine and serine residues in the *E. coli* lipoate acetyltransferase (H602C, S550C and S550A; Russell & Guest, 1990, 1991*b*) and in the bovine lipoate branched-chain acyltransferase (H391N and H391Q; Griffin & Chuang, 1990). However, comparable H427A and H427N substitutions in the yeast lipoate acetyltransferase had no effect on acyltransferase activity (Niu *et al.*, 1990), and in order to ensure that the inactivation conferred by the H602C substitution in the *E. coli* enzyme was due to elimination of the histidine rather than an irreversible acetylation of the cysteine substituent, further substitutions at this site have now been investigated.

In contrast, the *E. coli* E1p subunit exhibits a marked lack of sequence similarity with any of the known E1 subunits, including the E1o subunit of the *E. coli* 2-oxoglutarate dehydrogenase (ODH) complex (Darlison *et al.*, 1984; Matuda *et al.*, 1991; Wexler *et al.*, 1991). However, a 50-residue segment containing a $GXGX_{24-27}NN$ motif has been detected in several TPP-dependent enzymes including the E1 subunits of the 2-oxoacid dehydrogenase complexes, and it has been suggested that this motif could adopt a β–turn–α–turn–β secondary structure and be involved in TPP binding (Hawkins *et al.*, 1989). In order to investigate this possibility, site-directed substitutions of the second glycine residue have now been made in the E1p of *E. coli*.

In this paper an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible system for overproducing the PDH complex of *E. coli* is described, and it was used to characterize mutant complexes with site-directed substitutions in their E1p and E2p subunits.

MATERIALS AND METHODS

E. coli strains, bacteriophages and plasmids

The following *E. coli* strains were used as hosts for M13 and plasmid derivatives: TG1 (Δ*lac-proAB supE thi hsd*Δ*5*/F′*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*), NM522 (Δ*lac-pro hsd*Δ*5*/F′*proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*) and DH5α [*supE44* Δ*lac*U169 (φ80*lacZ*VM15) *hsdR17 recA1 gyrA96 endA1 thi-1 relA1*] for routine M13, phagemid and plasmid DNA preparations and transformations; BW313 (*dut ung thi-1 relA spoT1*/F′*lysA*) for the preparation of uracil-containing templates for mutagenesis (Kunkel, 1985); BMH71-18mutL (Δ*lac-proAB supE thi mutL*::*Tn10*/F′*traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*) for increased recovery of mutant M13 phages (Kramer *et al.*, 1984); and JRG1342 (Δ*aroP-lpd18 recA1 metB1 met-105 azi pox pps-1 relA rpsL*) the host strain used in growth tests for checking the Ace phenotype conferred by plasmids and in expression studies with plasmid-encoded PDH complexes (Guest *et al.*, 1985). The plasmids and bacteriophages used or constructed during this work are shown in Tables 1 and 2.

Media

The nutritional phenotypes of derivatives of the PDH complex null strain JRG1342 (Δ*aroP-aceEF-lpd*) carrying plasmids encoding wild-type and mutant PDH complexes, were determined as described previously (Russell & Guest, 1990). L Broth (Lennox, 1955) was used with glucose (0.1 %, w/v) and ampicillin

Table 1. Plasmids and phages used in cassette mutagenesis or as receptors

Plasmid or phage	Parent vector	Insert DNA or details	References for plasmid (vector)
pGS87	pBR322	Source of *aceE* cassette	Guest *et al.* (1985)
pGS350	pT7T3-19U	1.5 kb *Bam*HI–*Kpn*I (*aceE* cassette)	This work, Fig. 1; (Mead *et al.*, 1986)
M13BK350	M13mp19	1.5 kb *Bam*HI–*Kpn*I (*aceE* cassette)	This work, Fig. 1; (Norrander *et al.*, 1983)
pGS284	pJLA502	5.8 kb *Bam*HI–*Sal*I (*ace'EF-lpd*) (source of one-lip *ace'EF-lpd*)	Russell & Guest (1990)
pGS318	pGS284	Source of H602C *AceF* cassette	Russell & Guest (1990)
pGS333	ptac-85	5.8 kb *Bam*HI–*Sal*I (*ace'EF-lpd*) (from pGS284)	This work; (Marsh, 1986)
pGS367	pGS333	31 bp *Bam*HI *aceE* linker (*aceEF-lpd* expression)	This work, Fig. 1
pGS378	pT7T3-18U	1.04 kb *Sph*I–*Xho*I (*aceF* cassette)	This work, Fig. 1; (Mead *et al.*, 1986)
pGS400	pBR322	7.1 kb *Hin*dIII–*Sal*I (*aceEF-lpd*)	Russell & Guest (1991*b*)
pGS500	pGS367	*Sph*I site (ptac-85) removed	This work
pGS501	pGS500	2.5 kb *Sph*I–[*Xho*I]–*Sal*I (*ace'F-lpd*) (*Xho*I site from pGS400)	This work, Fig. 1

Table 2. Oligonucleotides and phages or plasmids carrying mutations in the *aceE* and *aceF* genes

Co-ordinates are corrected for the additional bases (1933–1935 in *aceE*). The *aceE* mutations were subcloned directly into the pGS367 expression plasmid.

Mutation	Oligonucleotide		Template for mutagenesis	Intermediate plasmid (pGS400)	Overexpression plasmid (pGS367 or pGS501‡)
	Number	Co-ordinates			
E1p-G231S	S146	1812–1794	M13BK350	–	pGS453
E1p-G231M	S147	1812–1794	M13BK350	–	pGS454
E1p-G231A	S145	1810–1789	pGS350	–	pGS455
E2p-H602G	S155	5584–5604	pGS378	pGS462	pGS475
E2p-H602A	S155	5584–5604	pGS378	pGS463	pGS476
E2p-H602C*	–	–	–	pGS318	pGS481
E2p-R603Q	S179	5586–5608	pGS378	pGS498	pGS502‡
E2p-R603V	S180	5586–5608	pGS378	pGS499	pGS503‡
E2p-Δ387–396†	S84	4933–4947 and 4978–4992	pGS378	pGS472	pGS477
E2p-Δ387–411	S85	4933-4947 and 5023–5037	pGS378	pGS473	pGS478
E2p-Δ387–422	S86	4933–4947 and 5056–5070	pGS378	pGS474	pGS479

* The construction of pGS318 encoding E2p-H602C has been described previously (Russell & Guest, 1990).
† The deletion co-ordinates are inclusive.
‡ Derivatives of pGS501 (and pGS500) contain only one *Sph*I site.

(50 μg/ml) as the rich medium for plasmid-carrying strains and 2TY (Sambrook *et al.*, 1989) was used for strains carrying bacteriophages.

Recombinant DNA techniques

Standard methods were used for M13 phage and plasmid DNA preparation, restriction enzyme digestion, isolation of DNA fragments, ligation, transformation and transfection (Glover, 1985; Sambrook *et al.*, 1989). Strain JRG1342 was transformed with plasmid DNA by the method of Chung *et al.* (1989).

Oligonucleotide-directed mutagenesis

A replaceable-cassette approach was used for constructing mutant *aceEF-lpd* operons. The cassettes, 1.5 kb *Bam*HI–*Kpn*I for *aceE* mutagenesis and 1.04 kb *Sph*I–*Xho*I for *aceF* mutagenesis (see Fig. 1), were subcloned in phage or phagemid vectors for mutagenesis and sequencing (Table 1) and mutant *aceEF-lpd* operons were then constructed by replacing the corresponding fragments of the receptor plasmids, generally pGS367 for E1p and pGS400 for E2p (Table 2). The mutagenic oligonucleotides used in this study, S84 (5'-ATCCCTGGCATGCTGGGTGAAATCGAAGAA-3'), S85 (5'-ATCCCTGGCATGCTGGGTGCGAACCTGAGC-3'), S86 (5'-CCTGGCATGCTGCCGCATGTTACTCAC-3'), S145 (5'-CATTTCAgCGTCACCGAGGAAC-3'), S146 (5'-TCCATTTCAgaGTCACCGA-3'), S147 (5'-TCCATTTCcatGTCACCGA-3'), S155 (5'-TCCTTCGACg(g/c)tCGCGTGATC-3'), S179 (5'-CTTCGACCACcagGTGATCGACG-3') and S180 (5'-CTTCGACCACgtgGTGATCGACG-3'), are identical with the corresponding *aceE* and *aceF* sequences (see Table 2 for co-ordinates) except for the mismatches (lower case) designed to direct amino acid substitutions.

Construction of *aceE* mutants. The 1.5 kb *Bam*HI–*Kpn*I *aceE* cassette was subcloned from the wild-type *aceEF-lpd* operon of pGS87 into pT7T3-19U (phagemid) and M13mp19 (phage) to produce pGS350 and M13BK350 respectively (Table 1; Fig. 1). Mutagenesis was performed as described by Kunkel (1985).

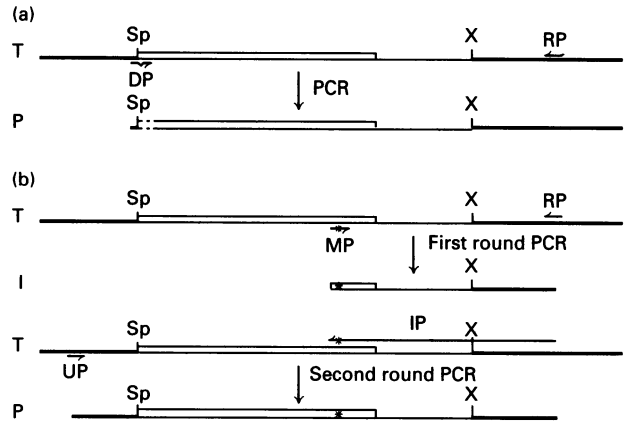

Fig. 2. PCR mutagenesis of the *aceF* gene

Deletions (*a*) and missense mutations (*b*) were introduced into a segment of the *aceF* gene encoding the catalytic domain using the phagemid, pGS378. Vector (thick lines), the *aceF* coding region (open boxes) and restriction sites (see legend to Fig. 1) are indicated. PCR primers are denoted by half-arrows above and below the templates and the arrowheads are placed at their 3′ ends. The DNA molecules are indicated as follows: T, template DNA (pGS378); P, PCR products; I, intermediate PCR product; RP, reverse sequencing primer; DP, deletion primers (S84, S85, S86); MP, missense primers (S155, S179, S180); IP, intermediate PCR product used as a primer; UP, universal sequencing primer. The approximate positions of the deletion mutations (broken line) and missense mutations (asterisk) are also indicated.

Mutant phages were enriched by transfecting BMH71-18mutL, and plaques were formed on TG1; mutagenized phagemid DNA was transformed directly into NM522. Several potential mutants were sequenced throughout the cassette region and one isolate carrying the desired mutation was used for replacing the wild-type cassette of the expression plasmid, pGS367 (Table 2; Fig. 1).

Construction of *aceF* mutants. The E2p catalytic domain is encoded by the 1.04 kb *Sph*I–*Xho*I *aceF* cassette and it was transferred to pT7T3-18U on a 1.1 kb *Eco*RI–*Hin*dIII fragment from M13mt120-SX377 (Russell & Guest, 1991*b*), to generate the phagemid, pGS378 (Table 1; Fig. 1), used in PCR mutagenesis (Fig. 2). Deletions were made by amplifying the *aceF* cassette with a mutagenic primer (S84, S85 or S86, which retain the *Sph*I site) and reverse sequencing primer, as shown in Fig. 2(*a*). Missense mutations were made by a two-stage procedure (Landt *et al.*, 1990) using a single mutagenic primer and flanking sequencing primers (Fig. 2*b*). In the first stage, mutagenic and reverse sequencing primers were used to amplify 0.5 kb fragments containing the desired mutations. These fragments were purified and used with universal sequencing primer in the second stage. In each case, the *aceF* cassettes were cleaved from the final PCR products, purified and subcloned for sequencing and expression. The cassettes were sequenced after recloning in the phagemid, pGS378, or by plasmid sequencing (Zhang *et al.*, 1988) after constructing mutant *aceEF-lpd* operons with pGS400 as the receptor (Table 1; Fig. 1). The pGS400 derivatives, listed as intermediate plasmids in Table 2, were used in nutritional tests in which the *aceEF-lpd* operons are expressed from the natural promoters. In most cases these intermediates also served as sources of the 5.8 kb *Bam*HI–*Sal*I fragments that were transferred to the *tac* expression plasmid, pGS367 (Fig. 1; Table 1). This is because pGS367 contains an extra *Sph*I site (derived from ptac-85) and lacks the engineered *Xho*I site, which precludes direct transfer of the 1.04 kb *Sph*I–*Xho*I *aceF* cassette. However, a more versatile expression plasmid, pGS501, capable of accepting the *Sph*I–*Xho*I *aceF* cassette was constructed and used with some of the later mutants. The extra *Sph*I site in pGS367 was removed by partial *Sph*I digestion, treatment with DNA polymerase (Klenow) and religation, followed by screening for a derivative (pGS500) which had lost the vector site. The *Xho*I site was then incorporated by replacing the 2.5 kb *Sph*I–*Sal*I *aceF-lpd* fragment of pGS500 with the corresponding *Sph*I–[*Xho*I]–*Sal*I fragment from pGS400 to produce pGS501 (Fig. 1). A derivative of pGS367 containing the H602C mutation (Russell & Guest, 1990) was constructed with the appropriate fragment of pGS318 (Tables 1 and 2).

Overproduction of PDH complexes

PDH complexes were overproduced in the *aceEF-lpd* deletion strain, JRG1342, transformed with derivatives of pGS367 encoding IPTG-inducible PDH complexes containing one lipoyl domain per E2p chain (Fig. 1, Table 1). Cultures (250 ml) in L Broth containing glucose (0.2%, w/v) and ampicillin (50 μg/ml) were grown at 37 °C with vigorous shaking to mid-exponential phase (A_{650} = 0.25–0.50) and induced by the addition of IPTG to 60 μM. After 6–8 h, cells were harvested, washed in buffer A [20 mM-potassium phosphate (pH 7.8) containing 2 mM-Na_2EDTA, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-benzamidine/HCl] and disrupted by French press or ultrasonic treatment in the same buffer. The crude extracts were clarified (100000 *g* for 30 min) and the PDH complexes were sedimented by further centrifugation (100000 *g* for 4 h). The PDH complexes were resuspended in buffer A and further purified by f.p.l.c. ion-exchange chromatography using a Mono Q column with a 0–700 mM-NaCl gradient in buffer A. Fractions containing PDH complex were pooled and concentrated by sedimentation as before. Purified complexes were stored in buffer A plus glycerol (50%, v/v) at −20 °C.

Enzymology and lipoylation studies

The PDH complex activities and the activities of their constituent E1p, E2p and E3 components were assayed as described previously (Russell & Guest, 1990). Specific activities are expressed in units/mg of protein where 1 unit represents 1 μmol of product formed, or substrate transformed, per min.

The degree of lipoylation of purified complexes was estimated by digesting samples containing 50 μg of PDH complex with trypsin (0.4%, w/w) for 1 h at 30 °C, and non-denaturing PAGE of the soluble proteins in 20% gels to resolve the modified and unmodified lipoyl domains (Ali & Guest, 1990; Brookfield *et al.*, 1991). The lipoylation of isolated PDH complexes with lipoate–protein ligase (kindly provided by D. E. Brookfield) has been described previously (Brookfield *et al.*, 1991).

PAGE

The purification of wild-type and mutant PDH complexes was monitored by PAGE (Laemmli, 1970) with 8% resolving gels and 4% stacking gels containing 0.1% SDS. Samples contained about 20 μg of crude extract protein or 5 μg of purified complex. Some gels were subjected to quantitative densitometric analysis after staining with Coomassie Brilliant Blue R250. Protein concentrations were estimated by the method of Lowry *et al.* (1951) with BSA as standard.

Materials

The [α-[^{35}S]thio]dATP (50 TBq/mmol) was from Amersham International. Restriction endonucleases, DNA polymerase I (Klenow fragment) and T4 DNA ligase were supplied by Bethesda Research Laboratories, Boehringer-Mannheim, Pharmacia LKB Biotechnology or Northumbria Biologicals (Cramlington, Northd., U.K.). Sequenase (modified T7 DNA

polymerase) was obtained from the United States Biochemical Corporation. Phagemids pT7T3-18U and pT7T3-19U were obtained from Pharmacia–LKB Biotechnology. BSA (fraction V; 0.7%, w/w, water), the protein standard, was from Sigma.

RESULTS

Overproduction of PDH complexes

Construction of pGS367. In previous studies, plasmids were constructed for the thermoinducible overproduction of wild-type and mutant PDH complexes because expression from the *ace* promoter was poor (Russell & Guest, 1990). The complexes were routinely enriched to 20–25% of soluble protein but the specific activity of the wild-type PDH complex was often 5-fold less than expected. This was attributed to the heat-shock induction procedure and proteolytic degradation of the E1p subunit (Russell & Guest, 1990). In order to improve the yield of *active* PDH complex, an IPTG-inducible expression system was investigated. The ptac-85 vector has a powerful promoter (*tac*, *trp-lac*) controlled by a plasmid-encoded *lac* repressor and a *lacZ* ribosome-binding site which is well placed for efficient translation of genes cloned at the multiple cloning site (Marsh, 1986). The 5.8 kb *Bam*HI–*Sal*I fragment encoding a truncated E1p, a one-lip E2p and E3, was transferred from the thermoinducible plasmid pGS284 to ptac-85 to give pGS333 (Table 1). A double-stranded oligonucleotide linker, encoding 10 *N*-terminal residues of E1p, was inserted in the desired orientation at the *Bam*HI site of pGS333 to give pGS367 (Fig. 1). The linker, formed from S133 (5′-GATCCACGTCGTTCGGGAAACGTTCAGACAT-3′) and S134 (5′-GATCATGTCTGAACGTTTCCCGAA-CGACGTG-3′), was designed to re-create the *Bam*HI site in the *aceE* coding region so that it could be used in cassette mutagenesis (Fig. 1).

Expression of the PDH complex from pGS367. It was previously shown that derivatives of JRG1342 (Δ*aroP-aceEF-lpd recA*) carrying thermoinducible *aceEF-lpd* operons are inviable at inducing temperatures in glucose minimal media (Russell & Guest, 1991*b*). This is apparently strain-specific because transformants of JRG590, a recombination-proficient strain with a smaller deletion (Δ*nadC-aceEF*), could grow under comparable conditions (Russell & Guest, 1990). It has also been shown that overexpression of a lipoyl domain subgene leads to incomplete lipoylation of the domain (Miles & Guest, 1987; Ali & Guest, 1990). Thus in defining conditions for efficient expression from pGS367, care was taken not to compromise the viability of the host strain and to ensure full lipoylation of the expressed complexes. Nutritional tests in glucose minimal media showed that pGS367 would complement the acetate requirement of JRG1342 at low IPTG concentrations (10–25 μM) but was lethal at higher concentrations (40–120 μM). In contrast, JRG1342-[pGS367] was viable at IPTG concentrations up to 120 μM in rich media. Studies on the effects of IPTG concentration and aeration showed that IPTG concentrations above 60 μM gave no further increase in PDH complex synthesis and that the primary effect of aeration was to alter the degree of lipoylation. Thus high aeration produced full lipoylation, decreased aeration gave partially lipoylated complexes and only a small proportion of the lipoyl domains were lipoylated in anaerobic cultures (not shown). Furthermore, it appeared that the anaerobically expressed complexes were otherwise intact since full lipoylation and a concomitant increase in PDH complex activity could be obtained by treatment with lipoate–protein ligase *in vitro* (Brookfield *et al.*, 1991). Under the standard conditions for growth and induction (see the Materials and methods section) the overproduced complexes were not underlipoylated.

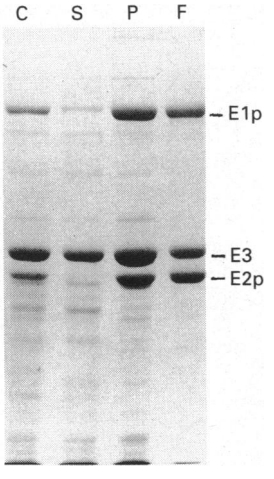

Fig. 3. Overproduction and purification of the pGS367-encoded PDH complex

A culture of JRG1342 [pGS367] was grown and induced as described in the Materials and methods section. Samples (μg of protein) at different stages in the purification were analysed by SDS/PAGE (8% gel stained with Coomassie Brilliant Blue): C, cell-free extract (15); S, 100000 *g* supernatant (15); P, 100000 *g* pellet (15); and F, f.p.l.c.-purified complex (7.5). The E1p, one-lip E2p and E3 subunits are indicated.

Purification and activity of overproduced PDH complexes. Wild-type and mutant PDH complexes were overproduced from derivatives of pGS367 (Materials and methods section), and the purities and yields were monitored at each stage by SDS/PAGE (Fig. 3) and by assaying the overall complex and subunit activities. Densitometric analyses indicated that some 35% of the protein in clarified extracts of JRG1342[pGS367] is PDH complex, and the proportion increased to about 65% in the 100000 *g* pellet and to more than 80% in the f.p.l.c.-purified material (Fig. 3). The crude extracts contained excess E3, presumably as a result of the combined expression from the *tac* and *lpd* promoters (Fig. 1). Much of this excess remained in the 100000 *g* supernatant, indicating that it is not bound to the complex (Fig. 3). The E1p/E2p/E3 polypeptide chain stoichiometry of the sedimented PDH complex was approximately 0.3:1.0:1.3 whereas that of the f.p.l.c.-purified material was 0.3:1.0:1.0, indicating that a fraction of the E3 subunits in the sedimented complex are less firmly bound. The relative deficiency in E1p may be a direct consequence of the excess of E3 since both bind to the surface of the E2p core.

The yields from 250 ml cultures of JRG1342[pGS367] were about 20 mg of purified complex, representing an 80% recovery from the clarified extracts. The yields obtained for inactive complexes were poorer and correspondingly less pure because the JRG1342 transformants grew less well (Fig. 4). Typical specific activities for the purified pGS367-encoded one-lip PDH complex and its components are shown in Table 3. Relative to pure PDH complex, the specific activities were 1.5–2-fold down for the complex and E1p, but the E2p and E3 activities were high. The low PDH complex and E1p activities may be directly related to the altered subunit stoichiometry, particularly the deficiency in the rate-limiting E1p subunit (Bates *et al.*, 1977). The purified pGS367-encoded PDH complex retained only 9% of its activity when TPP was omitted but it was fully re-activated by 5 μM-TPP, which is far less than is provided in the standard assay (200 μM). The requirement for TPP by f.p.l.c.-purified complexes could prove useful in TPP-binding studies with E1p mutants substituted in the putative TPP-binding fold.

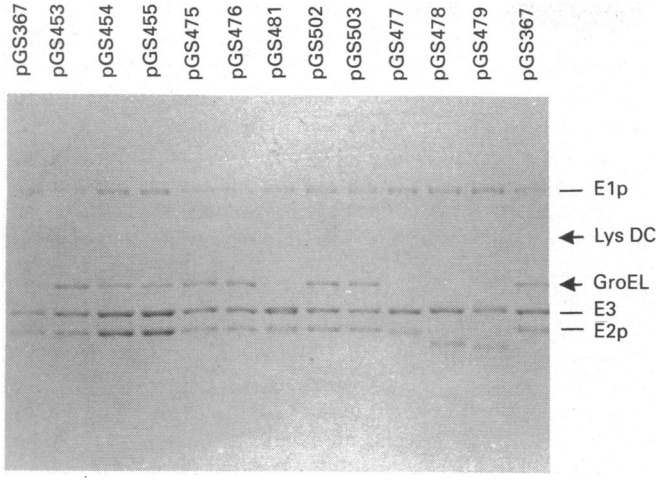

Fig. 4. SDS/PAGE analysis of mutant PDH complexes

Samples (2 μg of protein) of the f.p.l.c.-purified complexes encoded by the different plasmids were analysed by SDS/PAGE and stained with Coomassie Brilliant Blue. The positions of the E1p, one-lip E2p and E3 subunits are indicated, as are the positions of the contaminants, lysine decarboxylase (LysDC) and the chaperonin subunit (GroEL).

Oligonucleotide-directed mutagenesis of the *aceE* gene

The sequences containing the $GXGX_{24-27}NN$ motifs in the proposed TPP-binding sites could resemble the cofactor-binding folds of many adenine dinucleotide-linked dehydrogenases (Wierenga *et al.*, 1985) by forming comparable β–turn–α–turn–β structures (Hawkins *et al.*, 1989). The corresponding sequence in the E1p of *E. coli* is $GDGX_{26}NCN$ (residues 229–260), which lacks the NN consensus. In preliminary studies the following site-directed substitutions were made: G231A, G231S and G231M. Alanine and serine were chosen as minimal hydrophobic and polar replacements, and methionine, which should be locally disruptive at a turn, has a side chain which might be accommodated within the hydrophobic core of the protein, thus decreasing the possibility of wide range disruption. The replacements were intended to inflict various degrees of disruption of the E1p structure without necessarily removing its ability to fold.

Mutations were made in vectors carrying the 1.5 kb *Bam*HI–*Kpn*I *aceE* cassette (Fig. 1) and derivatives of pGS367 containing the mutant cassettes were used to express the corresponding one-lip PDH complexes: pGS453, E1p-G231S; pGS454, E1p-G231M; and pGS455, E1p-G231A (Tables 1 and 2). None of the plasmids was capable of complementing the acetate requirement of JRG1342 (with 20 μM-IPTG), indicating that the corresponding complexes had little or no activity. The three plasmids expressed sedimentable complexes which purified in the same way as the pGS367-encoded complex and had similar subunit stoichiometries (Fig. 4). The PDH complex and E1p activities of the mutant complexes were severely impaired but E2p and E3 were unaffected (Table 3). The order of increasing impairment, G231A > G231M > G231S, indicates that the hydrophobic substituents are tolerated better than the polar substituent.

During the sequencing of the 1.5 kb *Bam*HI–*Kpn*I *aceE* cassettes, an error was discovered in the nucleotide sequence of the *aceE* gene (Stephens *et al.*, 1983*a*). Two copies of the ATC sequence at co-ordinates 1931–1933 were detected and as a consequence, an isoleucine codon is added to the revised sequence: AAG-*ATC*-ATC (1928–1936), K-*I*-I (residues 273–275). The extra bases were clearly visible on the original autoradiographs of both DNA strands, but they had been deleted during sequence compilation. The relative molecular mass of the *aceE* product (E1p) is therefore increased by one isoleucine residue to 99598 (886 amino acid residues) and the original nucleotide co-ordinates (and residue positions) greater than 1933 (273) increase by 3 (1).

Oligonucleotide-directed mutagenesis of the *aceF* gene

In order to resolve the contradictory observations concerning the importance of the putative catalytic histidine residue in lipoate acyltransferases, two further substitutions (H602G and H602A) were made in the E2p of *E. coli*, the latter being chosen because it is identical with the apparently neutral substitution in yeast E2p (Niu *et al.*, 1990). The importance of the adjacent arginine residue in the highly conserved DHRXXDG motif was investigated with two substitutions (R603Q and R603V). Additionally, a set of nested deletions was designed to help define

Table 3. Biochemical characterization of overproduced PDH complexes

The specific activities refer to f.p.l.c.-purified complexes.

Mutation	Overexpression plasmid	Ace phenotype conferred	Specific activity (units/mg of protein)			
			PDH complex	E1p	E2p	E3
One-lip E2p	pGS367	+	14	0.07	7.7	183
E1p-G231S	pGS453	–	< 0.001	< 0.002	10.4	110
E1p-G231M	pGS454	–	0.003	< 0.002	10.1	129
E1p-G231A	pGS455	–	0.01	0.005	10.2	107
E2p-H602G	pGS475	–	0.007	0.017	< 0.07	218
E2p-H602A	pGS476	–	0.01	0.021	< 0.07	146
E2p-H602C	pGS481	–	< 0.001	0.030	< 0.07	194
E2p-R603Q	pGS502	+	5.3	0.044	8.6	154
E2p-R603V	pGS503	+	3.4	0.028	3.2	119
E2p-Δ387–396	pGS477	+	3.2	0.039	3.8	234
E2p-Δ387–411	pGS478	–	0.04	0.016	0.10	154
E2p-Δ387–422	pGS479	–	0.15	0.028	0.22	97
None*			25	0.1	7	50

* Values for pure wild-type PDH complex (Russell & Guest, 1990).

the catalytic domain boundary and to assess the importance of predicted secondary structural elements in a region of poor similarity with CAT (Guest, 1987; Guest *et al.*, 1989). The mutations and deletions were introduced by PCR mutagenesis, confirmed by sequencing and then subcloned (see the Materials and methods section; Fig. 2 and Table 2). The properties of the corresponding PDH complexes (overproduced by the pGS367 or pGS501 series of expression plasmids) and their abilities to complement the Ace$^-$ nutritional lesion of JRG1342 when expressed from both the natural promoter (pGS400-derived intermediate plasmids) and the *tac* promoter (pGS367 and pGS501 derivatives) were investigated. A pGS367 derivative containing the H602C substituent (Russell & Guest, 1990) was constructed and included for comparison.

E2p-H602 mutants. The importance of H602 was confirmed in growth tests with two sets of plasmids encoding the H602G, H602A and H602C substitutions in *aceEF-lpd* operons expressed from the *ace* promoter (pGS400 derivates) and the *tac* promoter (pGS367 derivatives). Both sets of substituted complexes were unable to complement the acetate requirement of JRG1342 and the IPTG-induced complexes had no detectable E2p activity (Tables 2 and 3). However, in the sensitive PDH complex assay, the H602G and H602A complexes retained 0.05–0.07% of the activity of the parental complex, but the H602C complex had no detectable activity (Table 3). In each case, a sedimentable complex, which could be purified in the same way as the pGS367-encoded complex, was assembled. Furthermore, the lipoyl domains of the mutant complexes served as substrates for E1p in reductive acetylation tests (not shown). These results strongly support the original suggestion that, as in CAT, H602 is an essential catalytic residue.

E2p-R603 mutants. The arginine residue next to H602 in the E2p of *E. coli*, R603, is highly conserved in a wide range of E2 sequences. In the only known variant, the MRP3 protein of *Neurospora crassa*, lysine occupies this position (Russell & Guest, 1991*a*). In CAT, this position is occupied most frequently by alanine, so the conservation of a basic residue seems to be an important feature of the lipoate acyltransferases. In order to test whether R603 performs an essential structural or catalytic role in the E2p of *E. coli*, polar (R603Q) and hydrophobic (R603V) substitutions were made (Table 2). In nutritional tests the corresponding complexes were able to complement the acetate requirement of JRG1342 regardless of whether they were expressed from the *ace* promoter (pGS498 and 499) or the *tac* promoter (pGS502 and 503; Table 3). The PDH complexes overproduced from pGS502 and pGS503 were sedimentable and could be purified by the same method as the parental complex (Table 3 and Fig. 4). The f.p.l.c.-purified R603Q- and R603V-based complexes had about 20–30% of the PDH complex activity of comparable preparations of the parental complex, and, whereas the R603V complex had only 30% of parental E2p subunit activity, the R603Q complex appeared to be unaffected in this respect (Table 3). These results imply that R603 does not play an essential role in E2p catalysis.

E2p deletion mutants. The predicted similarity between CAT and the catalytic domain of E2p is not strong in the region of the putative *N*-terminal boundary of the domain (Guest *et al.*, 1989). A set of nested deletions, which extend from the inner linker into the *N*-terminal region of the catalytic domain but leave the lipoyl and E3-binding domains intact, was constructed in order to assess the importance of this region. The deletions extend up to, through and into the putative equivalents of α_1 and β_A in CAT (E2p-Δ387–396, E2p-Δ387–411 and E2p-Δ387–422 respectively and inclusive). These structural elements contribute residues to the chloramphenicol-binding pocket in CAT (Leslie, 1990). The three deletions were made by PCR mutagenesis of the *Sph*I–*Xho*I *aceF* cassette in pGS378 (Fig. 2) and the corresponding *aceEF-lpd* operons were reconstructed in pGS400 and pGS367 (Table 2). In nutritional tests the E2p-Δ387–396 complex complemented the lesion of JRG1342 whereas the E2p-Δ387–411 and E2p-Δ387–422 complexes appeared to be inactive when expressed from the *tac* promoter under IPTG-inducing conditions (Table 3), but weakly active when expressed from the natural promoters in pGS473 and pGS474 (not shown). The overproduced complexes were sedimentable, implying that the residues responsible for core assembly and E1p binding are located outside the segments deleted from the catalytic domain. The enzymological properties of mutant PDH complexes paralelled the nutritional responses (Table 3; Fig. 4). Thus the smallest deletion (E2p-Δ387–396) suffered only modest decreases in PDH complex and E2p activities, whereas the larger deletions (E2p-Δ387–411 and E2p-Δ387–422) retained very little of either (Table 3). These results are consistent with the view that, as in CAT, residues in the *N*-terminal segment of the E2p catalytic domain are involved in catalysis.

DISCUSSION

The PDH complex of *E. coli* offers opportunities to investigate many structure–function aspects of the 2-oxoacid dehydrogenase complexes, particularly the assembly of the complex, the catalytic mechanisms of individual subunits, the molecular interactions associated with substrate specificity and cofactor binding, domain organization and conformational mobility. The *E. coli* PDH complex has the added advantage that engineered subunits and domains can be expressed independently or as part of a complex in a host that is devoid of PDH complex, and the effects can be assayed both *in vivo* and *in vitro*.

Compared with thermoinduction (Russell & Guest, 1990), the IPTG-inducible system developed here with pGS367 and its derivatives provided a more convenient and reproducible method for the overproduction of PDH complexes for rapid purification and 80% recovery. The yields of soluble complex were 1.5-fold higher and, in the case of the parental complexes, the specific activities of the purified material were 2–3-fold higher. Furthermore, the plasmids could be used in nutritional tests with transformants of JRG1342 in the presence of low concentrations of IPTG. Some 35% of the soluble protein in crude cell-free extracts was PDH complex and this could be purified almost to homogeneity by sedimentation and ion-exchange chromatography. The overproduced PDH complexes were deficient in E1p subunits and had an excess of E3 subunits (Fig. 3). Indeed, hyperproduction of the latter may limit the extent of E1p binding. The specific activities of the PDH complex were lower than maximal but consistent with the relative amounts of the rate-limiting E1p subunit (Table 3). The stoichiometries of the overproduced complexes might be improved if the internal *lpd* promoter is deleted, but this would have to be done without removing other potential transcriptional or translational signals in the *aceF-lpd* intergenic region (Stephens *et al.*, 1983*c*; Spencer & Guest, 1985). The newly constructed *aceEF-lpd* overexpression plasmid, pGS501, contains six unique restriction sites which provide convenient replaceable cassettes for mutagenesis in the *aceE*, *aceF* and *lpd* genes.

The purified complexes contained various amounts of two contaminating polypeptides (Fig. 4). *N*-Terminal sequence analyses, kindly performed by J. B. C. Findlay and J. N. Keen (S.E.R.C. Amino Acid Sequencing Facility, University of Leeds), showed that the minor contaminant (upper band) is identical with lysine decarboxylase over six residues (Watson *et al.*, 1992), and the major contaminant (lower band) is identical with the chaperonin, GroEL, over 30 residues (Hemmingsen *et al.*, 1988).

These contaminants copurify with the PDH complex but can be removed by gel filtration with Sepharose 6B (Angier, 1989). The presence of GroEL is interesting because it may be needed for folding the PDH subunits. However, it is not known whether its prominence here is related to either the overproduction of the PDH complex or the stress that may be caused by IPTG. Previously it was wrongly concluded that the GroEL contaminant was a proteolytic fragment of E1p (Russell & Guest, 1990), because a tryptic fragment of this subunit migrates with approximately the same mobility (Radford *et al.*, 1987; Texter *et al.*, 1988).

Replacing E1p-G231 with three different substituents caused dramatic decreases in E1p activity, consistent with the critical role in TPP binding proposed for this residue. The disruption of a turn in the proposed binding fold might be expected to have serious structural as well as catalytic consequences. However, each of the mutant subunits assembled into sedimentable complexes with stoichiometries similar to the parental complexes. This suggests either that substituting G231 does not sufficiently affect the conformation of E1p to prevent assembly, or that binding to the E2p core is mediated by an independently folding E1p domain. Thus the results reported here indicate that G231 is an important residue in E1p, but further studies will be needed to establish whether the substituted E1p subunits are impaired in their ability to bind TPP. Since the submission of this paper, it has been reported that substituting asparagine, threonine or glycine for the aspartate residue that is conserved between the glycine residues in the $GDGX_{24}NN$ motifs of four pyruvate decarboxylases abolishes activity and TPP binding of the *Zymomonas mobilis* pyruvate decarboxylase (Diefenbach *et al.*, 1992). Interestingly, the corresponding position is not universally conserved in the motifs of the 2-oxoacid dehydrogenases, but can be occupied by glutamate or glutamine as well as aspartate (Hawkins *et al.*, 1989).

In CAT, H195 functions as a general base in the acetylation of chloramphenicol and its replacement leads to enzyme inactivation (Burns & Crowl, 1987; W. V. Shaw, personal communication). Substitution of the equivalent histidine residue (H602) by glycine, alanine or cysteine in the highly conserved motif of E2p severely impaired lipoate acetyltransferase activity without affecting the assembly of the E2p core, the ability to bind E1p and E3 subunits or the acetylation of the lipoyl domain by E1p. These results confirm that H602 is critical for E2p activity and they are consistent with the view that this residue functions as a general base in the lipoate acetyltransferase reaction (Guest, 1987; Guest *et al.*, 1989). It is not clear why replacing the analogous residue, H427, had no effect on the activity of the catalytic domain in yeast E2p (Niu *et al.*, 1990). In view of the very high degree of conservation in all E2 subunits and CATs, it seems unlikely that the role of the histidine residue varies between lipoate acyltransferases from different sources, unless there are specific features of the E2p active site or the core symmetry in yeast which allow replacement of the invariant histidine. It also seems unlikely that potentially inactive yeast catalytic domains could have been contaminated with sufficient *E. coli* acetyltransferase during expression to have shown no impairment in activity. Substitution of another residue conserved in the E2 and CAT motifs, D606 in *E. coli* E2p and D431 in yeast E2p, decreased the acetyltransferase activities by up to 20-fold (Niu *et al.*, 1990; Russell & Guest, 1991*b*). In contrast, a basic residue which is conserved in the E2 motif (R603), but not in the CAT motif, could be replaced by glutamine and valine without inactivating E2p. This implies that R603 is conserved for some non-essential function.

The catalytic core of the *Azotobacter vinelandii* E2p has been crystallized (Schulze *et al.*, 1991) and the elucidation of its structure should identify the roles of the highly conserved residues and at the same time confirm or refute the predicted homology with CAT.

The approximate *N*-terminal boundary of the acetyltransferase domain of the *E. coli* E2p subunit can be estimated variously by limited proteolysis or sequence comparisons with other E2 subunits and CAT, as E372, W388, F396 or L404 (Packman & Perham, 1987; Guest, 1987; Guest *et al.*, 1989; Russell & Guest, 1991*a*). Both assessments depend on the unusual amino acid composition and structure of the interdomain linkers of the E2 subunits. In addition, previous studies with an *aceF* subgene (the *Sph*I–*Xho*I *aceF* cassette, Fig. 1), expressing an E2p catalytic domain starting at M385 from two thermoinducible λ promoters in pGS223, showed that a catalytically active inner core could be assembled from the *C*-terminal segment of E2p (Guest *et al.*, 1989; Angier, 1989). Unfortunately, this inner core became insoluble in the absence of E1p subunits. The properties of the three complexes containing the internal E2p deletions indicate that residues between M385 and G396 are not required for inner-core assembly or catalysis, which means that the functional catalytic domain boundary is probably distal but close to G396. These observations are consistent with the predicted structure–function similarities between E2p and CAT. It is not known why the E1p activities of the mutant E2p PDH complexes were lower than the unsubstituted complex. It could be due to variations in subunit stoichiometry, or the E1p subunits may be more susceptible to inactivation when E2p activity is impaired.

Further studies will be needed to identify the residues that interact with the lipoyl domains, those forming the interfaces between the eight E2 trimers proposed for the assembled core and those involved in E1 binding, since they have no counterparts in CAT. The elucidation of a structure for at least one E2, in concert with multiple sequence-alignment techniques, should also shed light on the different substrate specificities and E2-core symmetries exhibited by the lipoate acyltransferase subunits of the 2-oxoacid dehydrogenase complexes.

Note added in proof (received 17 July 1992)

The structure of the catalytic core of the *Azotobacter vinelandii* PDH complex has now been elucidated (Mattevi *et al.*, 1992).

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