Biochem. J. (1988) 256, 741–749 (Printed in Great Britain)

Overexpression and mutagenesis of the lipoamide dehydrogenase of *Escherichia coli*

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A 'split-gene' technique for the overexpression and mutagenesis of the gene encoding the lipoamide dehydrogenase of *Escherichia coli* was developed in order to overcome the instability problems encountered when attempting to mutate the intact gene. The lipoamide dehydrogenase gene, *lpd*, was dissected into two fragments which were separately subcloned into M13 vectors for mutagenesis *in vitro* followed by reconstitution in the pJLA504 expression vector under the transcriptional control of the λP_R and λP_L promoters and a temperature-sensitive λ repressor. After thermoinduction, *E. coli* cells transformed with the plasmid carrying the reconstituted *lpd* gene contained 4–5 times more lipoamide dehydrogenase activity than is normally found in the wild-type organism. The strategy was used to engineer a Glu-188 → Asp replacement in lipoamide dehydrogenase, and this generated an enzyme with markedly different kinetic properties.

INTRODUCTION

Lipoamide dehydrogenase [dihydrolipoamide dehydrogenase, EC 1.8.1.4 (formerly 1.6.4.3)] is an essential component (E3) of the pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase and branched-chain-2-oxoacid dehydrogenase multienzyme complexes, which catalyse the oxidative decarboxylation of the respective 2-oxo acids to acetyl-CoA, succinyl-CoA and branched-chain-fatty acyl-CoA (Reed, 1974; Williams, 1976; Guest, 1978; McCully *et al.*, 1986). The *E. coli* enzyme is typical in being a homodimeric flavoprotein of subunit M_r 51274 (including FAD) and in catalysing the NAD^+-dependent re-oxidation of the dihydrolipoamide cofactors that are covalently bound to the acyltransferase components (E2) of the multienzyme complexes. The bacterial and mitochondrial glycine-cleavage systems also contain a lipoamide dehydrogenase component that functions in the reversible oxidative decarboxylation of glycine (Kochi & Kikuchi, 1976). More recently, lipoamide dehydrogenase has been found in organisms which lack the 2-oxoacid dehydrogenase complexes, notably in the halophilic archaebacteria (Danson *et al.*, 1986) and in a membrane-associated state in the bloodstream form of the eukaryotic parasite *Trypanosoma brucei* (Danson *et al.*, 1987), but in both cases the function of the enzyme is unknown. Lipoamide dehydrogenase belongs to a family of related flavoprotein oxidoreductases each containing an active disulphide bridge that undergoes reversible oxidation or reduction during the catalytic cycle (Williams, 1976). Other members of this group include glutathione reductase (EC 1.6.4.2; Schulz *et al.*, 1978), mercuric reductase (EC 1.6.4.-; Fox & Walsh, 1982) and thioredoxin reductase (EC 1.6.4.5; Holmgren, 1980). These enzymes contain some very highly homologous regions of primary structure, especially around the cysteine residues of the active disulphide (Williams *et al.*, 1982).

The lipoamide dehydrogenase gene, *lpd*, is located at 2.6 min in the *E. coli* linkage map immediately distal to the *aceE* and *aceF* genes that encode the respective dehydrogenase (E1p) and acetyltransferase (E2p) components of the PDH complex (Guest, 1978; Fig. 1). The *lpd* gene has been cloned and its nucleotide sequence has been determined (Guest & Stephens, 1980; Stephens *et al.*, 1983). Furthermore, quantitative transcript mapping studies of the *ace–lpd* region have shown that the *lpd* gene can be transcribed by readthrough from the *ace* promoter during the synthesis of the PDH complex, and independently from its own promoter during the synthesis of the 2-oxoglutarate dehydrogenase complex (Spencer & Guest, 1985). The *lpd* gene can therefore be expressed both as a distal gene of the *ace* operon and as an independent gene which is co-ordinately regulated with the *sucABCD* operon encoding the dehydrogenase (E1o) and succinyltransferase (E2o) of the 2-oxoglutarate dehydrogenase complex and the β- and α- subunits of succinyl-CoA synthetase. These observations have explained how the expression of a single *lpd* gene can be coupled to *aceEF* and *sucAB* expression in order to supply E3 components for assembly into the two independently regulated complexes.

The primary structure of the *E. coli* lipoamide dehydrogenase, translated from the *lpd* structural gene, has been compared with the primary and three-dimensional structures of the human erythrocyte glutathione reductase (Krauth-Siegel *et al.*, 1982; Pai & Schulz, 1983), and this has revealed a remarkable degree of structural conservation in most domains, the major differences occurring only at the enzyme surface and the substrate-binding domain (Rice *et al.*, 1984). Of the 463 equivalenced residues, some 50% are either identical or conservatively

Abbreviations used: Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid); $APAD^+$, 3-acetylpyridine–adenine dinucleotide; IPTG, isopropyl β-thiogalactoside; PAGE, polyacrylamide-gel electrophoresis; PDH, pyruvate dehydrogenase.

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substituted. Such comparisons have allowed meaningful predictions to be made about the functions of specific amino acid residues in lipoamide dehydrogenase, despite the absence of a complete structure for this enzyme (Rice *et al.*, 1984; Guest & Rice, 1984). The aim of the present work was to devise a strategy for the overexpression and site-directed mutagenesis of the *E. coli* lipoamide dehydrogenase in order to define the functions of potentially important structural and catalytic residues.

METHODS AND MATERIALS

Strains of *E. coli*, plasmids and bacteriophages

Bacteriophages M13mp18, mp19 and their derivatives were propagated in strain JM101 (*ΔproAB-lac supE thi/ F′ traD36 proA⁺B⁺ lacIqZΔM15*) for preparing DNA templates for mutagenesis *in vitro* and sequence analysis (Messing, 1979). A PDH-complex-deletion strain, JRG-1342 (*ΔaroP-lpd*; Guest *et al.*, 1985) was used as the host for expressing plasmid-encoded lipoamide dehydrogenase. Plasmid pGS81 (Lpd^+Ap^R) which contains a 5.4 kb *Hin*dIII/*Eco*RI (*ace′F–lpd*) fragment was reconstructed from the pBR325 derivative pGS41 (*ace′E aceF lpd*; Guest *et al.*, 1983) by *Hin*dIII-promoted deletion and re-ligation (R. E. Roberts & J. R. Guest, unpublished work). The phagemid pUC119 (J. Messing & J. Vieira, unpublished work) and the expression vectors pJLA502 and pJLA504 (Schauder *et al.*, 1987) were kindly provided by Dr. J. Vieira and Dr. J. E. G. McCarthy respectively.

Oligonucleotide-directed mutagenesis

The Amersham oligonucleotide-directed *in vitro* mutagenesis kit (Taylor *et al.*, 1985) was used to create a *Sal*I site immediately downstream of the *lpd* terminator, a *Xho*I site 28 bp upstream of the *lpd* start codon and to change the Glu-188 codon (GAA) to an Asp codon (GAC) in the *lpd* structural gene. The synthetic oligonucleotides used were: S37, TTCCAGTCGACTTGC-TCC (7501–7519); S60, TGACCGCTCGAGATAAA (5969–5985); and S56, ATCGGTCTGGACATGGGC-ACCGTT (6556–6579). The nucleotide co-ordinates (in parentheses) refer to the *lpd* gene (Stephens *et al.*, 1983). Mutant products were detected and characterized by nucleotide sequence analysis.

Molecular cloning and nucleotide sequence analysis

Plasmid preparation, restriction-endonuclease digestion, DNA-fragment isolation, ligation and transformation were done by standard methods (Maniatis *et al.*, 1982). Primer extension cloning from M13 was performed as described by Miles & Guest (1987).

Single-stranded M13 templates were prepared and sequenced by the dideoxy chain-termination method using [α[^{35}S]thio]dATP and salt-gradient gels (Sanger *et al.*, 1980; Biggin *et al.*, 1983). Several synthetic oligonucleotides were used as primers for confirming the sequences of the M13 constructs [co-ordinates are those given by Stephens *et al.* (1983)]: S1 ('universal primer'); S55 (6150–6172); S42 (6426–6445); S59 (6933–6951); and S58 (7174–7190) (see Fig. 1).

Lipoamide dehydrogenase expression

Derivatives of the expression plasmids pJLA502 and pJLA504 carrying wild-type or mutated *lpd* genes were used to transform strain JRG1342 (*Δace-lpd*), which was grown at 28 °C in L broth (Lennox, 1955) containing glucose (2 g/l) and ampicillin (100 μg/ml, when required) using an inoculum (1:20) from an overnight culture. The cultures were shifted to 42 °C at an A_{650} of 0.4–0.5 and sampled at intervals for enzymic analysis and SDS/PAGE as described by Miles & Guest (1987). Transformants of JRG1342 containing phagemids (pUC119, derivatives) carrying the wild-type *lpd* gene were grown at 37 °C in L broth plus glucose (2 g/l), ampicillin (100 μg/ml) and IPTG (10 μg/ml), as required, and grown to an A_{650} of 0.8–1.0.

Growth tests

The nutritional phenotypes of plasmid-containing transformants of JRG1342 were described by Guest *et al.* (1985), and the presence of lipoamide dehydrogenase activity in transformants of JRG1342 was detected by using the following plate test. Glass plates containing L agar plus glucose (0.2%, w/v) were inoculated with small patches of the test strains, and grown at 37 °C for 24 h, and the cells were lysed by adding chloroform (0.5 ml) to the inverted plates and incubating for a further 8–10 h. The plates were then overlayered with molten agar (0.5%, 44 °C) containing NAD^+ (0.25 mM), NADH (0.5 mM), oxidized lipoamide (0.75 mM) and Nbs_2 (0.75 mM) in 40 mM-potassium phosphate buffer, pH 7.8. Cells containing lipoamide dehydrogenase activity rapidly developed an intense yellow coloration, whilst bacteria containing no enzyme activity remained pale. Cultures of JM101 and untransformed JRG1342 were used as positive and negative controls respectively.

Lipoamide dehydrogenase assay and PAGE

Lipoamide dehydrogenase activity was measured by monitoring the reduction of 3-acetylpyridine–adenine dinucleotide ($APAD^+$) at 366 nm in cell-free extracts of organisms as described by Langley & Guest (1977). In some cases, $APAD^+$ was replaced by NAD (1.5 mM) and the reaction was monitored at 340 nm. Absorption coefficients of 9.1×10^3 $M^{-1} \cdot cm^{-1}$ and 6.22×10^3 $M^{-1} \cdot cm^{-1}$ were used for APADH and NADH respectively. Enzyme activities are expressed as μmol of dihydrolipamide oxidized/h per mg of protein. Protein was measured in cell-free extracts as described by Lowry *et al.* (1951), with bovine serum albumin as standard. The proteins of cell-free extracts were also analysed by electrophoresis in 12% (w/v) polyacrylamide gels containing 0.1% SDS (Laemmli, 1970) and stained with Coomassie Brilliant Blue.

Materials

The [α-[^{35}S]thio]dATP (410 Ci/mmol) and the oligonucleotide-directed '*in vitro*' mutagenesis kit were supplied by Amersham International. Restriction endonucleases, DNA polymerase (Klenow fragment) and phage-T_4 DNA ligase were from either Bethesda Research Laboratories or Boehringer. Oligonucleotides were made with an Applied Biosystems DNA synthesizer (model 381A). Oxidized lipoamide and Nbs_2 were obtained from Sigma, and NAD^+ and $APAD^+$ were from Boehringer. M_r markers for SDS/PAGE were supplied by BDH. Purified lipoamide dehydrogenase from *E. coli* and dihydrolipoamide were kindly supplied by Ms. Jane Angier of J. R. G.'s Department.

RESULTS AND DISCUSSION

A strategy for lipoamide dehydrogenase amplification and mutagenesis

An important factor in developing a strategy for mutagenesis *in vitro* is to ensure that the mutated gene can be expressed at a high level in order to facilitate studies on the altered gene product. Possible approaches include using: (*a*) the versatile pUC118/9 phagemid vectors that contain a phage replication origin for generating single-strand templates for mutagenesis and sequencing, and a *lac* promoter for controllable expression (J. Messing & J. Vieira, unpublished work); or (*b*) M13mp18/19 as vectors for mutagenesis followed by subcloning the mutated gene into expression vectors such as pJLA502/4 which have tandem λP_R and λP_L promoters controlled by the thermosensitive repressor (*λcI857*), a highly efficient *atpE* ribosome-binding site, and an fd transcriptional terminator downstream of the structural-gene cloning site (Schauder *et al.*, 1987; Fig. 2). Owing to the paucity of suitable restriction sites flanking the *lpd* gene, and to the very high inherent instability of the 5.4 kb *Hin*dIII/*Eco*RI fragment (Fig. 1), both in M13 and in the single-stranded forms of the phagemid vectors, it proved impossible to clone the intact *lpd* gene in a form suitable for mutagenesis *in vitro*. It was therefore necessary to adopt a different strategy in which the *lpd* gene was dissected into two fragments that could be separately and stably cloned into M13 for mutagenesis and then reconstructed in the expression vector.

The most successful strategy for enzyme amplification and mutagenesis *in vitro* is illustrated in Fig. 2. This strategy is based on several observations, such as the favourable distribution of *Xho*II sites in the partially sequenced 5.4 kb *Hin*dIII/*Eco*RI fragment, and the presence of a nascent *Sal*I site, usefully located for deleting unwanted DNA downstream of the *lpd* transcriptional terminator (Fig. 1). There is only one *Xho*II site in the *lpd* coding region (AGATCC, position 6474), and this proved to be very useful for generating proximal (A) and distal (B) segments of the gene. It also had the advantage of containing a half-*Bam*HI site which recreates a *Bam*HI site when segment B is ligated into a vector *Bam*HI site (Fig. 2). The 5.4 kb *Hin*dIII/*Eco*RI fragment of pGS81 containing part of the *aceF* gene and all of the *lpd* gene was isolated and digested with *Xho*II. The 1.98 kb *Hin*dIII/*Xho*II fragment was then cloned into the *Hin*dIII and *Bam*HI sites of M13mp18 to yield a stable phage, 18LPDA, which provided the template for

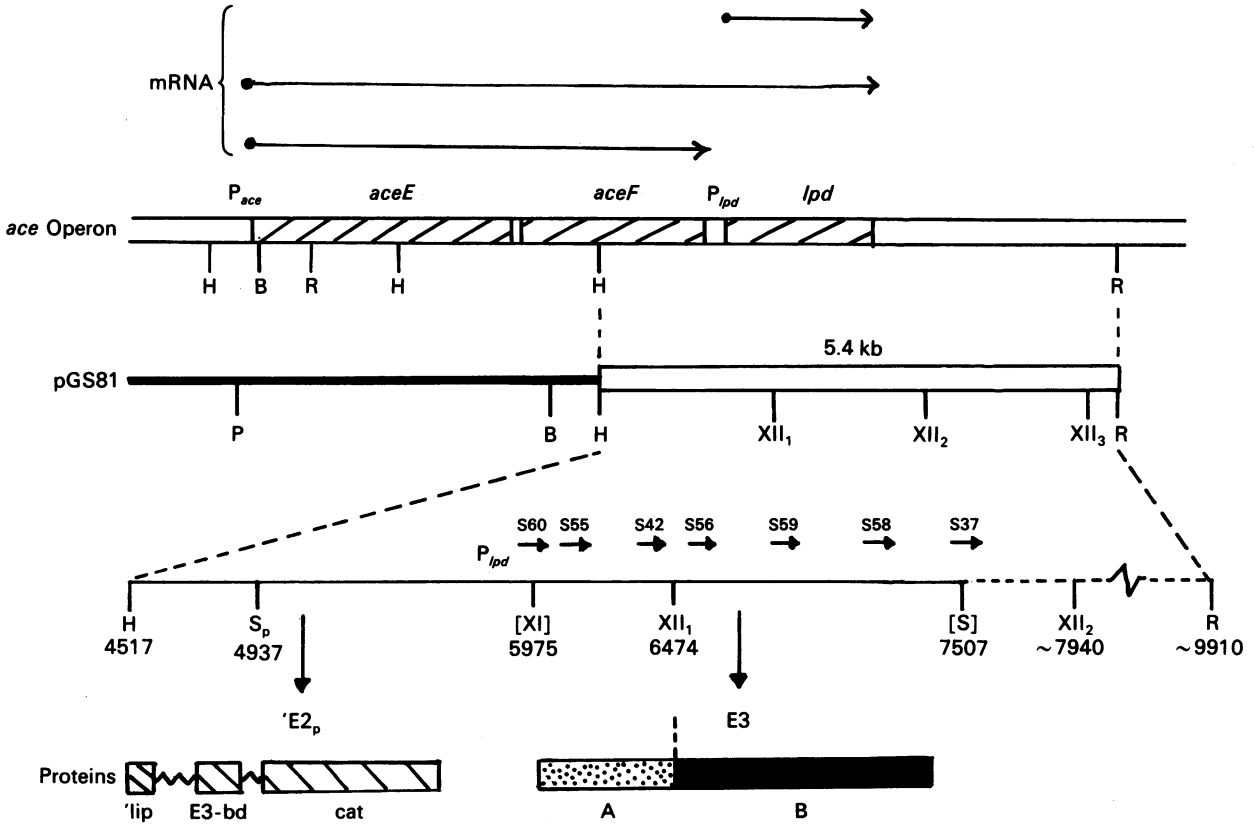

Fig. 1. Organization of PDH-complex genes of *E. coli*

The mRNA transcripts from the *ace* and *lpd* promoters are shown. The 5.4 kb *Hin*dIII/*Eco*RI cloned fragment of pGS81 (open bar) is expanded to show: the sequenced (——) and unsequenced (----) regions; the priming sites (→) of oligonucleotides used for sequencing and/or mutagenesis; and the restriction sites (H, *Hin*dIII; Sp, *Sph*I; XI, *Xho*I; XII, *Xho*II; S, *Sal*I; R, *Eco*RI; B, *Bam*HI; P, *Pst*I). Sites created during plasmid construction are shown in square brackets, and the proteins encoded by the 5.4 kb fragment of pGS81 are indicated by bars (bottom): 'lip, E3-bd, and cat denote part of the lipoyl domain, the E3-binding and the subunit-binding-plus-catalytic domains of a truncated E2p component; the two segments (A and B) of the lipoamide dehydrogenase, E3 are also shown.

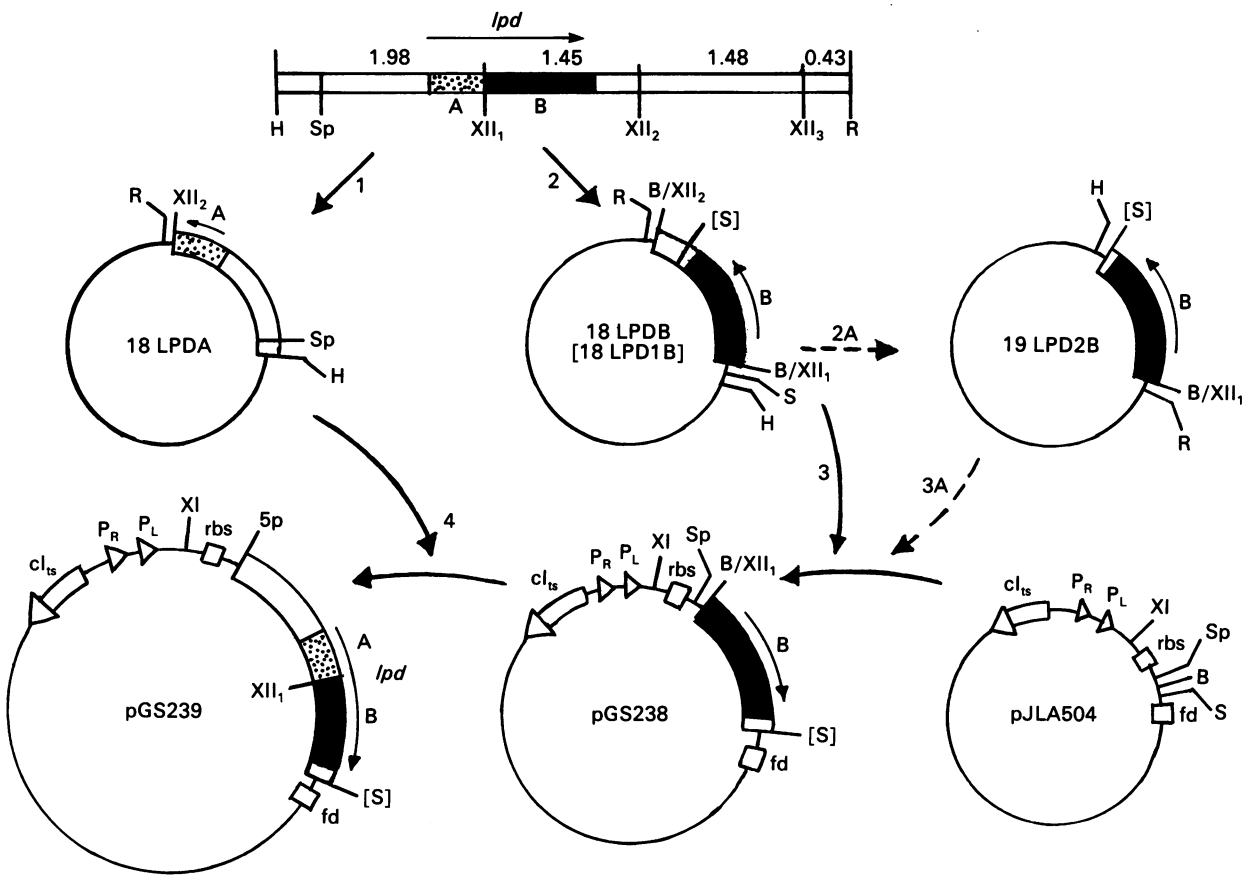

Fig. 2. Strategy used for overexpression and mutagenesis of the *lpd* gene

The 5.4 kb *Hin*dIII/*Eco*RI fragment of pGS81 is denoted by the open bar containing the *lpd* gene (stippled and shaded portions). Restriction sites employed in the cloning (H, *Hin*dIII; Sp, *Sph*I; XII, *Xho*II; B, *Bam*HI; S, *Sal*I; R, *Eco*RI) are indicated, and sites created by mutagenesis are in square brackets. The steps in the strategy are as follows. 1 and 2, Cloning the 1.98 kb *Hin*dIII/*Xho*II fragment (A) and the 1.45 kb *Xho*II fragment (B) into M13mp18 and subsequent creation of a *Sal*I site at the end of the *lpd* terminator; 3, subcloning the 1.03 kb *Bam*HI(*Xho*II$_1$)/*Sal*I fragment from 18LPD1B into the expression vector pJLA504 to create the receptor plasmid pGS238; 4, creation of the *lpd* expression plasmid (pGS239) by sub-cloning the 1.53 kb *Sph*I/*Xho*II fragment from 18LPDA into the receptor plasmid pGS238. A simpler version of 18LPDB containing single *Sal*I and *Bam*HI sites and a shorter and fully-sequenced insert (19LPD2B) was also used for mutagenesis of the distal portion (B) of the *lpd* gene and cloning into the expression vector; see 2A and 3A. Abbreviations: cI_{ts}, thermosensitive repressor; rbs, *atpE* ribosome-binding site; fd, terminator.

mutagenesis of the proximal segment of the *lpd* gene (A), that encodes 157 *N*-terminal amino acid residues (Fig. 2).

The 1.45 kb *Xho*II fragment (XII_1–XII_2 in Fig. 2) encoding 316 *C*-terminal residues of lipoamide dehydrogenase was cloned from the same digest into the *Bam*HI site of M13mp18, and one of the products having the insert in the desired orientation was designated 18LPDB (Fig. 2). Interestingly, both *Xho*II sites (XII_1 and XII_2) recreate *Bam*HI sites when this fragment is cloned. This M13 derivative was used to create a *Sal*I site at position 7507, GTCTTC → GTCGAC, as shown in Fig. 3. Three progeny phages were tested by *Sal*I restriction analysis of replicative-form DNA and shown to contain the desired mutation. Template DNA from one of these phages, 18LPD1B, was primer-extended using universal primer and the 1.03 kb *Bam*HI(*Xho*II$_1$)/*Sal*I fragment was subcloned into the expression vector pJLA504 to generate PGS238, and into M13mp19 to produce phage 19LPD2B (Fig. 2). Plasmid pGS238 contains the wild-type distal segment (B) of the *lpd* gene and served as a receptor of the proximal segment (A) for expression of the reconstructed *lpd* gene(s). Phage 19LPD2B provided template DNA for mutagenesis of the distal segment (B), having a shorter and fully sequenced insert, and single *Sal*I and *Bam*HI sites, to simplify subcloning. The proximal and distal segments of the *lpd* gene were combined in pGS239 (Fig. 2) by primer-extending 18LPDA and ligating the isolated 1.54 kb *Sph*I/*Xho*II fragment into the *Sph*I and *Bam*HI sites of the A receptor, pGS238. The resulting 2.57 kb *Sph*I/*Sal*I fragment of pGS239 encodes a truncated E2p chain that lacks the lipoyl and E3-binding domains, but forms a discrete core-forming and E1p-binding catalytic domain, in addition to lipoamide dehydrogenase (Fig. 1). These two products are both expressed from λ promoters of the expression vector, which effectively replaces the *ace* promoter, and the *lpd* gene is additionally expressed from its own promoter, which is retained in the construct. This procedure allows good amplification of lipoamide

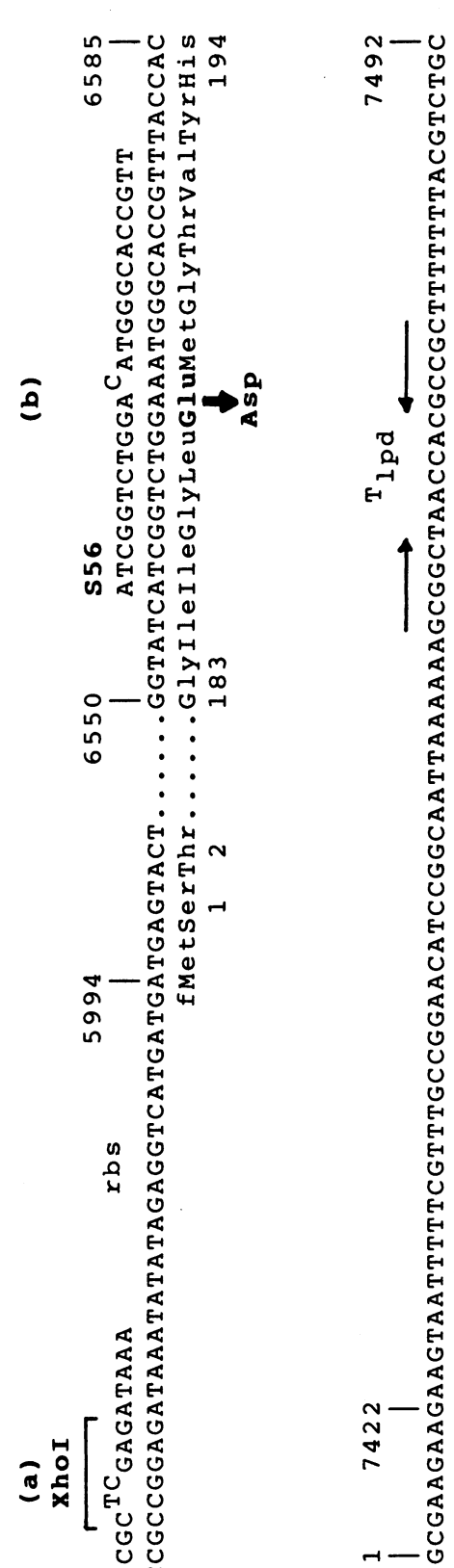

Fig. 3 Site-directed mutagenesis at three sites

Independent mutations were made of three sites: (a) creation of a *Xho*I site upstream of the coding region; (b) changing the Glu-188 codon to an Asp codon in the *lpd* coding region; and (c) creation of a *Sal*I site downstream of the *lpd* transcriptional terminator (T_{lpd}).

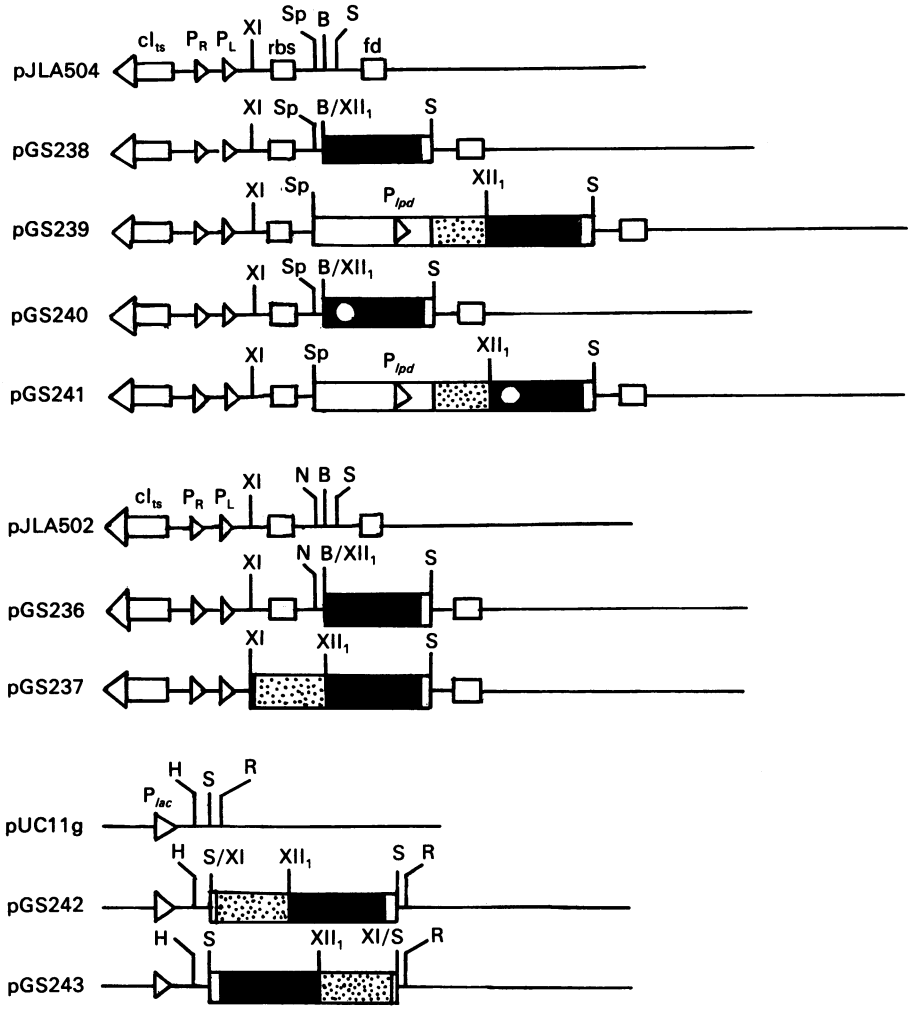

Fig. 4. Structures of the plasmid constructs

The bacterial DNA is denoted by bars with the *lpd* gene stippled (proximal segment, A) or shaded (distal segment, B). The vector DNA is represented by a solid line. Relevant promoters are indicated by arrowheads showing the direction of transcription. The *atpE* ribosome-binding site (rbs), terminator (fd) and important restriction sites (XI, *Xho*I; XII_1, *Xho*II; B, *Bam*HI; H, *Hin*dIII; N, *Nco*I; R, *Eco*RI; S, *Sal*I; Sp, *Sph*I) are also shown. The position of the Glu-188 → Asp codon change is denoted by the open circle within the shaded bar. cI_{ts} is the thermosensitive repressor.

dehydrogenase and a simple means of introducing mutations in the proximal segment of the *lpd* gene (via 18LPDA) before cloning in pGS238 for expression. However, alteration of the distal segment requires the reconstruction of a new receptor plasmid (equivalent to pGS238) following each mutagenesis in 18LPD1B or 19LPD2B.

A similar approach was used to construct a plasmid in which the *lpd* gene was solely under the control of the lambda P_R and P_L promoters. In this case the distal segment of the *lpd* gene (B) was transferred from 18LPD1B to pJLA502 to create the intermediate pGS236 (Fig. 4). A unique *Xho*I site was introduced into the A segment, immediately upstream of the *lpd* ribosome-binding site, by site-directed mutagenesis of 18LPDA template using an oligonucleotide (S60), as shown in Fig. 3. Three of the phage progeny were screened by *Xho*I restriction analysis of RF DNA and shown to contain the desired mutation. Template DNA from one of these phages, namely 18LPD1A, was primer-extended with universal primer, and the 0.5 kb *Xho*I/*Xho*II fragment (A) was subcloned into the *Xho*I and *Bam*HI sites of the receptor plasmid (pGS236) to create pGS237 (Fig. 4). This plasmid contains the entire coding region plus ribosome-binding site of a promoterless *lpd* gene, inserted downstream of the $\lambda P_R P_L$ promoters of the vector. It should be noted that the *atpE* ribosome-binding site is removed as a consequence of using the *Xho*I site of the expression vector in this construction.

The promoter-less *lpd* gene was transferred on the 1.53 kb *Xho*I/*Sal*I fragment of pGS237 into the *Sal*I site of the phagemid pUC119 in both the *lac* (pGS242) and *anti-lac* (pGS243) orientations for expression studies (Fig. 4). Both of these phagemid derivatives were stable, and it appears that the earlier problems may have been due to the large size of the inserted DNA.

Expression of lipoamide dehydrogenase in plasmid-containing strains

The *ace–lpd* deletion strain, JRG1342 (Ace^- Lpd^-) was chosen as the host for expression studies, since it provides

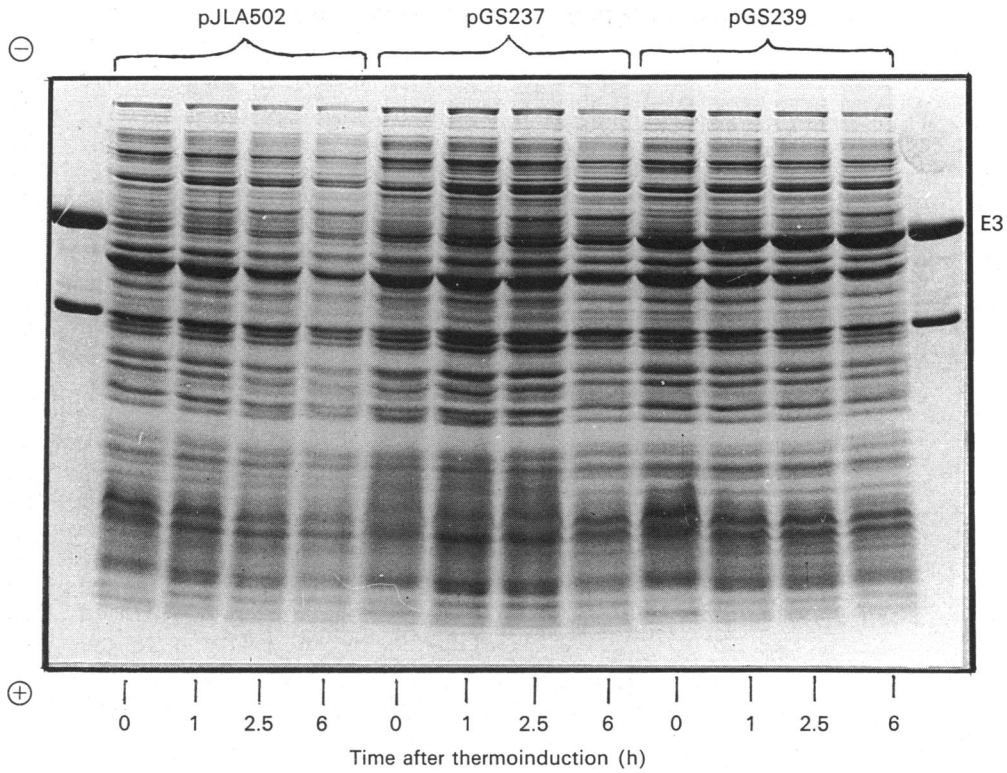

Fig. 5. Expression of lipoamide dehydrogenase in plasmid-containing derivatives of JRG1342 (Δ*ace–lpd*)

Cell-free extracts equivalent to 50 μg of protein were electrophoresed in an SDS/12%-polyacrylamide slab gel at a constant current of 30 mA. Coomassie Blue was used to stain the proteins. Purified lipoamide dehydrogenase (upper band) was used as a marker.

a background that is totally deficient in lipoamide dehydrogenase (Guest *et al.*, 1985). Ampicillin-resistant transformants containing each of the newly constructed plasmids were tested to determine whether the corresponding lipoamide dehydrogenases would restore an Lpd^+ phenotype. Plasmids pGS237, pG239 and pGS242 promoted growth on minimal acetate medium, and a single supplement of acetate was sufficient for growth with glucose or succinate as carbon and energy sources. This shows that the Lpd^+ phenotype is conferred by the three plasmids. The transformants synthesize an intact 2-oxoglutarate dehydrogenase complex utilizing chromosomally encoded E1o and E2o components in conjunction with plasmid-specified lipoamide dehydrogenase (E3), but they require acetate because they still lack a functional PDH complex. This contrasts with transformants containing pGS243, which retained the Lpd^- phenotype and, unlike the pGS237-, pGS239- and pGS242-containing strains, gave negative results in the lipoamide dehydrogenase plate tests. This result was expected, since pGS243 carries a promoter-less *lpd* gene orientated in such a way that it cannot be expressed from the *lac* promoter (Fig. 4).

Protein profiles obtained by SDS/PAGE of cell-free extracts of pG237 and pGS239 transformants of JRG1342 prepared at different times after thermoinduction are shown in Fig. 5. A prominent band of M_r identical with that of purified lipoamide dehydrogenase is evident at zero time for JRG1342(pGS239), and it increases in intensity relative to the other protein bands during the course of the experiment, but is not present in the vector control (pJLA502). No band corresponding to a truncated E2p polypeptide lacking lipoyl and E3-binding domains was detected in the cell-free extracts (Fig. 5). This was to be expected, since this inner-core polypeptide forms a high-M_r aggregate that is sedimented during extract preparation. The lipoamide dehydrogenase activity (6.8 μmol/h per mg) measured in samples removed from the JRG1342(pGS239) culture before thermoinduction is likely to be due to 'leakiness' of the λ promoters and to expression from the *lpd* promoter that is not controlled by the *λcI857* gene product. The lipoamide dehydrogenase activities increased in samples taken from the culture during incubation at 42 °C to a value of 10.3 μmol/h per mg, some 4–5 times greater than that normally found in an untransformed wild-type strain of *E. coli* (2.2 μmol of APAD reduced/h per mg). No activity was detected in untransformed JRG1342 or the vector control at any time during the experiment. A protein band of similar M_r to, but of much lower intensity than, that observed for JRG1342(pGS239) was apparent in samples of pGS237 transformants taken after thermoinduction (Fig. 5). Enzymic analysis confirmed that lipoamide dehydrogenase expression is rather low in samples taken after temperature shift (Table 1), and non-detectable at zero time.

High lipoamide dehydrogenase activities were found in extracts of JRG1342 transformed with pGS242, the phagemid with the *lpd* gene cloned in the *lac* orientation (Table 1). This contrasts with strains transformed with pGS243, the *anti-lac lpd* phagemid, which contained very little activity (Table 1). Adding IPTG to the medium

Table 1. Specific activities for lipoamide dehydrogenase in cell-free extracts of plasmid-containing derivatives of JRG1342 (*Δace–lpd*)

Extracts were prepared from strains containing pJLA504, pJLA502, pGS239, pGS241 and pGS237, 2.5 h after the 30 → 42 °C temperature shift (see the Methods and materials section). Extracts were prepared from strains containing pUC119, pGS242 and pGS243 during mid-exponential growth at 37 °C ($A_{650} = 0.7$). Details of the plasmid and phagemid structures are shown in Fig. 4. Enzyme assays were performed as described in the Methods and materials section, with $APAD^+$ as the substrate. Specific activities are expressed as μmol/h per mg of protein.

Plasmid		Specific activity
None		< 0.1
pJLA504	(vector)	< 0.1
pGS239	(wild-type)	10.3
pGS241	(Glu-188 → Asp)	77.0
pJLA502	(vector)	< 0.1
pGS237	(wild-type)	0.56
pUC119	(vector)	< 0.1
pGS242	(w.t., *lac* polarity)	4.1
pGS243	(w.t., *anti-lac* polarity)	0.4

produced a marginal increase in enzyme levels in JRG-1342(pGS242), but had no effect with JRG1342(pGS243) (results not shown). However, the level of expression obtained with JRG1342(pGS242) was not as great as that obtained after thermoinduction of the pGS239-containing strain (Table 1), and there was no sign of an amplified protein band in the E3 region of SDS/PAGE electrophoretograms of the phagemid-containing strain. Indeed, pGS242 appeared to express a high-M_r (~110000) product of unknown origin that was not investigated further. It was thus decided not to use pGS242 as the vehicle for mutagenesis of the *lpd* gene.

The overall amplification of lipoamide dehydrogenase was relatively low in all of the constructs. This might have been expected for pGS239, which retains the *lpd* promoter and other regulator loci associated with this promoter, but it was not expected for pGS237 and pGS242, where the *lpd* coding region and ribosome-binding site are directly linked to the respective λ and *lac* promoters. It has previously been suggested that *lpd* expression may be autoregulated by the uncomplexed E3 component (Guest, 1978). If so, the generally low expression might be due to the fact that none of the strains synthesize E2p components that are capable of binding and diminishing the level of uncomplexed E3. However this does not explain the very low expression observed with pGS237, which lacks most of the *aceF–lpd* intergenic region and presumably the site of auto-regulation. Indeed, this construct (pGS237) might have been expected to give very high levels of lipoamide dehydrogenase activity, especially as it contains the same *lpd* insert that is well-expressed from the *lac* promoter in the phagemid, pGS242. It is therefore concluded that poor expression is a consequence of poor positioning of the *lpd* gene and its ribosome-binding site relative to the $\lambda P_R P_L$ promoters in pGS237.

Site-directed mutagenesis of the *lpd* gene

In human erythrocyte glutathione reductase a salt bridge between Lys-66 and Glu-201 has been implicated in the repulsion of the positively-charged nicotinamide ring from its binding pocket after it has reduced the flavin coenzyme (Pai & Schulz, 1983). Both residues are in highly conserved regions in lipoamide dehydrogenase, but Lys-66 is replaced by Ser-52:

		66	67			201		
Glutathione reductase	P	K	K	V	.....	L	E	M
Lipoamide dehydrogenase	P	W	K	V	.....	V	E	M
		52	52			188		

It has been suggested that the adjacent residue, Lys-53, may form a displaced salt bridge with Glu-188 in lipoamide dehydrogenase, thereby diminishing the postulated repulsion of NAD^+ and promoting the channelling of reducing equivalents to the nicotinamide nucleotide rather than away from it, as occurs in glutathione reductase (Rice *et al.*, 1984). In order to investigate the kinetic consequences of altering the properties of the salt bridge an oligonucleotide (S56) was designed to change Glu-188 to an aspartate residue. This conservative change shortens the length of the side chain and may have the effect of diminishing the ion-pair interaction, making the position of the positive charge less exact. Alternatively, the polypeptide main chain carrying either Glu-188 or Lys-53 may accommodate the Glu → Asp change and maintain the ion-pair. An additional mutation of Lys-53 to Arg should re-establish the overall length of the ion-pair, but position the positive charge further from the flavin ring.

An oligonucleotide (S56) containing a single mismatch at position 6567 in the *lpd* gene was used to generate the codon change GAA → GAC (Glu-188 → Asp), using 19-LPD2B template DNA (Fig. 3). Four our of six progeny phages, screened by dideoxy sequencing with universal primer, contained the desired mutation, and one of these (19LPD3B) was sequenced across the entire *lpd* coding region, using primers S56, S59, S58 and universal primer, to ensure that no adventitious mutations had occurred. The Glu-188 → Asp mutation was transferred to pJLA-504 on the 1.03 kb *Bam*HI/*Sal*I fragment of primer-extended 19LPD3B to yield the intermediate plasmid pGS240. This in turn served as the receptor for the unmutated proximal portion of the *lpd* gene carried on the 1.54 kb *Sph*I/*Xho*II fragment of 18LPDA. The resultant plasmid, pGS241, is identical with pGS239 in all respects apart from the Glu-188 → Asp mutation carried by the former (Fig. 4).

The SDS/PAGE protein profiles obtained after thermo-inducing pGS241-containing transformants of JRG1342 were similar to those obtained for JRG1342(pGS239), i.e. a major protein band of M_r 56000 was present from zero time and it grew in intensity over the 6 h duration of the experiment (results not shown). Preliminary experiments with cell-free extracts prepared 2.5 h after thermoinduction indicated that the mutant lipoamide dehydrogenase possessed markedly altered enzymic properties. Whereas the wild-type enzyme was far more efficient in the catalysis of electron transfer from dihydrolipoamide to NAD^+ than to $APAD^+$, the mutant enzyme appeared to be more active with the analogue. The data shown in Table 1 indicated that the pGS241-encoded mutant enzyme had a specific activity 7–8 times greater than that of the wild-type enzyme expressed from

Table 2. Kinetic parameters determined for lipoamide dehydrogenase in cell-free extracts of plasmid-containing derivatives of JRG1342 (*Δace–lpd*)

The apparent K_m values are expressed as mM, $V_{max.}$ values are in arbitrary units (μmol/h per ml of extract), and the results are the averages of two determinations.

Plasmid	Substrate	K_m	$V_{max.}$
pGS239 (wild-type)	APAD$^+$	1.00	13
	NAD$^+$	1.82	4170
pGS241 (Glu-188 → Asp)	APAD$^+$	0.53	118
	NAD$^+$	0.81	14

pGS239. The apparent K_m values for APAD$^+$ (2.5 mM-dihydrolipoamide) showed only small (2-fold) differences for the wild-type and mutant enzymes (Table 2). Thus, the effect of mutation appeared to be primarily on $V_{max.}$.

Sequence comparisons of lipoamide dehydrogenase and the mechanistically similar glutathione reductase (Rice *et al.*, 1984; Williams *et al.*, 1982) showed strong homology in all domains. This indicated that the chain-fold of the two enzymes should be similar. The detailed three-dimensional structure of human erythrocyte glutathione reductase showed that the disulphide–dithiol interchange half-reaction occurred on the **si** side of the isoalloxazine ring, whereas the nicotinamide nucleotide half-reaction took place on the **re** side (Thieme *et al.*, 1981). Since the mutation affects a residue in the pyridine nucleotide binding site, it would therefore be predicted that the rate of reduction of the mutant enzyme by dihydrolipoamide should be normal, unless indirect conformational effects come into play.

The *lpd* gene of *E. coli* has now been cloned in a form suitable for mutagenesis, allowing reconstruction of the gene in a good expression system. This strategy avoids the serious problems of gene instability that were encountered when attempting to mutagenize the intact *lpd* gene. The construction of the Glu-188 → Asp mutant demonstrates the success of the procedure, and it is hoped that further work on the purified mutant enzyme will give a greater insight into the catalytic mechanism of lipoamide dehydrogenase. The same strategy has now been used to create further active-site mutants, for example: Cys-44 → Ser; Cys-49 → Ser; Ser-52 → Lys; Lys-53 → Arg; Ile-184 → Tyr and Cys; His-444 → Gln; and a double mutant, Lys-53 → Arg, Glu-188 → Asp (N. Allison, G. C. Russell & J. R. Guest, unpublished work).

We are grateful to Dr J. E. G. McCarthy for providing the pJLA502/4 expression vectors. This work was supported by a Science and Engineering Research Council project grant to J.R.G. The travel expenses of C.H.W. were borne by the Medical Research Service, U.S. Veterans Administration, and by grant no. Gm 21444 from the National Institute of General Medical Sciences.

Received 23 May 1988/3 August 1988; accepted 5 August 1988

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