Gene-Protein Relationships of the α-Keto Acid Dehydrogenase Complexes of Escherichia coli K12: Isolation and Characterization of Lipoamide Dehydrogenase Mutants

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SUMMARY

Ten independent lipoamide dehydrogenase mutants (lpd) of Escherichia coli were isolated by selecting strains which required supplements of acetate plus succinate for best growth on glucose. They would not grow on unsupplemented medium (except anaerobically) nor would they grow with single supplements of acetate or lipoate, but they responded slowly to lysine plus methionine or succinate. Bacteria-free extracts of the mutants had between 1 and 10% of parental lipoamide dehydrogenase activity and no activity for the pyruvate and α-ketoglutarate dehydrogenase complexes could be detected. Evidence that the mutants contained the dehydrogenase (E1) and transacylase (E2) components of the complexes and were deficient only in the lipoamide dehydrogenase (E3) components was obtained from studies with mixtures containing lpd mutant extracts and either extracts of other mutants having defined lesions or purified lipoamide dehydrogenases, e.g. overall pyruvate dehydrogenase complex could be reconstituted with extracts of aceE and F mutants and the α-ketoglutarate complex was similarly reconstituted with sucA and B extracts. Furthermore, both complexes could be restored by adding extract of an aceE, sucA double-amber mutant (which lacks both types of E1 and E2 component but has 30% of parental lipoamide dehydrogenase activity) or with purified bacterial and mammalian lipoamide dehydrogenases. The bacterial enzymes were several times more efficient than the mammalian enzyme for restoring pyruvate dehydrogenase complex activity.

Genetic studies indicated that the wild-type phenotype could be restored by single reversion or transduction events and they confirmed that the mutants are deficient only in lipoamide dehydrogenase. The mutant phenotype was introduced into a recipient strain by cotransduction with leu+. This indicates that there is a lipoamide dehydrogenase gene in the leu region of the Escherichia coli linkage map and strongly supports the view that the E3 components of both α-ketoacid dehydrogenase complexes are specified by a single lipoamide dehydrogenase gene (lpd).

INTRODUCTION

The pyruvate and α-ketoglutarate dehydrogenase complexes of Escherichia coli (pdh and kgdh complexes) are large multi-enzyme complexes containing three types of enzymatic component. These are the dehydrogenase (E1), the dihydrolipoamide transacylase (E2) and the lipoamide dehydrogenase (E3) components and they catalyse the oxidative decarboxylation of pyruvate or α-ketoglutarate by analogous sequences of reactions (Fig. 1) (Reed & Cox, 1970). The complexes can be resolved into, and reconstituted from, their respective components and they contain many molecules of each component, although only one is illustrated. Recent evidence indicates that the pdh complex (M about 4 × 10⁶) consists of a
core complex containing 16 polypeptide chains of each component which may in addition have an extra two or three pyruvate dehydrogenase (E1), dimers associated with it (Vogel, Hoehn & Henning, 1972). The kgdh complex is smaller (M about $2.3 \times 10^6$) and its exact composition has still to be established. The dehydrogenase (E1) and transacylase (E2) components are specific to each complex; they are not functionally interchangeable, nor do they form hybrid complexes (Mukherjee, Matthews, Horney & Reed, 1965); they may therefore be designated E1p, E2p and E1kg, E2kg. By contrast Pettit & Reed (1967) showed that the lipoamide dehydrogenase components are functionally interchangeable, so the E3 component purified from one complex can be used for reconstituting active pdh and kgdh complexes. Furthermore, the E3 components purified from each complex had identical sedimentation constants and flavin contents, the same electrophoretic mobility in polyacrylamide gel and they were immunologically indistinguishable. The lipoamide dehydrogenases (lpdh) are disulphide-containing flavoproteins and they catalyse the reoxidation of the dihydrolipoamide coenzyme which is covalently bound to the transacylase components (Fig. 1). The lpdh activity of crude cell-free extracts is conveniently assayed with model substrates by following the dihydrolipoate or dihydrolipoamide-dependent reduction of 3-acetylNAD:

$$\text{Lip(SH)}_2 + 3\text{-AcetylNAD}^+ \rightleftharpoons \text{LipS}_2 + 3\text{-AcetylNADH} + \text{H}^+.$$ 

Genetic studies with mutants requiring acetate or succinate for aerobic growth with glucose have established the existence of two pairs of closely linked structural genes, aceE and aceF (near leu) for E1p and E2p and sucA and sucB (near gal) for E1kg and E2kg (Henning & Hertz, 1964; Herbert & Guest, 1969). None of the acetate or succinate-requiring mutants lacked lpdh activity and this has raised the question of whether there are two lipoamide dehydrogenase genes (lpd) or whether the E3 components of both are supplied by a single gene. If there are two lpd genes, lesions in one would probably be complemented by the other,
Lipoamide dehydrogenase mutants of *E. coli* assuming that the gene products are functionally interchangeable in vivo as well as in vitro. Lipoamide dehydrogenase-less mutants would then represent a specific class of double mutant having lesions in both *lpd* genes and requiring a dual supplement of acetate plus succinate. On the other hand, with just one *lpd* gene, single lesions would probably result in the same dual requirement for acetate plus succinate. Attempts to screen for such mutants have generally yielded *lip* mutants which respond either to this dual supplement or to exogenous lipoate (Herbert & Guest, 1968). They lack activity for both complexes because the biosynthesis of the lipoate coenzyme is blocked.

We now describe the isolation and properties of lipoamide dehydrogenase-less mutants, together with genetic studies which indicate that there is a lipoamide dehydrogenase gene (*lpd*) in the *leu* region of the *Escherichia coli* linkage map. The evidence strongly supports the view that a single *lpd* gene specifies the E3 components of both multi-enzyme complexes. A preliminary account of some of this work has been published previously (Guest & Creaghan, 1972).

**METHODS**

*Organisms, media and preparation of cell-free extracts.* Lipoamide dehydrogenase mutations were induced in several strains of *Escherichia coli* K12: T3A58 (F*, trpE, trpA); AB1325 (F−, proA, lacY, gal, purB, his, str, mtl, xyl, thi); XG3 (F−, leu, ara, lac, proC, txy, purE, trp, str, xyl, thi); WGA (F−, gal, trpA); and H (Hfr, thi). Strains which have been used for other purposes include a leucine-requiring mutant of WGA (WGAleU) and the *suc* mutants, W1485-suca1, WSucB17, WGA-suca35 (a *suc-amber* mutant) and WGA-aceE64-suca35 (which contains amber mutations in the *aceE* and *suca* genes). Further details on the origin and properties of these strains have been described previously (Herbert & Guest, 1968, 1969; Creaghan & Guest, 1972). In addition, several *ace* mutants were very kindly provided by Dr U. Henning: *aceE2* (A2T3; *aceE*, trp), *aceF10* (A10; *aceF*) and an *ace-amber* mutant *aceE64* (k1−1; *aceE64*, HfrH, pps, thy, aceE, P1).

Minimal medium E of Vogel & Bonner (1956) was used in all experiments except for the selection of mutants when a citrate-free medium was used (Herbert & Guest, 1970). Substrates were glucose (0.2 or 0.4%), acetate or succinate (50 mM) and galactose (0.5%), plus bromothymol blue indicator at a final concentration of 0.002%. When necessary, minimal media were supplemented with acetate (2 mM), succinate (2 mM), l-lysine (40 µg/ml), l-methionine (20 µg/ml), D,L-lipoate (0.1 µg/ml) and other supplements to satisfy the growth requirements of mutant organisms. Media were solidified with 1.5% agar (Difco). L-Broth and L-agar (Lennox, 1955) were used as complete media.

Organisms grown with shaking in glucose minimal medium (500 ml/2 l Erlenmeyer flask) were harvested in late exponential phase, washed twice with potassium phosphate buffer (0.04 M, pH 7.0) and finally resuspended at a concentration of 0.2 g wet wt/ml of the same buffer. Bacterial suspensions were disrupted at 0 °C for two periods of 2 min with an ultrasonic cell disintegrator (M.S.E., 100 W), followed by centrifuging at 25000g for 15 min. Protein concentrations in the supernatant extracts were estimated according to Lowry, Rosebrough, Farr & Randall (1951). Samples of each culture were tested to ensure that reversion had not occurred.

*Enzyme assays.* All spectrophotometric assays were at 25 °C and specific activities (µmol substrate transformed/mg protein/h) were determined in the region of proportionality between initial reaction velocity and protein concentration.

Lipoamide dehydrogenase (*lpdh* or E3) was assayed by recording the dihydrolipoate-dependent reduction of 3-acetylNAD at pH 7.8 according to Creaghan & Guest (1972).
Pyruvate and \( \alpha \)-ketoglutarate dehydrogenase complexes were also assayed at 366 nm by recording the ketoacid-dependent reduction of 3-acetylNAD. The reaction mixture contained (\( \mu \)mol in 1 ml final volume): tris-HCl buffer, pH 8.5, 120; L-cysteine, HCl, 3; coenzyme A, 0.08; TPP, 0.2; 3-acetylNAD, 0.8; sodium pyruvate or sodium \( \alpha \)-ketoglutarate, 7.5 and extract containing up to 1 mg protein. After incubating for 2 to 3 min the reaction was started by adding ketoacid to one cuvette. Initially, this method was used to test for reconstitution of pdh and kgdh complex activities from different sources of components; the two sources of components (up to 2 mg total protein) were added to the reaction mixture for 5 min before adding 3-acetylNAD and then the ketoacid. Subsequently, a modified complementation assay was adopted; it gave the same results as the previous method, but was more economical on reagents and has been used in all the experiments described below. In this, mutant extracts or extract plus purified lpdh (0.2 to 0.8 mg total protein) were incubated in the same reaction mixture with 3-acetylNAD (1.6 \( \mu \)mol) for 10 min (approx.) and the extinction at 366 nm was measured versus water to ensure that endogenous reduction of 3-acetylNAD was complete and that an excess of this substrate remained, before adding the ketoacid.

\( \alpha \)-Ketoglutarate dehydrogenase (E1kg), the E1 component of the kgdh complex was assayed spectrophotometrically at 420 nm and pH 6.3 with ferricyanide as the electron acceptor (Hager & Kornberg, 1961).

SuccinylCoA synthetase was assayed spectrophotometrically at 230 nm according to Bridger, Ramaley & Boyer (1969).

Succinate dehydrogenase and dihydrolipoamide trans-succinylase (E2kg), the E2 component of the kgdh complex, were assayed by methods described previously (Creaghan & Guest, 1972).

Selection of lipoamide dehydrogenase mutants. Washed suspensions of log phase L-broth cultures of parental strains in tris-maleate buffer (10\(^8\) bacteria/ml of 0.1 M buffer, pH 6.0) were exposed to \( N \)-methyl-\( N' \)-nitro-\( N \)-nitrosoguanidine at 100 \( \mu \)g/ml for 20 min at 37 °C. After washing, a sample of each treated culture was diluted 20 times into citrate-free glucose minimal medium supplemented with acetate and succinate and incubated with shaking for 18 h at 37 °C. The expressed cultures were washed twice and samples giving a final concentration of 2 \( \times \) 10\(^8\) bacteria/ml (approx.) were inoculated into unsupplemented medium containing benzylpenicillin (300 units/ml) and incubated for 5 h at 37 °C. Several dilutions of the penicillin-treated cultures were spread on plates of glucose minimal medium plus acetate and succinate. Mutant colonies requiring acetate, succinate or both were detected by replicating to unsupplemented medium. Prospective mutants (12 to 24 from each experiment) were diluted in saline and tested directly for their ability to grow aerobically on glucose minimal medium with the following supplements (12 isolates/plate): acetate, succinate, lipoate, lysine + methionine and acetate + succinate. Their ability to grow on unsupplemented media containing glucose (aerobically or anaerobically), succinate and acetate as sole carbon and energy sources was also tested. The plates were examined carefully over 84 h and the phenotypes scored. Any isolates which responded better to acetate plus succinate than to acetate, succinate or lipoate alone were purified on the doubly supplemented medium and the screening procedure repeated. Typical ace, suc and lip mutants were tested at the same time for comparative purposes and only one isolate of a particular phenotype was retained per mutagen-treated culture. The acetate plus succinate-requiring mutants were further characterized by enzymological analysis, and they were not designated lipoamide dehydrogenase mutants (lpd) until a deficiency in lipoamide dehydrogenase, pyruvate and \( \alpha \)-ketoglutarate dehydrogenase complex activities had been established and the restoration...
of both complex activities by purified lipoamide dehydrogenase had been demonstrated.

**Transduction with phage PI.** Lysates of donor strains were prepared by confluent lysis using the media and methods of Lennox (1955). Transduction mixtures contained $10^9$ stationary-phase recipient organisms, $2 \times 10^9$ PI and CaCl$_2$ (5 μmol) in 1 ml of L-broth. The recipient cultures were preincubated with CaCl$_2$ for 30 min at room temperature followed by 20 min at 37 °C with the phage. Unadsorbed phage were removed by centrifuging and appropriate dilutions of the transduced culture were plated on selective media. All media used for initial selections, except glucose-based media, were enriched with nutrient broth (Difco) (0.2%, v/v). Recombinant colonies were counted after 2 to 4 days at 37 °C depending on the selective medium and purified on the same medium before scoring the inheritance of non-selective markers.

**Materials.** Purified pig heart lipoamide dehydrogenase was from the Sigma Chemical Company, London as a suspension containing 10 mg protein/ml of 70% (NH$_4$)$_2$SO$_4$ solution. A sample of bacterial lipoamide dehydrogenase was kindly provided by Dr C. H. Williams, Jun. (Department of Biochemistry, The University of Michigan, Ann Arbor, U.S.A.). This was purified from *Escherichia coli* B by the method of Williams, Zanetti, Arscott & McAllister (1967) and consequently represents a mixture of E3 components from both ketoacid dehydrogenase complexes. Both enzymes were dialysed versus phosphate buffer (20 mM, pH 7.8) before use, and the specific activities for lpdh in the preparations used here were 108 (pig heart enzyme) and 350 (*Escherichia coli* B enzyme; 600 when first received) using the assay described above. Neither preparation contained any detectable pdh or kgdh complex activity. The sources of some other materials were: DL-dihydrolipoic acid (Sigma) and 3-acetylNAD (Boehringer, Mannheim, Germany).

**RESULTS**

**Isolation of lipoamide dehydrogenase mutants (lpd)**

Mutants lacking lipoamide dehydrogenase activity would be expected to require exogenous acetate and succinate for aerobic growth on glucose if the only essential function of this enzyme in *Escherichia coli* is to serve as a component (E3) of the pyruvate and/or α-ketoglutarate dehydrogenase complexes, and if this function cannot be replaced by some other enzyme under physiological conditions. Failure to isolate lipoamide dehydrogenase mutants could be due to the existence of two genes with functionally interchangeable gene products. Alternatively, if there is only one lipoamide dehydrogenase gene (lpd), such mutants should be induced at normal frequencies in mutagen-treated cultures and the failure to isolate lipoamide dehydrogenase mutants could then be due to failure in detection. Recent experience with some genetically constructed ace, suc double mutants, which should exhibit the same phenotype (Creaghan & Guest, 1972), suggested that lpd mutants might be detected by a carefully controlled screen.

Using the procedure described in the Methods section and starting with parental strains which had been used successfully for isolating lip and suc mutants, 12 independent mutants having the desired phenotype were recovered from 16 mutagen-treated cultures. Of these, ten have been designated lpd mutants, and the remaining two have been tentatively identified as double mutants combining mutations in ace and suc genes. The parental strains for specific mutants are: T3A58 (lpd1, 2); AB1325 (lpd3); XG3 (lpd4); WGA (lpd5) and H (lpd6, 7, 8, 9, 10).

The basic nutritional characteristics of these mutants, observed by streaking faintly
Fig. 2. Growth of the lipoamide dehydrogenase mutant T3A58/lpd1 and the parental strain T3A58.
Cultures were shaken at 37 °C in 250 ml Erlenmeyer flasks fitted with side arms. Each flask contained
10 ml of medium E plus glucose (0.4 %, w/v) and L-tryptophan (30 μg/ml) inoculated with 0.2 ml of
a washed suspension of cells (grown aerobically for 16 h in the same medium plus acetate and
succinate) to give an initial extinction at 610 nm of 0.1 (equivalent to 10⁸ bacteria/ml). Anaerobic
growth was measured in sealed flasks containing an atmosphere of H₂ with 5 % CO₂.

Growth of the parental strain is indicated thus: · · ·, aerobic with or without acetate plus
succinate; · · ·, anaerobic without supplements. Aerobic growth of the lpd mutant is shown with
supplements: ●, acetate plus succinate; ○, acetate; ●, succinate; △, lysine plus methionine; ▲,
lipoate; and ■, unsupplemented medium. Anaerobic growth of the mutant on unsupplemented
medium is indicated thus: ○.

turbid suspensions on solid media, were as follows. They failed to grow aerobically with
glucose, acetate or succinate as sole carbon and energy sources. On glucose medium, they
responded best to a combined supplement of acetate plus succinate, producing good
colonies in two days at 37 °C; no growth was observed with single supplements of acetate or
lipoate. They gave an intermediate response to supplements of succinate alone and lysine
plus methionine (which was marginally better). Growth was slow at first but good colonies
were generally produced after 4 days, without any signs of reversion. In this respect they
resembled ace, suc double-mutants and lip mutants. Anaerobically, both mutant and paren-
tal strains grew slowly on unsupplemented glucose medium as would be expected, because
acetate and succinate can be produced endogenously by lpdh-independent routes which are
derepressed under these conditions. The same growth responses were observed with citrate-
free media and media containing citrate.

A similar pattern of responses was observed for growth in liquid media (Fig. 2). The
mutants clearly needed acetate plus succinate for best aerobic growth on glucose. The extent
of growth was significantly less than for the parental strain but this presumably reflects the
mutants’ inability to utilize the substrate fully. The weak but significant response to succinate
Lipoamide dehydrogenase mutants of *E. coli*

Table 1. Enzyme activities of extracts of lpd mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>lpdh</th>
<th>pdhc</th>
<th>kgdhc</th>
<th>E1kg</th>
<th>E2kg</th>
<th>scs</th>
<th>sdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lpd+</td>
<td>2.60</td>
<td>2.20</td>
<td>0.70</td>
<td>3.8</td>
<td>0.14</td>
<td>9.0</td>
<td>10.3</td>
</tr>
<tr>
<td>lpdI</td>
<td>0.02</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>4.0</td>
<td>0.14</td>
<td>1.0</td>
<td>3.4</td>
</tr>
<tr>
<td>lpd5</td>
<td>0.26</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>1.8</td>
<td>0.13</td>
<td>2.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

and lysine plus methionine was observed as well as the masking of the mutant phenotype under anaerobic conditions.

Enzymological studies

Ultrasonic extracts of all the mutants were assayed for lipoamide dehydrogenase (lpdh), pyruvate dehydrogenase (pdh complex) and α-ketoglutarate dehydrogenase complex (kgdh complex) activities. The results for two representative mutants, *lpdI* and *lpd5*, are shown in Table 1, together with the specific activities obtained with typical parental strains (*Lpd+*). Seven of the mutants, *lpdI*, 2, 3, 6, 7, 8 and 10, had about 1% of parental lpdh activity whereas the remaining three, *lpd4*, 5 and 9, had approximately 10%. No pdh complex or kgdh complex activity could be detected in any of the mutants. Several of the mutants were also examined for the presence of the dehydrogenase and trans-succinylase components of the ketoglutarate dehydrogenase complex (E1kg and E2kg) by direct assays; succinylCoA synthetase and succinate dehydrogenase were also assayed. Substantial amounts of the two components of the kgdh complex were always found (Table 1) and, although the specific activities of the other two enzymes were rather low, this is frequently observed with strains lacking kgdh complex activity.

To determine whether the absence of both complex activities was due solely to the lack of lpdh (the common E3 component), the effects of adding extracts of *ace* and *suc* mutants, known to be lacking one or more enzymatic components but possessing lpdh activity, were investigated (Table 2). The overall pdh complex could be reconstituted by mixing extracts of mutant *lpdI* with those of *aceE2*, which lacks pyruvate dehydrogenase activity (component E1p); *aceF10*, which lacks dihydrolipoyl trans-acetylase activity (component E2p) or *aceE64*, an amber mutant which lacks both E1p and E2p and synthesizes a reduced amount of the E3 component. This complementation clearly indicates that *lpdI* contains active E1p and E2p components. Similar experiments with *sucA1*, *sucB17* and *sucA35* (an amber mutant), which lack E1kg, E2kg and both of these activities respectively, confirm that the *lpd* mutant contains active E1kg and E2kg components (Table 2). In other experiments extracts of a double-amber mutant, *wgaaceE64sucA35*, were used. This mutant was constructed from the two polar *ace* and *suc* mutants and in addition to lacking both complex activities it contains neither type of E1 or E2 component but has a specific activity of 0.8 μmol/mg protein/h for lpdh, i.e. approximately 30% of wild-type. This mutant would not complement *aceE*, *aceF*, *sucA* or *sucB* mutants for pdh and kgdh complex activities, but both complexes were restored when mixed with *lpd* mutants (Fig. 3). Thus we conclude that mutants *lpdI* and *lpd5* contain active E1-E2p and E1-E2kg partial complexes and that the absence of overall complex activities is simply due to a deficiency in lpdh (E3 components).
Fig. 3. Complementation between extracts of lpd mutants and \textit{wga}aceE64sucA35; a double-amber mutant for overall pyruvate (●) and α-ketoglutarate (○) dehydrogenase complex activities. Different amounts of the double-amber mutant were added to 0.2 mg protein from extracts of T3A58lpdt (···), WGAlpds (----) or WGAlpdt (-----) and assayed for α-keto acid-dependent reduction of 3-acetylNAD (μmol/h) by the complementation assay (see Methods).

Table 2. Complementation between extracts of lpd and ace or suc mutants

The pdh complex and kgdh complex activities of mixtures of crude extracts containing 0.4 mg protein of T3A58lpdt plus an equal amount from the ace or suc mutants in 1 ml of reaction mixture were measured by the complementation assay (see Methods). Extracts of the complementing strains all contained lipoamide dehydrogenase (specific activities between 1.2 and 2.5 μmol/mg protein/h) but no pdh or kgdh complex could be detected for the ace or suc mutants respectively; + indicates the presence of corresponding complex in the complementing strain.

<table>
<thead>
<tr>
<th>Complementing strain</th>
<th>pdh complex (μmol/h)</th>
<th>kgdh complex (μmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>aceE2</td>
<td>0.39</td>
<td>+</td>
</tr>
<tr>
<td>aceF10</td>
<td>0.29</td>
<td>+</td>
</tr>
<tr>
<td>aceE64</td>
<td>0.19</td>
<td>+</td>
</tr>
<tr>
<td>sucA1</td>
<td>+</td>
<td>0.10</td>
</tr>
<tr>
<td>sucB17</td>
<td>+</td>
<td>0.17</td>
</tr>
<tr>
<td>sucA35</td>
<td>+</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Several other mutants showed positive complementation with ace, suc and ace, suc double-mutants but these tests were not extended to all the mutants ultimately designated lpd.

Probably the most convincing demonstration that the lpd mutants are deficient only in lpdh comes from studies with purified lipoamide dehydrogenases. The pig heart enzyme restored both dehydrogenase complex activities to extracts of lpd mutants; typical results are
Lipoamide dehydrogenase mutants of E. coli

Pig heart lipoamide dehydrogenase (μg protein)

Fig. 4. Complementation of extract of lpdt mutant for overall pyruvate (●) and α-ketoglutarate (○) dehydrogenase complex activities by purified pig heart (----) or Escherichia coli B (-----) lipoamide dehydrogenases. The mutant used was WGA lpdt I derived from WGA leu and T3A58lpdt (WGA, LT11 of Table 4) and duplicate samples containing extract (0.2 mg protein) were mixed with purified enzyme and assayed for the overall complexes by the complementation method.

Illustrated in Fig. 4. The activities of both complexes increased with increasing lpdh concentration and the ratios of pyruvate complex to α-ketoglutarate complex varied from 4:1 to 2:1 with different extracts. The purified enzyme would not complement any of the ace, suc or ace, suc mutants for the missing complexes, and, although its ability to complement lpd mutants was unaffected by exhaustive dialysis, it was completely inactivated by heating (100 °C for 3 min). The pdh and kgdh complex activities of all ten lpd mutants were restored by pig heart lpdh and this was used as an important criterion for identifying lpd mutants. The reason for some mutants with approximately 10% of parental lpdh activity not showing any detectable activities for the overall complexes has not been investigated. Their E3 components may be defective in complexing with the other components as well as lacking in catalytic activity. On the other hand, the mutants may make less of a fully active E3 component in which case it could be concluded that co-operation between E3 components occurs in the production of complex activity. However, many other explanations could be proposed.

Purified Escherichia coli B lpdh also restored both α-ketoacid dehydrogenase complex activities to the lpd mutant extracts and it was more effective than the pig heart enzyme (Fig. 4).

In the complementation studies illustrated in Fig. 3 and 4, the same extract of strain WGA lpdt and the same assay conditions were used with the three different sources of lpdh,
purified pig heart and *Escherichia coli* B enzymes and the extract of the ace, suc double-
amber mutant which represents a crude source of bacterial enzyme. An estimate of the
relative complementation efficiencies of these lpdh preparations may therefore be obtained
from the slope of the curves relating activity of pdh complex restored to amount of lpdh
added. These give values of 90 for *E. coli* B enzyme, 10 for pig heart enzyme and 1 for the
double-amber extract when complementation is expressed in terms of μmol/h of pdh complex
activity restored/mg of protein in the lpdh preparation. However, after correcting for the
specific activities of the lpdh preparations, these complementation efficiencies become 0.26
(*E. coli* B), 0.09 (pig heart) and 1.2 (double-amber extract), in terms of units of pdh complex
activity recovered per unit of lpdh added. Clearly, the mammalian enzyme is the least effi-
cient and the difference between the crude and purified bacterial enzymes could in part be
due to progressive inactivation of the latter, because the sample used in these experiments
had lost at least 40% of its original activity. Also, the maximum activity for kgdh complex
reconstituted with purified bacterial enzyme was twice that obtained with the other sources of
lpdh, whereas the source of lpdh made no difference to the maximum for pdh complex
activity (Fig. 4). This effect was particularly apparent with the extract of r3A58/lpd1 where
the ratio of 2 observed for pdh:kgdh complex activities with the double-amber mutant
(Fig. 3) and the pig enzyme became 1:3 with purified *E. coli* B lpdh. Generally the pdh com-
plex activity was 2 to 4 times greater than kgdh complex at saturation, depending on the
mutant extract. This was thought to reflect the relative activities of the two complexes
normally found in glucose-grown parental strains. The greater yields with *E. coli* B enzyme
are difficult to interpret unless it has become modified in some way during purification so that
it increases the catalytic activity of the kgdh complex.

**Genetic studies**

Spontaneous revertants of several lpd mutants were selected by spreading $2 \times 10^8$ bacteria
on plates of glucose medium with either acetate or succinate as supplement and on unsupple-
mented glucose and acetate media. Some 5 to 50 revertant colonies generally appeared on
the plates within 2 to 5 days at 37 °C and, after purification on the same medium, the vast
majority grew on unsupplemented glucose and acetate media regardless of the type of
selection used initially. Enzymatic analysis of selected revertants showed that they had regained
activities of lpdh and both dehydrogenase complexes apparently in a single step (Table 3).
Wild-type derivatives of mutant lpd1 were also selected by Pr-mediated transduction.
Using a lysate of WGA as the donor strain, Lpd+ transductants were selected on the same
media used for the reversion studies. The frequencies of complete transduction ranged from
18 to 24/10^4 Pr and higher frequencies of abortive transduction were observed with all
selective media. All the transductants grew on unsupplemented glucose medium and enzym-
atic analysis with selected transductants again showed simultaneous restoration of lpdh
and both complexes (Table 3). The succinylCoA synthetase and succinate dehydrogenase
activities of revertants and transductants were also restored to values comparable to the
parental strain.

These studies indicate that single genetic events (reversion and recombination) can restore
a wild-type phenotype to the lpd mutants. They confirm that the mutants are lacking only in
lipoamide dehydrogenase otherwise many of the revertants would require either acetate or
succinate. However, these observations do not indicate whether *Escherichia coli* contains
one or more lpd genes, because restoration of one wild-type lpd gene would be sufficient to
restore a wild-type phenotype. Proof that there is but a single lpd gene would depend on
whether or not all primary-site reversion and recombination events were confined to one
Table 3. Properties of spontaneous revertants and Lpd⁺ transductants of the lipoamide dehydrogenase mutant IpdI

The enzymes were assayed as described in the Methods section. Revertant RSI was isolated as a succinate-independent revertant of T3A58/pd1 on glucose minimal medium plus acetate and RAI was likewise isolated as an acetate-independent revertant on medium supplemented with succinate but not acetate. TI was a typical transductant isolated after P1-mediated gene-transfer from the donor strain (WGA) to T3A58/pd1; transductants isolated on different selective media gave similar results.

<table>
<thead>
<tr>
<th>Enzyme specific activities (μmol/mg protein/h)</th>
<th>Strain</th>
<th>pdh complex</th>
<th>kgdh complex</th>
<th>lpdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent and donor (WGA)</td>
<td>2·20</td>
<td>0·70</td>
<td>2·60</td>
<td></td>
</tr>
<tr>
<td>Mutant lpdI and recipient</td>
<td>&lt; 0·01</td>
<td>&lt; 0·01</td>
<td>0·02</td>
<td></td>
</tr>
<tr>
<td>Revertant: RSI</td>
<td>1·46</td>
<td>0·66</td>
<td>1·55</td>
<td></td>
</tr>
<tr>
<td>Revertant: RAI</td>
<td>1·43</td>
<td>0·75</td>
<td>1·68</td>
<td></td>
</tr>
<tr>
<td>Transductant: TI</td>
<td>1·80</td>
<td>0·42</td>
<td>1·81</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Cotransduction of the Lpd⁻ phenotype with Leu⁺

Leu⁺ transductants were selected on leucine-free glucose medium supplemented with acetate plus succinate and of 124 which were purified and tested 10 (8%) required both supplements (represented by LT5 and 11) and the remainder (represented by LT7 and 12) required neither. Average specific activities for the two pairs of transductants are shown together with values for the donor and recipient strains processed at the same time for comparison.

<table>
<thead>
<tr>
<th>Specific activity (μmol/mg protein/h)</th>
<th>Strain</th>
<th>pdh complex</th>
<th>kgdh complex</th>
<th>lpdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor: T3A58/pd1 (Leu⁺, Lpd⁻, Gal⁺)</td>
<td>&lt; 0·01</td>
<td>&lt; 0·01</td>
<td>0·03</td>
<td></td>
</tr>
<tr>
<td>Recipient: WGAleu (Leu⁻, Lpd⁻, Gal⁻)</td>
<td>2·40</td>
<td>0·07</td>
<td>2·60</td>
<td></td>
</tr>
<tr>
<td>Transductants: WGA.LT7 and 12 (Leu⁺, Lpd⁺)</td>
<td>1·76</td>
<td>0·67</td>
<td>2·39</td>
<td></td>
</tr>
<tr>
<td>Transductants: WGA.LT5 and 11 (Leu⁻, Lpd⁻)</td>
<td>&lt; 0·01</td>
<td>&lt; 0·01</td>
<td>0·03</td>
<td></td>
</tr>
</tbody>
</table>

small segment of the chromosome, and this was not tested. Instead, attempts were made to introduce the Lpd⁻ phenotype into a recipient strain by P1 transduction using T3A58/pd1 as the donor. Strain WGAleu was chosen as the recipient since it contained ace and suc-linked markers (leu and gal) where there is the highest probability of finding the lpd gene(s). The frequencies of Leu⁺ and Gal⁺ transduction were 15 and 5/10⁶ P1 respectively using glucose and galactose-based selective media supplemented with acetate plus succinate. In subsequent tests, none (< 1%) of the Gal⁺ transductants required acetate, succinate or both, but 8% of the Leu⁺ transductants required both acetate and succinate. Enzymological analysis of selected Leu⁺ transductants confirmed that the acetate- and succinate-requirers lacked lpdh activity and both complexes (Table 4). These lpdI derivatives resemble the original T3A58/pd1 mutant in all respects and one of them WGA/pd1 (WGA.LT11) was used in some of the experiments chosen here to illustrate complementation. Thus it can be concluded that there is an lpd gene in the leu region and that there is unlikely to be another elsewhere.

DISCUSSION

The results clearly demonstrate that the problem of isolating lipoamide dehydrogenase mutants of Escherichia coli is one of detecting them against a background of ace, suc, lip, ppc and other mutants which respond to the same group of nutritional supplements. The earlier failures are almost certainly due to their requirement for acetate, in addition to succinate,
being insufficiently pronounced on solid medium and, unlike the lip mutants, these mutants have no other obvious nutritional features which reveal their identity. On the other hand, their response to succinate alone is apparently too poor for them to have been recovered as a distinct class of succinate-requirers. The lipoamide dehydrogenase-less mutants cannot be defined by nutritional criteria alone, so the demonstration of a deficiency in this enzyme was essential. Further, to exclude the possibilities of the mutants being strongly polar ace and suc mutants, or lpd mutants with additional lesions in ace or suc genes, which would be hidden by the phenotype, another essential criterion for defining the mutants was to show that cell-free extracts could be complemented for both \( \alpha \)-ketoacid dehydrogenase complexes by purified lipoamide dehydrogenase.

All the evidence indicates that the mutants are simply deficient in lipoamide dehydrogenase and their existence confirms that the only essential function of this enzyme is as a component of the pdh and kgdh complexes and that it cannot be replaced \((in \ vivo)\) by any other enzyme.

Proof that there is only one \( lpd \) gene depends upon whether the present mutants contain a single genetic lesion. Despite a rather difficult screening procedure, 10 out of 16 mutant hunts yielded \( lpd \) mutants and since 13 gave suc mutants, 4 (lip), 2 (suc) and 2 (ace, suc double mutants), the \( lpd \) mutants are by no means as rare as might be expected if they were double mutants. Also the existence of a single \( lpd \) gene is favoured by demonstrating that the mutant phenotype can be transferred into an \( Lpd^+ \) recipient by transduction, and by extensive linkage analyses and three-point reciprocal crosses in which one \( lpd \) mutation has behaved like a single genetic marker located extremely close to \( aceF \) at the distal end of the ace region (Guest & Creaghan, 1972; and unpublished observations). These observations establish that there is an \( lpd \) gene between the \( aceF \) and \( pan \) genes and they also indicate that there are no other equivalent \( lpd \) genes outside this region. However, the remote possibility that there are two \( lpd \) genes in this small segment of the linkage map and that the \( lpd \) mutants have lesions in both has still to be excluded. But for the unusual dual role of lpdh and the history of the problem, this possibility would rarely be considered. Nevertheless, the simplest interpretation of the evidence is that the E3 components of the pdh and kgdh complexes are specified by a single \( lpd \) gene. These results are completely consistent with the biochemical evidence of Pettit & Reed (1967) although their criteria may not have discriminated between two very similar proteins, especially if the E3 components of the two complexes were derived from a single pool. Even if the E3 components of both complexes are subsequently shown to be identical in all respects, there would still be a finite possibility that they are specified by two identical genes. Alternatively, slight differences between the two components could be due to subsequent modifications of a single gene product. By contrast to Escherichia coli, pig heart mitochondria contain two major molecular forms of lpdh, Fp-I the main flavoprotein component of kgdh complex, Fp-II the corresponding component of pdh complex and uncomplexed lpdh appears to be a mixture of both. They are functionally interchangeable and immunochemically indistinguishable, though distinct from the \( E. \ coli \) enzyme. However, the differences between the two forms may be conformational rather than structural (Sakurai, Fukuyoshi, Hamada, Hayakawa & Koike, 1970) and further electrophoretic heterogeneity may be due to proteolytic modification during purification (Wilson, 1971). If there is just one \( lpd \) gene for each organism, then the requirement for interaction with two partial complexes may impose considerable limitations on the extent of structural changes in lpdh during divergent evolution. This is supported by the complementation studies which indicate that the mammalian enzyme can couple with bacterial E1–E2 partial complexes to generate the catalytic activities of the overall complexes, although it is not known whether hybrid
components (pseudo-polarity) or a combination of translational polarity (in sucA amber communication) complexes is rather unusual and here it raises a number of interesting questions concerning the expression of the pdh complex syntheses which would more easily be explained by having two lpd genes in the distal regions of ace and suc operons. For example, the polarity exhibited by both aceE and sucA amber mutants appears to extend to lpdh synthesis (Henning, Dennert, Hertel & Shipp, 1966; Creaghan & Guest, 1972), but this could be a consequence of the lack of E1 and E2 components (pseudo-polarity) or a combination of translational polarity (in ace) and pseudo-polarity (in suc). Also, the syntheses of pdh complex and kgdh complex appear to be controlled by independent regulatory mechanisms since the respective complexes are induced by pyruvate and α-ketoglutarate (or acetate) and the ratio of pdh complex:kgdh complex in crude cell-free extracts can vary between 4:1 and 1:2 for organisms grown with different substrates. A somewhat similar situation has recently been described in Bacillus subtilis where a single gene, trpX, specifies the glutamate-binding protein of p-aminobenzoate synthetase and anthranilate synthetase (Kane, Holmes & Jensen, 1972). The trpX gene is not in the trp operon and its expression is repressed semico-ordinately by tryptophan.

We wish to thank Dr U. Henning for kindly supplying the ace mutants used in this work and Dr C. H. Williams, Jun. for a sample of Escherichia coli B lipoamide dehydrogenase.

REFERENCES


