Purification of a New Dihydrolipoamide Dehydrogenase from Escherichia coli

GILBERT RICHARME
Institut Jacques Monod, Tour 43, 2 Place Jussieu, 75251 Paris Cedex 05, France

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I purified a new dihydrolipoamide dehydrogenase from a lpd mutant of Escherichia coli deficient in the lipoamide dehydrogenase (EC 1.6.4.3) common to the pyruvate dehydrogenase (EC 1.2.4.1) and 2-oxoglutarate dehydrogenase complexes. The occurrence of the new lipoamide dehydrogenase in lpd mutants, including a lpd deletion mutant and the immunological properties of the enzyme, showed that it is different from the lpd gene product. The new dihydrolipoamide dehydrogenase had a molecular weight of 46,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was expressed in low amounts. It catalyzed the NAD\(^+\)-dependent reduction of dihydrolipoamide with a maximal activity of 20 \(\mu\)mol/min per mg of protein and exhibited a hyperbolic dependence of catalytic activity on the concentration of both dihydrolipoamide and NAD\(^+\). The possible implication of the new dihydrolipoamide in the function of 2-oxo acid dehydrogenase complexes is discussed, as it is its relation to binding protein-dependent transport.

Dihydrolipoamide dehydrogenases catalyze the NAD\(^+\)-dependent oxidation of dihydrolipoamide (reviewed by Williams [29]; dihydrolipoamide + NAD\(^+\) \xrightarrow{\text{enzyme}}\) lipoamide + NADH + H\(^+\)). The most important function of dihydrolipoamide dehydrogenases as a component of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (16) is their implication in the oxidative decarboxylation of pyruvate and 2-oxoglutarate. In Escherichia coli, a single lipoamide dehydrogenase, the lpd gene product, has been discovered; it has been found to be common to both multienzyme complexes (15). The pyruvate dehydrogenase complex consists of pyruvate dehydrogenase (EC 1.2.4.1; aceE gene product), dihydrolipoamide acetyltransferase (EC 2.3.1.12; aceF gene product), and lipoamide dehydrogenase (EC 1.6.4.3; lpd gene product); the 2-oxoglutarate dehydrogenase complex consists of 2-oxoglutarate dehydrogenase (EC 1.2.4.2; sucA gene product), dihydrolipoamide succinyltransferase (EC 2.3.1.61; sucB gene product), and lipoamide dehydrogenase (EC 1.6.4.3; lpd gene product).

A second important function of the dihydrolipoamide dehydrogenases as a component of branched-chain keto acid dehydrogenase complexes is related to their implication in the oxidative decarboxylation of branched-chain keto acids. These enzyme complexes are encountered in bacteria which can utilize branched-chain amino acids for growth (they perform an essential step in the catabolism of these amino acids after they are transaminated to keto acids). The bacterium *Pseudomonas putida*, which can use branched-chain amino acids for growth, possesses a branched-chain keto acid dehydrogenase complex which contains (in addition to its dehydrogenase and transacylase components) a specific lipoamide dehydrogenase (lpd Val) that is different from the lipoamide dehydrogenase (lpd Glc) of the pyruvate and 2-oxoglutarate dehydrogenase complexes (24). Branched-chain keto acid dehydrogenase activities have also been found in bacteria, such as *Bacillus subtilis*, which synthesize branched-chain fatty acids (from branched-chain keto acids) for maintenance of their membrane fluidity (13). A dihydrolipoamide dehydrogenase (lpd Glc) has also been shown to participate in glycine oxidation in *P. putida* as a component of glycine decarboxylase (23). The bacterium *Escherichia coli*, which cannot use branched-chain amino acids for growth and does not synthesize significant amounts of branched-chain phospholipids (11), does not appear to contain branched-chain keto acid dehydrogenase activities (28).

Dihydrolipoamide dehydrogenases have also been discovered in several microorganisms which do not seem to possess lipoamide-dependent 2-oxo acid dehydrogenase complexes. A dihydrolipoamide dehydrogenase has been purified from the halophilic archae bacterium *Halobacterium halobium*, although this bacterium catalyzes pyruvate and 2-oxoglutarate oxidation by ferredoxin-linked oxidoreductases (4); similarly, a dihydrolipoamide dehydrogenase has been isolated from the African parasite *Trypanosoma brucei* (2); these discoveries raise the question of whether dihydrolipoamide dehydrogenases have a cellular function, in addition to their established role in the 2-oxo acid dehydrogenase complexes.

In studies concerning the possible implication of lipoamide dehydrogenase in the function of binding protein-dependent transport systems, I have discovered a residual dihydrolipoamide dehydrogenase activity in lpd mutants of *E. coli* which were deficient in the lipoamide dehydrogenase component of the pyruvate and 2-oxo glutarate dehydrogenase complexes (including the deletion mutant JRG599 [21]). The purification and characterization of this second lipoamide dehydrogenase are reported here.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* strains K-12, JRG301 (F\(^−\) lpd-I trpA trpE), T3A58 (F\(^+\) trpA trpE), JRG599 [Hfr Δ(aroP-lpd)74 pps-I melA1 thyA56 metB1 azi-14 ton-54 tss-87 met-105], and K-11 [Hfr pps-I melA1 thyA56 metB1 azi-14 ton-54 tss-87 met-105] (5-7) were kindly provided by J. R. Guest (Sheffield University, Sheffield, United Kingdom). Strains LA5732 (F\(^−\) lpd-I trpA trpE mgl-512 zee-700::Tn10) and *E. coli* LA5732 (F\(^−\) lpd-I trpA trpE mgl-512 zee-700::Tn10) transformed with plasmid pHG30 (containing the mgl operon of *Salmonella typhimurium*) (12) were provided by H. G. Heine; they were originally from W. Boos (Fachbereich Biologie, Konstanz University, Konstanz, Federal Republic of Germany). Unless otherwise indicated, the bacteria were grown aerobically at 37°C in LB rich medium that was...
supplemented with 4 mM each acetate and succinate for strains that were deficient in the *lpd* gene product. Strain LA5732 carrying plasmid pHG30 was grown in LB rich medium supplemented with 4 mM each of acetate and succinate and 0.4% glucose (in order to repress the *mgl* genes) to an *A*$_{600}$ of 0.5 and then transferred to the same medium but without glucose and was kept there for 2 h to obtain expression of the *mgl* genes.

**Purification.** The purification procedure was done at 4°C and started with 30 g of bacteria (strain LA5732 transformed with plasmid pHG30). The bacteria were washed with 100 ml of 100 mM Tris hydrochloride (pH 7.2) and suspended in 30 ml of 40 mM potassium phosphate (pH 6.8)–5 mM EDTA–0.5 mM dithiothreitol. The cell suspension was sonicated for 5 times for 15 s each time with a sonifier (settled at a power of 25 W; Branson Sonic Power Co., Danbury, Conn.); the suspension of broken cells was centrifuged for 10 min at 20,000 × *g*, and the supernatant was centrifuged for 30 min at 180,000 × *g*. The 180,000 × *g* supernatant was applied to a DEAE cellulose column (DE52; 4 by 34 cm) that was equilibrated in 20 mM potassium phosphate (pH 6.8)–2 mM EDTA–0.5 mM dithiothreitol; the column was washed with 200 ml of the preceding buffer and eluted with 500 ml of linear gradient of 0 to 0.5 M NaCl in the same buffer. The lipoamide dehydrogenase activity eluted as a single peak, and the pooled fractions were loaded onto a lipopolysaccharide column (1.5 by 20 cm) that was equilibrated in 20 mM potassium phosphate (pH 6.8)–2 mM EDTA–0.5 mM dithiothreitol–15 M NaCl, which was prepared by coupling 200 mg of lipoic acid (oxidized form) dissolved in dioxane to 8 g of AH Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) with 1-ethyl-3-dimethylaminopropyl)carbodiimide, as recommended by the manufacturer. The column was washed overnight with 1 liter of the same buffer, and it was eluted with 100 ml of 0 to 2 M NaCl in the same buffer; the lipoamide dehydrogenase activity eluted as a single peak at 0.6 M NaCl. The pooled fractions were diluted 10-fold in 20 mM potassium phosphate (pH 6.8)–2 mM EDTA–0.5 mM dithiothreitol and loaded onto a Blue-Trisacryl column (1.4 by 7 cm; IBM France) equilibrated in the preceding buffer; the column was washed with 50 ml of the same buffer and eluted with 40 ml of a linear gradient of 0 to 0.5 M NaCl in the same buffer.

**Enzyme assays.** Dihydrolipoamide dehydrogenase was assayed as described previously (6) by following the dihydrolipoate-dependent reduction of NAD at 340 nm (or 3-acetyl-NAD at 366 nm where indicated). To 500 μl of 100 mM Tris hydrochloride (pH 8.0) containing 5 mM EDTA, 1 mM NAD (or 3-acetyl-NAD), 10 μl of 100 mM dihydrolipoate (dissolved in ethanol), and 50 μl of protein fraction were added; the rate of increase of the *A*$_{340}$ (or *A*$_{366}$) as a function of time during the first minute was taken as the activity of dihydrolipoamide dehydrogenase; no variation in absorbance was observed in the absence of dihydrolipoate on the addition of 10 μl of ethanol. The lipoate-dependent oxidation of NADH by purified protein was measured by assaying the oxidation of NADH in a medium containing 100 mM Tris hydrochloride (pH 8.0), 5 mM EDTA, 1 mM lipoate, and 50 μM NADH. The activities of the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and branched-chain keto acid dehydrogenase complexes were measured as described previously (25).

NADPH ubiquinone oxidoreductase was assayed as described previously (14) by following the ubiquinone (coenzyme Qo)-dependent oxidation of NADH at 340 nm. NADPH glutathione oxidoreductase was assayed as described previously (3) by following the glutathione-depen-
TABLE 1. Purification of the new lipoamide dehydrogenase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Total protein (mg)</th>
<th>Total enzyme (arbitrary units)</th>
<th>Sp act (μmol of NADH produced/min per mg of protein)</th>
<th>% Recovery</th>
<th>Overall purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>180,000 × g supernatant*</td>
<td>40</td>
<td>1,500</td>
<td>4.5</td>
<td>0.003</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Pool from DEAE cellulose column</td>
<td>40</td>
<td>145</td>
<td>2.9</td>
<td>0.02</td>
<td>64</td>
<td>6.6</td>
</tr>
<tr>
<td>Pool from lipoyl Sepharose</td>
<td>12</td>
<td>1.9</td>
<td>1.9</td>
<td>1.0</td>
<td>42</td>
<td>330</td>
</tr>
<tr>
<td>Heated enzyme from preceding step</td>
<td>12</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>33</td>
<td>330</td>
</tr>
<tr>
<td>Pool from Blue-Trisacryl column</td>
<td>5</td>
<td>0.030</td>
<td>0.6</td>
<td>20</td>
<td>13</td>
<td>6,600</td>
</tr>
</tbody>
</table>

* NAD reduction was not detectable in the 180,000 × g supernatant, and it was estimated by measuring the reduction of 3-acetyl NAD with a correction for the maximal velocity of the enzyme for both substrates.

measured by the NADH-dependent decrease of the A_340. They were localized at the interface between 60 and 25% sucrose. The dihydrolipoamide dehydrogenase activity was measured by following the dihydrolipoamide-dependent reduction of 3-acetyl NAD at 366 nm.

**Protein determination.** Protein was determined by the assay of Bradford (1).

**RESULTS**

Purification of the dihydrolipoamide dehydrogenase. Dihydrolipoamide dehydrogenase was purified from a lpd mutant that was transformed with a plasmid containing the mgl operon coding for the binding protein-dependent galactose transport system. In fact, as observed in previous studies (21), transformation of a strain deleted for the mgl genes with a plasmid containing the mgl operon leads to the stimulation of lipoamide dehydrogenase activity in crude extracts from this strain (although the new lipoamide dehydrogenase is not a mgl gene product [see below]). *E. coli* LA5732 (a derivative of the lpd mutant JRG 301 deleted for the mgl genes) transformed with plasmid pHG30 (a derivative of pBR322 containing the complete mgl operon [mglB mglA mglC mglE] of *S. typhimurium* [12]) was used. The results of a typical purification are shown in Table 1. The bacteria were broken by ultrasonic disruption as described above. The supernatant after the ultracentrifugation step (180,000 × g for 30 min) contained more than 85% of the dihydrolipoamide dehydrogenase activity and was used for the purification. The dihydrolipoamide dehydrogenase eluted as a single peak from the Blue-Trisacryl column at 100 mM NaCl and gave a single band when analyzed by SDS-gel electrophoresis (Fig. 1; see below). When the heat step was omitted, the dihydrolipoamide dehydrogenase copurified with several other polypeptides, including the aceF gene product (the dihydrolipoyltransacetylase of the pyruvate dehydrogenase complex) and the mglC gene product (the 29,000-dalton component of the binding protein-dependent galactose transport). These polypeptides were characterized on immunoblots of bacterial extracts from strains containing (or deleted for) the ace

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**FIG. 1.** Chromatography of the new dihydrolipoamide dehydrogenase on a Blue-Trisacryl column.
and mgl genes with antibodies raised against the purified complex (unpublished data).

Characterization by SDS-gel electrophoresis and immunoblotting. Electrophoresis of the purified dihydrolipoamide dehydrogenase on SDS-polyacrylamide gels gave a single protein band (Fig. 2), confirming the homogeneity of the preparation. Comparison of its electrophoretic mobility with those of standard proteins that were run simultaneously indicated that it had a molecular weight of 46,000; the lpd gene product of E. coli had a molecular weight of about 56,000, as determined by SDS-gel electrophoresis (29). Electrophoresis of the purified protein on native polyacrylamide gels is shown in Fig. 3; the protein band (revealed by silver staining) (Fig. 3A) and the dihydrolipoamide dehydrogenase activity band (revealed by NADH fluorescence), after incubation for 15 min in the presence of substrates (Fig. 3B), migrated at the same positions, indicating that the single-protein band is responsible for the lipoamide dehydrogenase activity. No other activity bands appeared as a result of the longer incubation times, and the band shown in Fig. 3B became stronger and subject to significant spreading.

Further characterization of the new dihydrolipoamide dehydrogenase was performed by immunoblotting experiments. Crude extracts from several E. coli strains were tested by immunoblotting with antibodies raised against the purified lipoamide dehydrogenase (Fig. 4A) and with antibodies raised against the lpd gene products (Fig. 4B) (the latter were generously provided by J. R. Guest, Sheffield University). Antibodies raised against the lipoamide dehydrogenase recognized a single protein band with a molecular weight of about 46,000 in crude extracts from a wild-type strain (Fig. 4A, lane 1) with respect to the lpd gene product and in crude extracts from the lpd deletion mutant JRG599 (Fig. 4A, lane 2). In contrast, as shown in Fig. 4B, antibodies raised against the lpd gene product recognized a single band (molecular weight, 52,000) in the wild-type strain and did not react with the lpd deletion mutant JRG599. These experiments show that the new lipoamide dehydrogenase described here is distinct from the lpd gene product. Since several results were obtained that showed an increase in the dihydrolipoamide dehydrogenase activity in crude extracts from strains containing the mgl genes coding for the binding protein-dependent galactose transport system, immunoblotting experiments with antibodies raised against the new lipoamide dehydrogenase were performed with crude extracts from a strain that contained the mgl genes (strain LA5732 transformed with plasmid pHG30) and with extracts from a mutant that was unable to synthesize any of the mgl gene products (strain LA5732 [12]). Antibodies recognized in crude extracts from both strains a single protein band at a molecular weight of 46,000 (Fig. 4A, lanes 3 and 4), indicating that the lipoamide dehydrogenase is not a mgl gene product. The higher-molecular-weight bands which were seen in lanes 1 and 3 of Fig. 4A were not detected reproducibly.

Kinetics constants. The velocity of the NAD-dependent dihydrolipoamide oxidation showed a hyperbolic dependence on the NAD and dihydrolipoamide concentrations (data not shown). Michaelis constants of 150 μM for NAD
and 25 μM for dihydrolipoamide were determined. A maximal velocity of 20 μmol/min per mg of protein was determined in the direction of NAD reduction, compared with a specific activity of 10 μmol/min per mg of protein for the purified E. coli B lipoyl dehydrogenase (7). The maximal velocity in the direction of NADH oxidation was low. The purified enzyme possessed a low NADH-ubiquinone oxidoreductase activity (3% of the dihydrolipoamide dehydrogenase activity) and did not display NADPH-glutathione oxidoreductase activity (data not shown).

Molecular weight of the native protein. The molecular weight of the native protein was determined by gel filtration of the dihydrolipoamide dehydrogenase on a Bio-Gel P200 column as described above. A molecular weight of 88,000 was determined for the native protein. This value, compared with the molecular weight of 46,000 determined by SDS-gel electrophoresis, suggests that the protein (like most other dihydrolipoamide dehydrogenases studied) exists as a dimer in its native state.

Cellular location. The following results shed some light on the cellular location of the new lipoyl dehydrogenase. A total of 80% of the lipoyl dehydrogenase activity was found in the supernatant after ultracentrifugation of bacterial ultrasonic extracts for 30 min at 180,000 × g (the 20% found in the pellet could have been either membrane bound or could have represented aggregates of the dehydrogenase). Lysis of bacteria in the presence of Triton X-100 did not produce significantly greater amounts of dihydrolipoamide dehydrogenase in the supernatant fraction (data not shown). Ultracentrifugation of crude extracts from cells that were broken by ultrasonic disruptions on a sucrose density gradient as described above showed that most of the dihydrolipoamide dehydrogenase was found in fractions containing soluble proteins, whereas less than 10% sedimented in the membrane-containing fraction (as determined by their NADH oxidase activities). These results suggest that the lipoyl dehydrogenase described here is probably located in the cytoplasm, with a possible interaction of a fraction of the enzyme molecules with the cytoplasmic membrane. This cellular location is consistent with that of most lipoyl dehydrogenases studied. These enzymes are generally considered to be soluble cytoplasmic enzymes, but their possible interaction with membranes has been suggested in several cases (2, 26).

Cellular expression of the new lipoyl dehydrogenase. The dihydrolipoamide dehydrogenase described here was expressed in low amounts in E. coli. Calculation of the amount of enzyme (30 μg) recovered from 30 g of bacteria (with a 20% recovery of enzyme activity and assuming 5 × 10¹¹ cells per g [wet weight] of bacteria) suggests that it is produced at a level of about 200 molecules per cell. In contrast, the lpd gene product, which represents 0.3% (estimated by immunological studies) of total protein in ultrasonic extracts of E. coli (7), would be produced at greater than 10,000 copies per cell. The low level of expression of the new lipoyl dehydrogenase explains why it has been overlooked in previous studies. Cellular expression of the protein in bacteria grown in several media has been studied by the immunoblotting technique. The bacteria were grown in minimal medium M63 (10) containing 0.4% glucose, 0.4% glycerol, or 0.4% glycerol plus 0.05% isoleucine and 0.3% valine. The amount of lipoyl dehydrogenase (estimated from three independent experiments) was similar in crude extracts from bacteria that were grown in glucose or glycerol medium and was found at a three-times-higher level in crude extracts from bacteria that were grown in medium containing glycerol, isoleucine, and valine.

Miscellaneous. I tried to complement crude extracts from the lpd mutant (which was deficient in the lpd gene product but which contained the aceE, aceF, sucA, and sucB gene products in a functional form with the purified lipoyl dehydrogenase, but no restoration of pyruvate dehydrogenase or 2-oxoglutarate dehydrogenase activity was obtained with the new lipoyl dehydrogenase [in contrast to that obtained with the purified lpd gene product, which is able to complement these extracts (6)]. I was not able to detect any branched-chain keto acid dehydrogenase activity in crude extracts from E. coli (as reported previously [28]), even when these extracts were supplied with the purified new lipoyl dehydrogenase.

DISCUSSION

The results of this study indicate the occurrence of a second lipoyl dehydrogenase in E. coli. Its low abundance (about 200 molecules per cell) explains why it has been overlooked in previous studies (10, 27). The molecular weight of the new dihydrolipoamide dehydrogenase (46,000), its occurrence in lpd mutants (including a deletion mutant), and its immunological properties indicate that it is different from the lpd gene product, the lipoyl dehydrogenase of the pyruvate dehydrogenase, and 2-oxoglutarate dehydrogenase complexes.

It is not clear whether the new lipoyl dehydrogenase functions in association with the pyruvate dehydrogenase (aceE gene product) and dihydrolipooyl transacetylase (aceF gene product) components of the pyruvate dehydrogenase complex (or with the 2-oxoglutarate dehydrogenase [sucA gene product] and dihydrolipoyltrans succinylase [sucB gene product] of the 2-oxoglutarate dehydrogenase complex). The failure of the lpd mutant JRG301 (containing the aceE, aceF, sucA, and sucB gene products and the new lipoyl dehydrogenase) to grow aerobically in minimal medium that was not supplemented with acetate or succinate (6) suggests that this is not the case. Also, the failure of the purified dihydrolipoamide dehydrogenase to complement the aceE and aceF gene products in crude extracts for the restoration of the pyruvate dehydrogenase complex activity and the sucB and sucA gene products for restoration of the 2-oxoglutarate dehydrogenase complex activity (this study) led to the same conclusion. However, it cannot be excluded that these failures were due to, respectively, the low abundance of the new dihydrolipoamide dehydrogenase and the partial denaturation of the purified enzyme. In contrast, the partial copurification of the present dihydrolipoamide dehydrogenase with the aceF gene product, when the heat step of the purification procedure was omitted (unpublished data), and the ability of the lpd mutant JRG301, containing the aceE and aceF gene products and the new lipoyl dehydrogenase, to sustain high binding protein-dependent transport activity compared with those of the aceE and aceF mutants (19) suggests that a functional interaction may exist between the aceE and aceF gene products and the new lipoyl dehydrogenase.

Several properties of the new lipoyl dehydrogenase are similar to those of lpd Val, the second lipoyl dehydrogenase of P. putida. Both proteins have a similar molecular weight (about 47,000), whereas the lpd gene product of E. coli and the lpd Glc protein of P. putida have molecular weights of about 56,000. The new lipoyl dehydrogenase was overproduced when cells were grown in the presence of
isoleucine and valine. \( \text{lpd Val} \) was also induced by these compounds. Furthermore, antibodies raised against the purified new lipoamide dehydrogenase gave a faint reaction in immunoblots with crude extracts of \( \text{P. putida} \) producing \( \text{lpd Val} \) (unpublished data).

The new dihydrolipoamide dehydrogenase was revealed by studies that examined the implication of lipoic acid and 2-oxo acid dehydrogenases in the function of binding protein-dependent transport systems. The strong reduction of binding protein-dependent transport as a result of lipoic acid deprivation of a mutant deficient in lipoic acid synthesis or in strains deficient in the \( \text{aceE} \) and \( \text{aceF} \) gene products, but not in the \( \text{lpd} \) strain (deficient in the \( \text{lpd} \) gene product but containing intact \( \text{aceE} \) and \( \text{aceF} \) gene products [6]), led to the discovery of the new dihydrolipoamide dehydrogenase and to the proposal of its implication in binding protein-dependent transport (17–19). The present study showed that the new dihydrolipoamide dehydrogenase is not a product of the \( \text{mgl} \) operon. However, several results, such as the increased activity of the lipoamide dehydrogenase in a strain containing the \( \text{mgl} \) operon (this study; 21) and the partial copurification of the \( \text{mgl} \) gene products with the lipoamide dehydrogenase (20; unpublished data), suggest an interaction between the \( \text{mgl} \) gene product and the new lipoamide dehydrogenase. However, supplemental work is necessary to determine the exact roles of lipoamide dehydrogenases, the proton motive force, and ATP in binding protein-dependent transport.

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LITERATURE CITED


