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Isolation of a Third Lipoamide Dehydrogenase from *Pseudomonas putida*

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Pseudomonads are the only organisms so far known to produce two lipoamide dehydrogenases (LPDs), LPD-Val and LPD-Glc. LPD-Val is the specific E3 component of branched-chain oxoacid dehydrogenase, and LPD-Glc is the E3 component of 2-ketoglutarate and possibly pyruvate dehydrogenases and the L-factor of the glycine oxidation system. Three mutants of *Pseudomonas putida*, JS348, JS350, and JS351, affected in *lpdG*, the gene encoding LPD-Glc, have been isolated; all lacked 2-ketoglutarate dehydrogenase, but two, JS348 and JS351, had normal pyruvate dehydrogenase activity. The pyruvate and 2-ketoglutarate dehydrogenases of the wild-type strain of *P. putida* were both inhibited by anti-LPD-Glc, but the pyruvate dehydrogenase of the *lpdG* mutants was not inhibited, suggesting that the mutant pyruvate dehydrogenase E3 component was different from that of the wild type. The lipoamide dehydrogenase present in one of the *lpdG* mutants, JS348, was isolated and characterized. This lipoamide dehydrogenase, provisionally named LPD-3, differed in molecular weight, amino acid composition, and N-terminal amino acid sequence from LPD-Glc and LPD-Val. LPD-3 was clearly a lipoamide dehydrogenase as opposed to a mercuric reductase or glutathione reductase. LPD-3 was about 60% as effective as LPD-Glc in restoring 2-ketoglutarate dehydrogenase activity and completely restored pyruvate dehydrogenase activity in JS350. These results suggest that LPD-3 is a lipoamide dehydrogenase associated with an unknown multienzyme complex which can replace LPD-Glc as the E3 component of pyruvate and 2-ketoglutarate dehydrogenases in *lpdG* mutants.

Most organisms produce only one lipoamide dehydrogenase, which is the E3 component of the keto acid dehydrogenase multienzyme complexes, pyruvate, 2-ketoglutarate, and branched-chain ketoacid dehydrogenases. The pyruvate and 2-ketoglutarate dehydrogenases of *Escherichia coli* have a common E3 component which is encoded by the *lpd* gene. The *lpd* gene is closely associated with *aceE* and *aceF*, the structural genes for the E1 and E2 components of pyruvate dehydrogenase, but is separated from *aceF* by 324 bases (18). Expression of *lpd* occurs either in conjunction with that of *aceE* and *aceF* (17) or separately from its own promoter (18). Mutants of *E. coli* affected in the expression of *lpd* lose both pyruvate and 2-ketoglutarate dehydrogenase activities (7). Evidence from the study of the human genetic disease lactic acidosis supports the belief that humans also possess a single lipoamide dehydrogenase. The defect in this disease is in the formation of lipoamide dehydrogenase, and these patients are deficient in pyruvate, 2-ketoglutarate, and branched-chain ketoacid dehydrogenases (12, 21).

Given the above background, it was surprising to find that *Pseudomonas putida* and *Pseudomonas aeruginosa* were capable of forming two lipoamide dehydrogenases (LPDs), LPD-Glc and LPD-Val (10, 14). LPD-Glc was the only lipoamide dehydrogenase found in *P. putida* grown in glucose synthetic medium, while both LPD-Glc and LPD-Val were recovered from cells grown in valine-containing medium. The two lipoamide dehydrogenases not only differed in physical properties such as molecular weight, amino acid composition, and UV spectrum (4, 10, 14) but were encoded by separate genes (16, 19). They also differed in function: LPD-Val was the specific E3 component of branched-chain ketoacid dehydrogenase, while LPD-Glc was the E3 component of 2-ketoglutarate and probably pyruvate dehydrogenases (16) and the L-factor of the glycine oxidation system (13). The structural gene for LPD-Val was found to be part of a tightly linked operon encoding all the components of the branched-chain ketoacid dehydrogenase complex (20). The nucleotide sequence of the LPD-Val gene has been determined and compared with the sequences of other lipoamide dehydrogenases (G. Burns, T. Brown, K. Hatter, and J. R. Sokatch, Eur. J. Biochem., in press).

During the study of the chromosomal location of genes encoding the components of pyruvate, 2-ketoglutarate, and branched-chain ketoacid dehydrogenases of *P. putida*, a mutant was isolated which was affected in *lpdG*, the structural gene for LPD-Glc (19). This mutant, *P. putida* JS348, lacked 2-ketoglutarate dehydrogenase, as expected, but had a normal amount of pyruvate dehydrogenase. The behavior of JS348 suggested the possibility of a third lipoamide dehydrogenase in *P. putida*. The objective of the present research was to explain the presence of pyruvate dehydrogenase activity in *P. putida* JS348. This communication reports the isolation of two additional *lpdG* mutants, their behavior, and isolation of a third lipoamide dehydrogenase from *P. putida* JS348.

MATERIALS AND METHODS

Bacterial strains and media. *P. putida* PpG2 is a wild-type strain which was originally obtained from I. C. Gunsalus. Strain JS348 was isolated after treatment of *P. putida* PpG2 with nitrosoguanidine by selection for mutants unable to grow in medium with 2-ketoglutarate as the carbon source (19). *P. putida* strains JS350 and JS351 were isolated in this study by the same procedure.

GAS medium (10 mM glucose, 2 mM acetate, and 2 mM succinate) was devised for mutants lacking both pyruvate

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and 2-ketoglutarate dehydrogenases, since these mutants frequently require acetate and succinate for growth (19). Valine-isoleucine medium was described before (9).

Enzyme assays. The assays for pyruvate, 2-ketoglutarate, and branched-chain ketoacid dehydrogenases (15) and for lipoamide dehydrogenase (16) were described before. The assays for the E1 and E2 components of the ketoacid dehydrogenases were described before (16).

Purification of LPD-3. LPD-3 was purified from 88.7 g of *P. putida* JS348 cells grown in GAS synthetic medium. The cells were suspended in two 150-ml batches of 50 mM potassium phosphate buffer containing 1 mM EDTA and 0.5 mM dithiothreitol (phosphate-EDTA-DTT buffer). The cell suspensions were sonicated for four 10-min periods, each in an ice bath with a Heat Systems model W-225R sonic oscillator at a power setting of 8. The broken cell suspension was centrifuged for 1 h at 90,000 × *g*. The supernatant solution was then centrifuged for 3 h at 176,000 × *g*. The pellet from this step was dissolved in the minimum amount of phosphate-EDTA-DTT buffer, total volume 83 ml, and added to a column of Sepharose CL-4B (5.0 by 90 cm) equilibrated with phosphate-EDTA-DTT buffer. Fractions (240 drops) were collected. Pyruvate dehydrogenase and lipoamide dehydrogenase were eluted simultaneously and recovered in fractions 52 to 75. The pool from the Sepharose CL-4B column was added to a DEAE-Sepharose CL-6B column (2.5 by 20 cm) equilibrated in phosphate-EDTA-DTT buffer. The column was washed with 240 ml of phosphate-EDTA-DTT buffer. A linear gradient of 0.0 to 0.6 M sodium chloride was used to elute protein from the column. Fractions (11 ml) were collected, and again pyruvate dehydrogenase and lipoamide dehydrogenase were eluted simultaneously at about 0.25 to 0.35 M sodium chloride. Fractions 70 to 90 were pooled and heated at 65°C for 5 min. This treatment inactivates the E1 component but not E2 and E3. The heat-treated fraction was dialyzed against two changes of 0.01 mM potassium phosphate (pH 7.0)–1 mM EDTA–5 mM 2-mercaptoethanol. The dialyzed, heat-treated fraction (210 ml) was loaded onto an Affi-Gel Blue column (1.5 by 17 cm) equilibrated with 10 mM potassium phosphate (pH 7.0)–1 mM EDTA–0.5 mM DTT. LPD-3 was eluted with a linear gradient of 0.0 to 0.5 M sodium chloride, again at about 0.25 to 0.35 M sodium chloride. Fractions (3 ml) were collected, and fractions 66 to 87 were pooled.

Methods used for characterization of LPD-3. The preparation of antibody against LPD-Glc was described before (16). Methods for digestion of LPD-3 with chymotrypsin and electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels were described before (3, 16). The N-terminal sequences of LPD-3 and LPD-Glc were determined by the Molecular Biology Resource Facility of the Saint Francis Hospital of Tulsa Medical Research Institute with a model 470A gas-phase protein sequencer equipped with a model 120A in-line phenylthiohydantoin amino acid analyzer (Applied Biosystems Inc.). Alignment of the N-terminal amino acid sequences was done by the use of the Bionet program GENALIGN (8). The spectra of LPD-3 and LPD-Glc were obtained with a Gilford Response automatic recording spectrophotometer.

RESULTS

Properties of *lpdG* mutants of *P. putida*. Like *P. putida* JS348, the two additional *lpdG* mutants isolated in this study, strains JS350 and JS351, were unable to grow in a medium containing 2-ketoglutarate as the carbon source. All three *lpdG* mutants were unable to use acetate as a sole carbon source, which is the classic test of mutants with a nonfunctional tricarboxylic acid cycle. However, these mutants did not possess the usual growth characteristics of *E. coli* mutants with a defective tricarboxylic acid cycle (5). For example, their growth on substrates metabolized by the tricarboxylic acid cycle intermediates, such as arabinose, glutamate, or lactate as the sole carbon source, was only slightly restricted. All three of the *lpdG* mutants had normal activities of the E1 and E2 components of 2-ketoglutarate dehydrogenase activities. Mutants JS348 and JS350 did not produce a precipitate with anti-LPD-Glc in Ouchterlony plates, while JS351 produced a band with a spur, suggesting that JS351 forms a modified LPD-Glc.

TABLE 1. Pyruvate and 2-ketoglutarate dehydrogenase activities of *lpdG* mutants of *P. putida*[a]

Enzyme and addition	Activity (nmol/min per mg of protein) in *P. putida* strain:			
	PpG2	JS348	JS350	JS351
Pyruvate dehydrogenase	45	42	0	19
+ LPD-Glc	47	55	36	40
+ Anti-LPD-Glc	5	39	—[b]	17
2-Ketoglutarate dehydrogenase	54	0	0	0
+ LPD-Glc	173	324	171	202
+ Anti-LPD-Glc	2	—	—	—

[a] The amounts of protein from each strain used in the above experiments were: PpG2, 330 μg; JS348, 540 μg for the pyruvate dehydrogenase assay and 135 μg for the 2-ketoglutarate dehydrogenase assay; JS350, 200 μg of protein; JS351, 200 μg of protein. LPD-Glc was used at 0.42 U when added. For the antibody inhibition experiments, 400 μg of each strain was incubated for 30 min with a 1:16 dilution of antiserum. A portion equal to 180 μg of enzyme protein was withdrawn and assayed for pyruvate and 2-ketoglutarate dehydrogenase activity.

[b] —, Not done.

It was previously reported that JS348 lacked 2-ketoglutarate dehydrogenase but had normal levels of pyruvate dehydrogenase (19) (Table 1). This was an unexpected finding, since *lpd* mutants of *E. coli* lack both pyruvate and 2-ketoglutarate dehydrogenases (7). Additional *lpdG* mutants of *P. putida* were isolated to see whether the behavior of JS348 was atypical. One of the newly isolated *lpdG* mutants, JS350, did indeed lack pyruvate as well as 2-ketoglutarate dehydrogenase, but the other, JS351, was similar to JS348 (Table 1). The pyruvate dehydrogenase activity of JS350 was restored by the addition of purified LPD-Glc (Table 1). This finding suggested that LPD-Glc was the E3 component of pyruvate as well as 2-ketoglutarate dehydrogenase. Furthermore, anti-LPD-Glc but not anti-LPD-Val inhibited both pyruvate and 2-ketoglutarate dehydrogenases of the wild-type strain, *P. putida* PpG2 (Table 1), which seemed to strengthen this conclusion. However, anti-LPD-Glc did not inhibit the pyruvate dehydrogenase of JS348 or JS351, which suggested that there was a third lipoamide dehydrogenase in *P. putida* which was being used by JS348 and JS351 as the E3 component of pyruvate dehydrogenase. If this were the case, however, it is not obvious why JS350 lacks pyruvate dehydrogenase. Since these mutants were obtained after nitrosoguanidine treatment, it is possible that JS350 has a second mutation which prevents the third lipoamide dehydrogenase from being assembled in the complex.

Purification and properties of LPD-3. The lipoamide dehydrogenase in JS348 was provisionally named LPD-3. LPD-3 was isolated from pyruvate dehydrogenase of JS348 and

TABLE 2. Purification of LPD-3 from *P. putida* JS348

Fraction	Protein (mg)	Activity	
		U	U/mg of protein
90,000 × *g* soluble	7,517	519	0.069
176,000 × *g* pellet	2,033	291	0.14
Sepharose CL-4B	415	232	0.56
DEAE-Sepharose CL-6B	35	157	4.5
Heat-treated DEAE-Sepharose	33	132	4.0
Affi-Gel Blue	1.5	100	68

purified to homogeneity by the procedure shown in Table 2. LPD-3 migrated as a single band in SDS-polyacrylamide gel electrophoresis and was separable from LPD-Glc. The molecular weight of LPD-3 determined by SDS-polyacrylamide gel electrophoresis was 53,000, in between those of LPD-Val (48,949) (Burns et al., in press) and LPD-Glc (56,000) (14). The absorption spectrum of LPD-3 was typical of lipoamide dehydrogenases, with a maximum at 455.6 nm. The previously reported absorption maximum for LPD-Glc was 455 to 456 nm and for LPD-Val it was 460 nm (4), the latter being somewhat higher than usual for a lipoamide dehydrogenase. Based on an M_r of 53,000, there were 1.4 mol of flavin adenine dinucleotide (FAD) per subunit of LPD-3, which is in fair agreement with the expected value of 1.0 mol of FAD per subunit.

The amino acid composition of LPD-3, not shown here, was clearly different from that of LPD-Val but similar to that of LPD-Glc. Since the molecular weight of LPD-3 is lower than that of LPD-Glc, we considered the possibility that LPD-3 was a truncated version of LPD-Glc caused by premature termination. However, no precipitate was formed between LPD-3 and anti-LPD-Glc in Ouchterlony plates. The enzymatic activity of LPD-3 was not inhibited by anti-LPD-Glc under conditions in which LPD-Glc was inhibited 93%. When purified LPD-3 and LPD-Glc were digested with chymotrypsin, the patterns of peptides formed from the two proteins were very different, showing that LPD-Glc and LPD-3 were different proteins (Fig. 1). A similar result was obtained with peptides from cyanogen bromide cleavage of LPD-3 and LPD-Glc.

Conclusive proof that LPD-Glc and LPD-3 are different proteins was obtained from comparison of amino acid sequences of their N-termini (Fig. 2). There were nine differences in 23 amino acids, which clearly shows that these two proteins are the products of separate genes. This region of lipoamide dehydrogenase contains the binding site for the adenine of FAD, beginning at residue 6 of LPD-3, and is highly conserved in all five lipoamide dehydrogenases shown in Fig. 2. The high degree of similarity in this region suggests that LPD-3 is a lipoamide dehydrogenase. The mercuric reductases of gram-negative bacteria are also redox-active disulfide flavoproteins, but are unlike the other proteins in this group since they have approximately 90 amino acid residues in front of the adenine-binding region (1, 2, 11). Since LPD-3 lacks this N-terminal segment, it is unlikely to be a mercuric reductase.

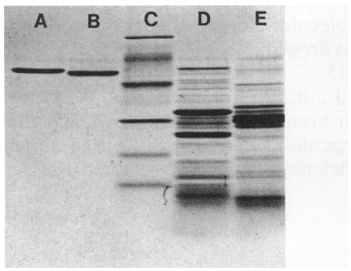

FIG. 1. Digestion of LPD-3 and LPD-Glc by chymotrypsin. Lanes: (A) 3 μg of LPD-glc, (B) 3 μg of LPD-3, (C) protein molecular weight standards, (D) digested LPD-Glc, (E) digested LPD-3. The protein standards were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

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E. coli          steiKtqVvVlGAGPaGYsAAfrcAdLGLeTvivErYntl
                   | | | |||| || ||   | ||| |   | |
A. vinelandii    msqKfdViVIGAGPGGYVAAIksAQLGLkTaLiEkYkgke
                   || |||||||||   |||  | | |
LPD-val          mqqtiqttllIIGGGPGGYVAAIRaGQLGipTVLvEgqal
                   |||||||| |||| ||||  ||
LPD-3              mksyDVVIIGGGPGGYnAAIRXGQLGl TVaxvq
                      ||| || ||| | |||   ||
LPD-glc            tqkfDVVvIGaGPGxYvAAIkXaQL
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FIG. 2. Alignment of the N-terminal amino acid sequences of lipoamide dehydrogenases from *E. coli*, *Azotobacter vinelandii*, LPD-Val, LPD-3, and LPD-Glc by GENALIGN. Unidentified amino acids are shown with an x. Uppercase and lowercase letters indicate identical and nonidentical amino acids, respectively.

Glutathione reductase is another redox-active disulfide flavoprotein which has distinct amino acid sequence similarity to *E. coli* lipoamide dehydrogenase (7) and LPD-Val (Burns et al., in press). Purified LPD-3 had no glutathione reductase activity, but glutathione reductase was detected in enzyme extracts of *P. putida* PpG2 at a specific activity of 50 nmol/min per mg of protein. Therefore, LPD-3 is not a glutathione reductase which has been recruited as a lipoamide dehydrogenase.

Functions of LPD-3. Purified LPD-3 was used to complement keto acid dehydrogenase activity of the mutants used in this study in an attempt to identify the function of LPD-3. LPD-3 was as effective as LPD-Glc in restoring the pyruvate dehydrogenase activity of JS350 (Table 3), but there was no clear-cut effect of LPD-3 on the pyruvate dehydrogenase of JS348 and JS351 (Table 3). LPD-3 was also able to restore 2-ketoglutarate dehydrogenase activity to JS348, JS350, and JS351 but was only about 60% as effective as LPD-Glc. This result suggests that LPD-3 is not the natural E3 component of the 2-ketoglutarate dehydrogenase of *P. putida*.

DISCUSSION

The data in this study explain the anomalous behavior of JS348, provide further evidence about the function of LPD-

TABLE 3. Complementation of pyruvate and 2-ketoglutarate dehydrogenases by LPD-Glc and LPD-3[a]

Enzyme and addition	Activity (nmol/min per mg of protein) in *P. putida* strain:			
	PpG2	JS348	JS350	JS351
Pyruvate dehydrogenase	82	42	1	21
+ LPD-Glc	86	38	34	48
+ LPD-3	80	47	35	51
2-Ketoglutarate dehydrogenase	24	0	0	0
+ LPD-Glc	40	268	173	203
+ LPD-3	52	152	121	135

[a] For each assay, there were 100 μg of protein from each strain and 0.42 U of each lipoamide dehydrogenase.

Glc, and identify a third lipoamide dehydrogenase in *P. putida*. Because of the extensive inhibition of pyruvate and 2-ketoglutarate dehydrogenases of wild-type *P. putida* by anti-LPD-Glc (Table 1), it seems likely that LPD-Glc is the E3 component of both of these multienzyme complexes in wild-type *P. putida*. The restoration of pyruvate and 2-ketoglutarate dehydrogenase activities in JS350 by LPD-Glc (Table 3) agrees with this conclusion. In this respect, JS350 behaves like a typical *E. coli lpd* mutant (7). However, the behavior of mutants JS348 and JS351 was unexpected, since they clearly have the *lpdG* mutation yet have nearly normal pyruvate dehydrogenase activity. Also, the lipoamide dehydrogenase isolated from JS348 was LPD-3, not LPD-Glc.

The question of the natural role of LPD-3 in *P. putida* is perplexing. Clearly LPD-3 is the product of a separate structural gene and not a truncated form of LPD-Glc or the result of a missense mutation. From the data in Tables 1 and 3, it appears that LPD-3 acts in place of LPD-Glc in *lpdG* mutants. LPD-3 is apparently preferentially assembled with the pyruvate dehydrogenase in JS348 and JS351, since these mutants have pyruvate but not 2-ketoglutarate dehydrogenase activity. We expect that LPD-3 will prove to be rather closely related to LPD-Glc based on their amino acid compositions and the fact that LPD-3 can replace LPD-Glc to a large extent. Since LPD-3 is not as effective as LPD-Glc in complementing 2-ketoglutarate dehydrogenase activity in JS348 and JS351 (Table 3), LPD-3 is probably not the E3 component of 2-ketoglutarate dehydrogenase. What then is the normal role of LPD-3? LPD-3 is almost certainly a lipoamide dehydrogenase because of the N-terminal sequence (Fig. 2). Since LPD-Glc is the E3 component of 2-ketoglutarate and probably pyruvate dehydrogenases and the L-factor of the glycine oxidase complex (13) and LPD-Val is the E3 component of branched-chain ketoacid dehydrogenase (10, 14; Burns et al., in press), that leaves very few possibilities for LPD-3. One possibility is that LPD-3 is the E3 component of 2-ketoadipate dehydrogenase. This complex has not been studied extensively, but it is used in the oxidation of D-lysine and pipicolic acid by pseudomonads (6).

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