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Mutations Affecting Lipoamide Dehydrogenases of *Pseudomonas putida*

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Pseudomonas putida grown on valine produces two lipoamide dehydrogenases, LPD-glu (M_r, 56,000 and LPD-val (M_r, 49,000). The 49,000-dalton protein is used by *P. putida* for branched-chain keto acid dehydrogenase, whereas the 56,000-dalton protein is presumably used for pyruvate and 2-ketoglutarate dehydrogenases. The objective of this study was to isolate and characterize mutants of *P. putida* with mutations affecting lipoamide dehydrogenases in order to study the relationship of these two proteins. Mutant JS287 lacked LPD-val, the lipoamide dehydrogenase which is induced by growth on valine and is specific for branched-chain keto acid dehydrogenase, and had normal amounts of LPD-glu, the lipoamide dehydrogenase which is formed during growth on glucose and which is probably used by both pyruvate and 2-ketoglutarate dehydrogenases. Mutant JS94 was a pleiotropic mutant with defects in 2-ketoglutarate, branched-chain, and lipoamide dehydrogenases. Proteolysis of LPD-glu and LPD-val produced completely different digestion products, suggesting that these two proteins are products of separate structural genes. Antisera prepared against LPD-glu reacted only with LPD-glu, whereas antisera prepared against LPD-val reacted with LPD-val and cross-reacted with LPD-glu. Although mutant JS94 did not produce active lipoamide dehydrogenase, cell-free extracts of this mutant contained a protein which cross-reacted with anti-LPD-val.

Branched-chain keto dehydrogenase is a common enzyme in the metabolism of valine, leucine, and isoleucine by bacteria (15) and mammals (4). In mammals, there are significant alterations in the metabolism of the branched-chain amino acids in individuals with diabetes (25) and during starvation (4). Branched-chain keto acid dehydrogenase is also of interest as the enzyme which is affected in maple syrup urine disease, a rare genetic disease (2).

Until recently, relatively little was known about the structure of branched-chain keto acid dehydrogenase, but this enzyme was assumed to be a multienzyme complex similar to pyruvate and 2-ketoglutarate dehydrogenases, with a dehydrogenase-decarboxylase subunit (E1 subunit), a transacylase (E2 subunit), and a lipoamide dehydrogenase (E3 subunit) (20). Pettit et al. (18) purified branched-chain keto acid dehydrogenase from bovine kidneys and found that the purified complex consisted of four polypeptides. We have also purified branched-chain keto acid dehydrogenase from *Pseudomonas putida*, where it is induced by growth on branched-chain amino acids (24), although the specific inducers are the branched-chain keto acids (13, 14).

The branched-chain keto acid dehydrogenase of *P. putida* has two unusual features; the activity of the complex is stimulated by L-valine (21), and the E3 subunit functions only with branched-chain keto acid dehydrogenase (23). The latter feature was completely unexpected since *Escherichia coli* produces a single lipoamide dehydrogenase which is shared by pyruvate and 2-ketoglutarate dehydrogenases and whose structural gene, *lpd*, is located contiguous to the structural genes for the E1 and E2 subunits of pyruvate dehydrogenase (5, 6, 17). *P. putida* grown on glucose produces a single lipoamide dehydrogenase (M_r, 56,000), whereas *P. putida* grown on valine produces two lipoamide dehydrogenases (M_r, 56,000 and 49,000) (23). The larger enzyme, which we have designated LPD-glu, functions with 2-ketoglutarate dehydrogenase and probably pyruvate dehydrogenase (22), whereas the smaller enzyme, LPD-val, functions only with branched-chain keto acid dehydrogenase (23). The objective of this study was to isolate and characterize mutants with mutations affecting the lipoamide dehydrogenases of *P. putida* to determine whether these mutants shed any light on the relationship of LPD-glu to LPD-val.

MATERIALS AND METHODS

Organisms and growth conditions. *P. putida* strain PpG2 was originally obtained from I. C. Gunsalus and was grown as described previously (13) by using the synthetic medium of Jacobson et al. (10) supplemented with 0.3% L-valine and 0.05% L-isoleucine. Keto acid dehydrogenase mutants were grown in GAS medium, which is the basal medium of Jacobson et al. (10) supplemented with 10mM D-glucose as the carbon and energy source and 0.2 mM sodium acetate and 0.2 mM sodium succinate as growth supplements for pyruvate and 2-ketoglutarate dehydrogenase mutants, respectively (6). To induce branched-chain keto acid dehydrogenase in mutants which were unable to use branched-chain amino acids as sole carbon sources, 25 mM L-valine was added to GAS medium (GASV medium).

Enzyme assays. Pyruvate, 2-ketoglutarate, and branched-chain keto acid dehydrogenases were assayed as described previously (24). The assay mixture for lipoamide dehydrogenase contained 150 mM potassium phosphate buffer (pH 7.0), 0.3 mM NAD, 0.1 mM NADH, and 3.0 mM lipoamide in a volume of 1.0 ml. The reaction was started with enzyme. Lipoamide was prepared as a 0.05 M solution in ethanol or acetone when crude extracts were assayed (23). The specific activities of these enzymes are expressed as micromoles of NAD reduced per minute per milligram of protein or micromoles of NADH oxidized per minute per milligram of protein.

Proteolysis of lipoamide dehydrogenases. The method of Cleveland et al. (1) was used to digest lipoamide dehydrogenases to demonstrate the degree of relatedness. The digestion products were separated by electrophoresis in vertical slab gels (thickness, 2 mm) by the method of Laemmli and Favre (11), except that the gels were composed of a 10 to 25% gradient of polyacrylamide. The yeast and pig heart lipoamide dehydrogenases used as controls were obtained from Sigma Chemical Co.

Isolation of mutants. A 1.2-ml portion of an overnight culture of strain PpG2 grown in L-broth (12) was used to inoculate 24 ml of fresh L-broth in a sidearm flask. Growth was allowed to continue until the absorbance at 660 mm reached 0.6 to 0.8 (2 to 3 h). Then 4.0 ml of the fresh culture was centrifuged, and the cells were suspended in 4.0 ml of 0.9% sterile saline. This suspension was centrifuged and suspended in 4.0 ml of nitrosoguanidine (100 μg/ml) in 0.05 M citrate buffer (pH 6.0). The suspension was shaken for 30 min, which resulted in about a 2-log kill. The cell suspension was then centrifuged and suspended in 2.0 ml of the Basal G solution of Jacobson et al. (10). Tubes containing L-broth (4.0 ml) were inoculated with 0.2 ml of the suspended cells and incubated overnight.

Two procedures were used to isolate mutants. In the first procedure, penicillin selection was used to enrich the mutants; this method was based on the methods of Guest and Creaghan (7) and Martin et al. (14). An overnight culture in L-broth was centrifuged and suspended in 4.0 ml of 0.9% sterile saline, and 0.1 ml was used to inoculate 5.0 ml of synthetic medium supplemented with 10 mM glucose as the energy source. The culture was incubated with shanking for 3 h, and then 30 mg of potassium benzylpenicillin per ml was added. The suspension was incubated for an additional 3 h, washed, and suspended in 5.0 ml of physiological saline; 10^{-1} and 10^{-2} dilutions were plated onto GAS agar. Replica plates were made onto glucose agar, and colonies which grew on GAS agar but not on glucose agar were picked. Mutant JS94 was isolated by this method.

The second procedure was based on the method of Ornston (16). This procedure did not utilize penicillin enrichment and was used to isolate mutant JS287. In this case, an overnight culture of strain PpG2 was plated onto a medium which contained 25 mM L-valine and 1 mM glucose as the energy sources. Mutants that were unable to use L-valine formed tiny colonies, which were picked. This procedure was used to isolate branched-chain keto acid dehydrogenase mutants by selecting for strains which grew on isobutyrate, isovalerate, and 2-methylbutyrate but not on valine, leucine, and isoleucine. The tiny colonies were picked, transferred to L-agar plates, and replica-plated onto synthetic medium supplemented with 10 mM valine or isobutyrate as the energy source. Mutants which grew on isobutyrate agar but not on valine agar were kept.

Immunological methods. The immunizing antigens used were purified preparations of LPD-glu and LPD-val. Each antigen was mixed with incomplete Freund adjuvant to a final protein concentration of 100 μg/ml. The mixture was chilled and homogenized by using a Sorvall Omnimixer at full speed for 1.5 min. Preimmune sera from the two young male New Zealand white rabbits used for immunization did not contain antibodies that reacted either by inhibition of enzyme activity or by precipitation. Initially each rabbit received a 0.5-ml injection in each footpad and 0.5 ml subcutaneously on each side of the neck. Then 16 days after the first injection, both rabbits received a second set of 0.5-ml injections intramuscularly in both thighs and 0.5 ml at each of four subcutaneous sites along the back. The rabbits were bled by cardiac puncture at 2, 4, and 5 weeks after the first injections. Antisera were collected by spinning clot-free blood in a clinical centrifuge and pipetting off the clear serum.

The gel for the double-diffusion plates was prepared by heating 0.9% agarose in 5 mM Tris-hydrochloride (pH 7.5)–0.9% sodium chloride–0.9% sodium azide. The agarose solution was poured onto Gel Bond film (0.2 mm; Marine Colloids) at a concentration of 0.25 ml/cm^2. The wells were 4 mm in diameter and held a total of 20 μl. There was 0.5 μg of purified lipoamide dehydrogenase, 400 μg of a 90,000-×-*g* supernatant, or 20 μl of a 1:2 dilution of antiserum in each well. The plates were incubated for 24 h in a moist chamber and then washed with two changes of 0.9% sodium chloride and one wash of deionized water. The container was gently rotated, and the solutions were changed every 24 h. The film was dried at 37°C, stained with Coomassie blue for 20 min, and destained with 5% methanol–7.5% acetic acid.

Enzyme inhibition studies were performed as follows. A 1-μg portion of LPD-val or 3.8 μg of LPD-glu was incubated with 200 μl of buffer (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.5 mM dithiothreitol) or 200 μl of a 1:16 dilution of either anti-LPD-val or anti-LPD-glu. After 30 min exactly one-half of each incubation mixture was added to the reagents for the lipoamide dehydrogenase assay described above.

RESULTS

Nutritional characteristics of mutants. In *E. coli*, *lpd* mutations affect both pyruvate and 2-ketoglutarate dehydrogenases, and such mutants require both acetate and succinate for growth (6). Mutant JS94 was isolated by a procedure which selected for mutants that required acetate or succinate or both for growth (see above). Mutant JS94 grew well in GAS medium and was able to grow with succinate but not with acetate as the sole carbon source, indicating that the tricarboxylic acid cycle is incomplete in this mutant (Table 1). This mutant did not have an absolute requirement for either acetate or succinate, but the lag phase of growth on glucose was shortened by addition of succinate to the medium. Mutant JS94 did not grow in media supplemented with any of the branched-chain amino acids or branched-chain fatty acids as the sole carbon source.

Mutant JS287 was isolated by a procedure which selected for strains which grew with branched-chain fatty acids but not with branched-chain amino acids as the carbon source, as described above. Only three enzymes are involved in the catabolism of branched-chain L-amino acids to branched-chain fatty acids (15); these are branched-chain amino acid transaminase, glutamate dehydrogenase, and branched-chain keto acid dehydrogenase. Since branched-chain keto acid dehydrogenase is a complex with at least three polypeptides, mutants with this phenotype could have a lesion in any of one of these five proteins. A total of 15 mutants were isolated by this procedure; 6 of these were identified as branched-chain keto acid dehydrogenase mutants and will be described elsewhere, 1 appeared to be a branched-chain amino acid transaminase mutant, and 7 did not appear to be either of these types but have not been identified yet. The remaining mutant, mutant JS287 was identified as a mutant that was not able to produce LPD-val.

TABLE 1. Ability of strains used in this study to grow in synthetic media supplemented with single carbon sources

Carbon source	Growth of strain:		
	PpG2	JS94	JS287
Valine	+	−	−
Leucine	+	−	−
Isoleucine	+	−	−
Isobutyrate	+	−	+
Isovalerate	+	−	+
2-Methylbutyrate	+	−	+
Acetate	+	−	+
Succinate	+	+	+

TABLE 2. Dehydrogenase activities of *P. putida* strains grown in GASV medium

Enzyme	Sp act in strain:		
	PpG2	JS94	JS287
Pyruvate dehydrogenase	0.113	0.039	0.152
2-Ketoglutarate dehydrogenase	0.064	0.000	0.112
Branched-chain keto acid dehydrogenase			
−LPD-val	0.052	0.000	0.006
+LPD-val	0.101	0.016	0.246
Lipoamide dehydrogenase	0.565	0.090	0.406

Keto acid dehydrogenase content of mutants. Since none of the mutants grew in synthetic media supplemented with branched-chain amino acids as the sole carbon source, it was necessary to devise a medium that did cause the induction of pyruvate, 2-ketoglutarate, and branched-chain keto acid dehydrogenases. GASV medium (see above) served this purpose. Glucose was the energy source, and acetate and succinate were supplied to satisfy the nutritional requirements of pyruvate and 2-ketoglutarate dehydrogenase mutants, respectively (6). Mutants lacking branched-chain keto acid dehydrogenase but possessing branched-chain amino acid transaminase should oxidize L-valine to 2-ketoisovalerate, which is the inducer of branched-chain keto acid dehydrogenase (14).

To identify mutants lacking LPD-val, all strains were assayed for branched-chain keto acid dehydrogenase with and without purified LPD-val (Table 2). The activity of mutant JS287 was stimulated markedly by purified LPD-val, which suggested that this organism lacked LPD-val but had normal E1 and E2 subunits (Table 2). Mutant JS94 was pleiotropic mutant which completely lacked 2-ketoglutarate dehydrogenase and had significantly less branched-chain keto acid dehydrogenase and lipoamide dehydrogenase than strain PpG2 (Table 2). Addition of purified LPD-val to cell-free extracts of mutant JS94 increased the level of branched-chain keto acid dehydrogenase to approximately 15% of the wild-type level measured under the same conditions. The content of pyruvate dehydrogenase in mutant JS94 also appeared to be lowered.

Mutant JS287 lacks LPD-val. The data shown in Table 2 suggested that mutant JS287 lacked LPD-val and that mutant JS94 lacked both LPD-glu and LPD-val. Because of the importance of a mutant which lacked LPD-val, we isolated lipoamide dehydrogenase from these two mutants to verify the nature of the lipoamide dehydrogenase produced. Each preparation started with 10 g (wet weight) of cells grown in GASV medium.

TABLE 3. Lipoamide dehydrogenases recovered from cell-free extracts of strains PpG2, JS94, and JS287 grown on GASV medium

Strain	Amt of lipoamide dehydrogenase recovered (U)	
	LPD-glu	LPD-val
PpG2	107	97
JS94	0	0
JS287	121	0

LPD-glu and LPD-val were completely separated on the DEAE-Sepharose column used to purify these two proteins (23), and the amount of enzyme in each pool was taken as an indication of the amount of lipoamide dehydrogenase produced. Strain PpG2 yielded both LPD-glu and LPD-val (Table 3). Cell-free extracts of mutant JS94 taken to the same stage yielded neither LPD-glu nor LPD-val; mutant JS287 yielded 121 U of LPD-glu and no LPD-val. To confirm the identification of the lipoamide dehydrogenase produced by mutant JS287, purification was completed. LPD-glu and LPD-val prepared from strain PpG2 grown in GASV medium were single polypeptides with molecular weights of 56,000 and 49,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). Lipoamide dehydrogenase from mutant JS287 eluted from Affi-Gel Blue in the same position as LPD-glu (23) and consisted of one protein with a molecular weight of 56,000 (Fig. 1).

To confirm the identity of the lipoamide dehydrogenase of mutant JS287 as LPD-glu, lipoamide dehydrogenase purified from mutant JS287 was used to supplement preparations of 2-ketoglutarate dehydrogenase and branched-chain keto acid dehydrogenase deficient in the E3 subunit prepared from *P. putida* (25). 2-Ketoglutarate dehydrogenase responds only to LPD-glu, and branched-chain keto acid dehydrogenase responds only to LPD-val. Table 4 shows that lipoamide dehydrogenase from mutant JS287 stimulated only 2-ketoglutarate dehydrogenase, which further identified the enzyme as LPD-glu.

Relationship of LPD-glu to LPD-val. LPD-glu and LPD-val could be the products of two separate structural genes, or LPD-val could be produced from LPD-glu by proteolysis. To distinguish between these two possibilities, both proteins were digested with trypsin, and the resulting peptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1). If LPD-val was a product of LPD-glu proteolysis, then the two enzymes should have yielded a number of identical polypeptides after digestion. Lipoamide dehydrogenases from

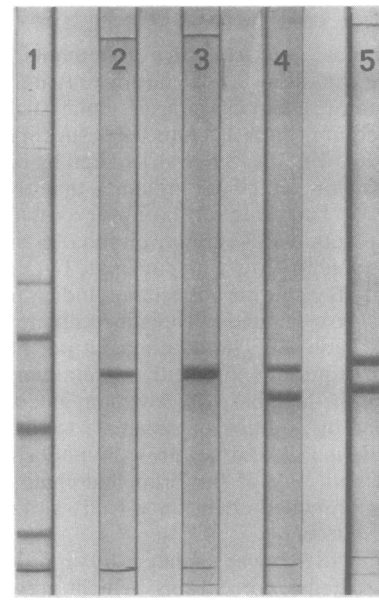

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lipoamide dehydrogenase from mutant JS287. Tube 1, protein standards (phosphorylase *b* [molecular weight, 94,000], bovine serum albumin [67,000], ovalbumin [43,000], carbonic anhydrase [30,000], soybean trypsin inhibitor [20,100]); tube 2, lipoamide dehydrogenase from mutant JS287; tube 3, lipoamide dehydrogenase from mutant JS287 plus purified LPD-glu; tube 4, lipoamide dehydrogenase from mutant JS287 plus purified LPD-val; tube 5, purified LPD-glu plus LPD-val. The gels contained 3 μg of each lipoamide dehydrogenase.

TABLE 4. Stimulation of 2-ketoglutarate dehydrogenase by lipoamide dehydrogenase from mutant JS287[a]

Assay	Addition	Change in optical density (U/min)
2-Ketoglutarate dehydrogenase	None	0.015
	LPD-glu	0.104
	LPD-val	0.015
	LPD-JS287[b]	0.078
Branched-chain keto acid dehydrogenase	None	0.005
	LPD-glu	0.001
	LPD-val	0.068
	LPD-JS287	0.003

[a] A 6.5-μg portion of 2-ketoglutarate dehydrogenase (specific activity, 2.7) (25), 8.0 μg of branched-chain keto acid dehydrogenase (specific activity, 9.02) (26), and 0.4 U of each lipoamide dehydrogenase were added to the reaction mixtures.

[b] LPD-JS287, Lipoamide dehydrogenase from JS287.

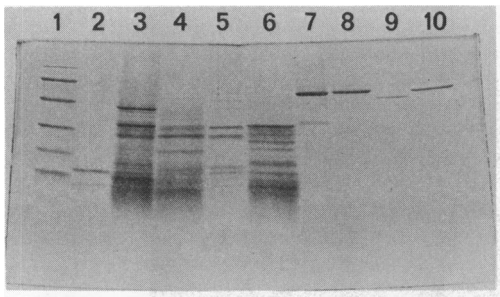

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chymotrypsin digests of lipoamide dehydrogenases. Lane 1 contained the protein standards described in the legend to Fig. 1 plus α-lactalbumin (M_r, 14,000), which is not resolved by the gel system in Fig. 1. Lane 2, 4 μg of chymotrypsin; lane 3, digest of lipoamide dehydrogenase from yeast; lane 4, digest of lipoamide dehydrogenase from pig heart; lane 5, digest of LPD-val; lane 6, digest of LPD-glu. The remaining lanes contained undigested samples of lipoamide dehydrogenases from yeast (lane 7) and pig heart (lane 8), LPD-val (lane 9), and LPD-glu (lane 10). Each digestion mixture contained 40 μg of lipoamide dehydrogenase plus 4 μg of chymotrypsin. The samples were digested for 30 min, and the entire contests were applied to the gel. Lanes 7 through 10 contained 2 μg of each undigested lipoamide dehydrogenase.

yeast and pig heart were included for comparison. Figure 2 shows that few, if any, of the peptides produced from LPD-glu and LPD-val by chymotrypsin matched; therefore, it appears that these two proteins are the products of separate structural genes. Similar results were obtained by proteolysis with papain and trypsin.

We also prepared antisera against LPD-glu and LPD-val and tested these sera for inhibition of lipoamide dehydrogenase. LPD-val was inhibited only by its specific antiserum; however,

TABLE 5. Inhibition of LPD-glu and LPD-val by immune sera[a]

Prepn	Change in optical density (U/min)	% Of control
LPD-val	0.180	100
LPD-val + anti-LPD-glu	0.177	98
LPD-val + anti-LPD-val	0.032	18
LPD-glu	0.121	100
LPD-glu + anti-LPD-glu	0.034	25
LPD-glu + anti-LPD-val	0.079	65

[a] There was no inhibition of lipoamide dehydrogenase by preimmune serum from either rabbit.

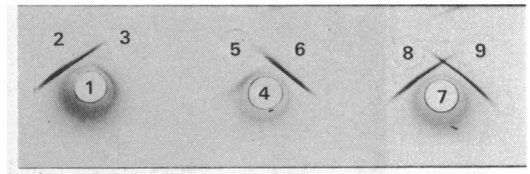

FIG. 3. Reaction of purified lipoamide dehydrogenase with anti-LPD-glu and anti-LPD-val. Well 1, anti-LPD-glu; well 4, anti-LPD-val; well 7, anti-LPD-glu plus anti-LPD-val; wells 2, 5, and 8, LPD-glu; wells 3, 6, and 9, LPD-val. Precipitates were stained with Coomassie blue.

LPD-glu was inhibited by both antisera (Table 5). The inhibition of LPD-glu by anti-LPD-val was not as strong as the inhibition by the homologous antiserum. These findings were corroborated by double-diffusion studies of antigen-antibody reactions (Fig. 3). Antisera against LPD-glu produced a precipitate only with LPD-glu (Fig. 3, wells 1 through 3), whereas antisera against LPD-val produced a precipitate with both LPD-val and LPD-glu (wells 4 through 6); however, the precipitate with LPD-val was more intense. When the center well contained antibodies against both antigens, spurs of nonidentity were formed (Fig. 3, wells 7 through 9). In a separate experiment, purified lipoamide dehydrogenase from mutant JS287 reacted strongly with anti-LPD-glu (Fig. 4, well 3), but in this case there was no detectable cross-reaction with anti-LPD-val (well 7) by either LPD-glu or lipoamide dehydrogenase from JS287.

Antisera were used to probe cell-free extracts of mutants JS94 and JS287 to search for cross-reacting proteins (Fig. 5). Sonic extracts were prepared from strains PpG2, JS94, and JS287 grown in GASV medium and clarified by centrifugation at 90,000 × g, as described previously (23). The supernatant from strain PpG2 reacted with both anti-LPD-glu and anti-LPD-val (Fig.

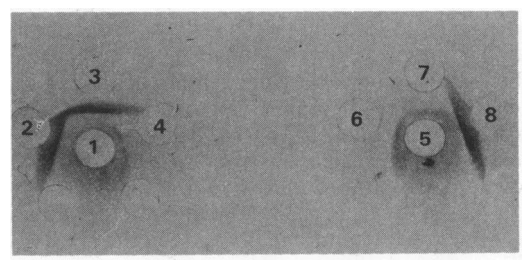

FIG. 4. Reaction of purified lipoamide dehydrogenase from mutant JS287 (wells 3 and 7) with anti-LPD-glu (well 1) and anti-LPD-Val (well 5). Purified LPD-glu was placed in wells 2 and 6, and purified LPD-val was placed in wells 4 and 8. Precipitates were stained with Coomassie blue.

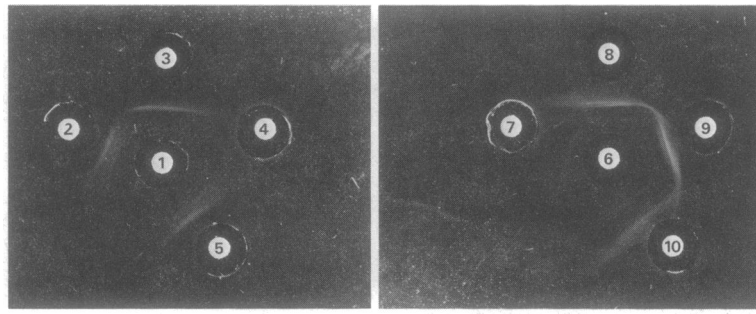

FIG. 5. Reaction of 90,000-×-*g* supernatant fractions from strains PpG2, JS94, and JS287 grown on GASV medium. Well 1 contained anti-LPD-glu, and well 6 contained anti-LPD-val. Wells 2 and 7 contained supernatant from mutant JS287. Wells 3 and 8 contained purified LPD-glu. Wells 4 and 9 contained supernatant from mutant JS94. Wells 5 and 10 contained supernatants from strain PpG2. Precipitates were not stained.

5, wells 5 and 10). Reaction of anti-LPD-glu antisera with supernatant from strain PpG2 produced at least two precipitin bands, which could be explained by reactions with free and complex-bound lipoamide dehydrogenase. The supernatant from mutant JS94 reacted with anti-LPD-val (Fig. 5, well 9) but not with anti-LPD-glu (well 4). Since mutant JS94 contains virtually no lipoamide dehydrogenase, the cross-reacting protein is probably the result of a nonsense or missense mutation in the structural gene for LPD-val. There was the suggestion of a faint precipitin band when mutant JS94 was tested against anti-LPD-glu, but we are not yet certain that there is a product of the LPD-glu structural gene in such extract. Cell-free extracts of mutant JS287 precipitated only with anti-LPD-glu (Fig. 5, well 2), and it appeared that there was no polypeptide formed by the LPD-val structural gene in these extracts (well 7).

DISCUSSION

The most interesting and significant result of this study was the isolation of mutant JS287, which lacks LPD-val but produces normal amounts of LPD-glu. At the very least, the existence of this mutant provides strong additional support for the data showing that the branched-chain keto acid dehydrogenase of *P. putida* utilizes a unique lipoamide dehydrogenase. This mutant also grows normally on acetate, which means that the tricarboxylic acid cycle is intact and, therefore, the lack of LPD-val has no effect on 2-ketoglutarate dehydrogenase. However, more important are the possibilities which this mutant provides for studying the biochemical genetics of *P. putida* branched-chain keto acid dehydrogenase. From the partial proteolysis study (Fig. 2), it appears that LPD-glu and LPD-val are products of separate structural genes rather than products of a single *lpd* gene with posttranslational modification of LPD-glu to LPD-val. It will be informative to determine where the LPD-glu and LPD-val structural genes are located in relation to pyruvate and 2-ketoglutarate dehydrogenases and to each other.

The properties of mutant JS94 are something of an enigma since so many proteins are affected. It is interesting that this mutant produces a protein which cross-reacts with LPD-val. Presumably this is a nonsense or missense mutation which affects enzyme activity but does not affect antigenicity. Mutant JS94 does not grow with acetate as the sole carbon source; therefore, it lacks the tricarboxylic acid cycle and resembles *sucA* and *sucB* mutants of *E. coli*, which have mutations in the structural genes for E1 and E2 subunits of 2-ketoglutarate dehydrogenase (6, 8). Mutant JS94 resembles a 2-ketoglutarate dehydrogenase mutant of *Rhizobium meliloti* which also grows well on succinate but not on acetate (3). Mutant JS94 differs from *sucA* and *sucB* mutants of *E. coli* since it grows well on succinate, whereas *E. coli* mutants grow poorly on succinate (6), presumably because succinate cannot be metabolized by the tricarboxylic acid cycle. If mutant JS94 is a polar mutant, its phenotype suggests that *sucA* and *sucB*, the structural genes for LPD-glu, LPD-val, and the E1 (*bkdA*) and E2 (*bkdB*) subunits of branched-chain keto acid dehydrogenase, are close together.

There is one curious feature of the mutants isolated by the procedure used to isolate mutant JS94, which was a procedure for selecting mutants requiring acetate or succinate or both for growth. A certain number of pyruvate dehydrogenase mutants were expected to result from this procedure, yet we failed to detect any such mutants. Perhaps pyruvate dehydrogenase mutants of *P. putida* do not require acetate for

growth and other selection procedures should be used. In this connection, it should be noted that acetate and succinate are not nutritional requirements for mutant JS94; this is different from the situation in *E. coli*, where *lpd* mutants require both of these supplements for growth with glucose (6). In mutant JS94, these supplements shorten the lag period of growth, which makes it possible to detect mutants on plates after 24 h but not after 2 or 3 days. It is possible that there are additional routes for acetate and succinate production in *P. putida* that are not available to *E. coli*.

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