Biochem. J. (1986) 233, 737–742 (Printed in Great Britain)

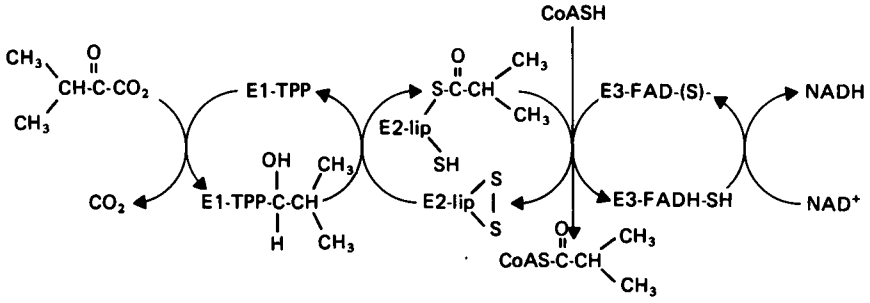
Resolution of branched-chain oxo acid dehydrogenase complex of *Pseudomonas aeruginosa* PAO

Vicki McCULLY, Gayle BURNS and John R. SOKATCH
Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190, U.S.A.

Branched-chain oxo acid dehydrogenase was purified from *Pseudomonas aeruginosa* strain PAO with the objective of resolving the complex into its subunits. The purified complex consisted of four proteins, of M_r 36000, 42000, 49000 and 50000. The complex was resolved by heat treatment into the 49000 and 50000-M_r proteins, which were separated by chromatography on DEAE-Sepharose. The 49000-M_r protein was identified as the E2 subunit by its ability to catalyse transacylation with a variety of substrates, with dihydrolipoamide as the acceptor. *P. aeruginosa*, like *P. putida*, produces two lipoamide dehydrogenases. One, the 50000-M_r protein, was identified as the specific E3 subunit of branched-chain oxo acid dehydrogenase and had many properties in common with the lipoamide dehydrogenase LPD-val of *P. putida*. The second lipoamide dehydrogenase had M_r 54000 and corresponded to the lipoamide dehydrogenase LPD-glc of *P. putida*. Fragments of *C*-terminal CNBr peptides of LPD-val from *P. putida* and *P. aeruginosa* corresponded closely, with only two amino acid differences over 31 amino acids. A corresponding fragment at the *C*-terminal end of lipoamide dehydrogenase from *Escherichia coli* also showed extensive homology. All three peptides had a common segment of eight amino acids, with the sequence TIHAHPTL. This homology was not evident in any other flavoproteins in the Dayhoff data base which suggests that this sequence might be characteristic of lipoamide dehydrogenase.

INTRODUCTION

Branched-chain oxo acid dehydrogenase of *Pseudomonas putida* PpG2 is an inducible enzyme formed during growth on branched-chain oxo acids (Marshall & Sokatch, 1972). The enzyme is part of a common pathway for the catabolism of valine, leucine and isoleucine (Martin *et al.*, 1973). The subunits of branched-chain oxo acid dehydrogenase correspond to those of other oxo acid dehydrogenases (Reed, 1974), and are El, the dehydrogenase, E2, the transacylase, and E3, lipoamide dehydrogenase (Fig. 1). Thiamin pyrophosphate is the cofactor for E1, and this subunit is responsible for the oxidative decarboxylation of oxo acid substrates. Lipoic acid is the cofactor for the E2 subunit, which is reductively acylated in a reaction catalysed by E1. E2 catalyses transacylation of the branched-chain fatty acyl group to CoA. E2 also catalyses transacylation between itself and dihydrolipoamide, which is the basis of the E2 assay used in the present paper and by Chuang *et al.* (1984). E3 is lipoamide dehydrogenase, which catalyses the oxidation of reduced lipoic acid bound to E2 or of free dihydrolipoamide. The complex purified from *P. putida* PpG2 was found to consist of four peptides, of M_r 37000, 39000, 46000 and 49000 (Sokatch *et al.*, 1981*b*). The 49000-M_r peptide was identified as lipoamide dehydrogenase. Branched-chain oxo acid dehydrogenases purified from mammalian tissues also consisted of four peptides, including lipoamide dehydrogenase (Pettit *et al.*, 1978; Odessey, 1982; Paxton & Harris, 1982; Heffelfinger *et al.*, 1983; Cook *et al.*, 1985). Mammalian branched-chain oxo acid dehydrogenases are regulated by phosphoryla-



Net reaction: 2-Oxoisovalerate + NAD^+ + CoASH → isobutyryl-CoA + CO_2 + NADH

Fig. 1. Reactions catalysed by the subunits of branched-chain oxo acid dehydrogenase

Abbreviations: TPP, thiamin pyrophosphate; lip, lipoic acid.

tion of the E1 subunit (Fantania *et al.*, 1981; Odessey, 1982; Patel & Olson, 1982; Paxton & Harris, 1982), but there is no evidence for phosphorylation of the complex from *P. putida* PpG2. Rather, the activity of the complex from *P. putida* is stimulated by L-valine, which increases the affinity of the complex for oxo acid substrates (Sokatch *et al.*, 1981*b*). In addition, the complex from *P. putida* required a specific lipoamide dehydrogenase, LPD-val, the 49000-M_r subunit, which was formed only during growth on branched-chain amino acids. A second lipoamide dehydrogenase LPD-glc, of M_r 56000, the only lipoamide dehydrogenase produced during growth in glucose minimal medium, was specific for 2-oxoglutarate dehydrogenase (Sokatch *et al.*, 1981*a*). LPD-glc and LPD-val were subsequently shown to be products of separate structural genes, and appear to be only distantly related (Sokatch *et al.*, 1983; Delaney *et al.*, 1984). This appears to be the only published report of structurally and functionally separate lipoamide dehydrogenases in the same organism (Sokatch *et al.*, 1983). Although we were able to purify LPD-val from *P. putida*, we had limited success in separating the other components of the complex. The objective of the present research was to attempt to resolve the subunits of branched-chain oxo acid dehydrogenase from *Pseudomonas aeruginosa*, a species closely related to *P. putida*.

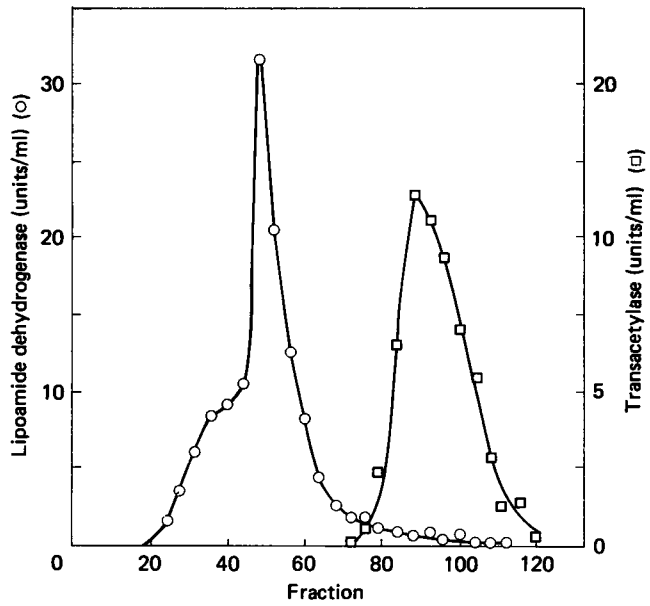


Fig. 2. Separation of E2 and E3 by column chromatography on DEAE-Sepharose

For full details, see the text.

EXPERIMENTAL

Organisms and growth conditions

P. aeruginosa strain PAO was obtained from the American Type Culture Collection, strain A.T.C.C. 15692, and was grown as described in Sokatch *et al.* (1983).

Enzymic methods

Branched-chain oxo acid dehydrogenase was assayed and purified essentially as described in Sokatch *et al.* (1981*b*). Lipoamide dehydrogenase was assayed as described in Sokatch *et al.* (1983).

The E1 assay was performed as described in Sykes *et al.* (1985), except that 1 mM-2-oxo[1-^{14}C]isovalerate (sp. radioactivity 130000 c.p.m./μmol) was the substrate. 2-Oxo[1-^{14}C]isovalerate was prepared from L-[1-^{14}C]valine (New England Nuclear) by the method of Meister (1957). A unit is 1 μmol of CO_2 evolved/15 min.

The procedure for the E2 assay was also described in Sykes *et al.* (1985), except that the substrate was 1 mM-isobutyryl-CoA; this was prepared as described by Stadtman (1957). The concentration of isobutyryl-CoA was determined by the hydroxamic acid assay described in the same reference, with redistilled isobutyric anhydride as the standard.

The E2 and E3 subunits were resolved from the complex by heat treatment. The purified complex was treated at 65 °C for 5 min, which completely inactivated branched-chain oxo acid dehydrogenase complex activity, but left over 90% of E2 and E3 activities. The heat-treated sample was then concentrated to 0.4 ml by using a Millipore CX-10 cartridge, and dialysed against 1 litre of 50 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-L-valine, 1 mM-EDTA, 0.5 mM-dithiothreitol and 0.5 mM-thiamin pyrophosphate (buffer A). The dialysed sample was applied to a column of Sephacryl S-400 (1.6 cm × 86 cm) and eluted with buffer A at a flow rate of 0.4 ml/min. Fractions (1 ml) were collected, and subunits E2 and E3 were found in a single peak between fractions 81 and 100, which were pooled. Electrophoresis in SDS/7.5%-polyacrylamide gels revealed the presence of two proteins, of M_r 49000 and 50000 (Laemmli & Favre, 1973). The pool from the Sephacryl column was then applied to a column of DEAE-Sepharose (1.5 cm × 6 cm), which had been equilibrated with buffer A. Protein was eluted with 40 ml of buffer A in the receiving flask and 40 ml of buffer A containing 350 mM-NaCl in the reservoir flask. Lipoamide dehydrogenase was eluted between fractions (1 ml) 32 and 64, and E2 was eluted between fractions 80 and 108 (Fig. 2).

The *C*-terminal peptide of enzyme LPD-val was prepared by CNBr cleavage. Samples were reduced and carboxymethylated (Crestfield *et al.*, 1963) and then digested with CNBr as described by Steers *et al.* (1965), except that 6 M-guanidine hydrochloride was used instead of 8 M-urea and the reaction was incubated for 1 h instead of 15 min. The digest was freeze-dried and then dissolved in 1 ml of 0.1 M-NH_4HCO_3, pH 8.5. The dissolved digest was applied to a column of Sephadex G-75 (superfine grade; 0.9 cm × 100 cm), equilibrated with 0.1 M-NH_4HCO_3, pH 8.5. The pooled fractions were freeze-dried and then redissolved in 0.11% (v/v) trifluoroacetic acid in water (solvent A). The h.p.l.c. system consisted of two Waters M-45 pumps, a U6K injector, model 441 absorbance detector set at 214 nm and Waters Automated Gradient Controller, The column was Waters μBondapak C_{18}, 3.5 mm × 300 mm. After sample injection, the column was washed for 10 min with solvent A, and then elution of peptides was accomplished with a linear gradient over 50 min ending with 100% solvent B, 10% trifluoroacetic acid in 50% acetonitrile. The peaks were pooled and analysed for amino acid content and sequenced by the Protein Studies Research Program at the Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.

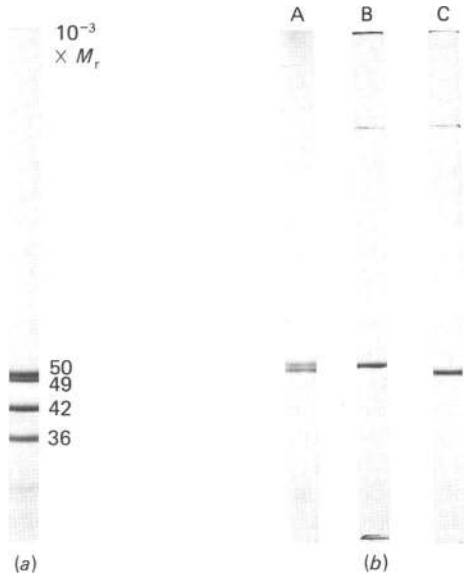


Fig. 3. Electrophoresis of (*a*) 13 μg of purified branched-chain oxo acid dehydrogenase of *P. aeruginosa* PAO and (*b*) purified E2 and E3 subunits of branched-chain oxo acid dehydrogenase, on SDS/7.5%-polyacrylamide gels

Lanes in (*b*): A, pool from Sephacryl S-400 column (4 μg of protein); B, pool of E3 activity isolated from DEAE-Sepharose column (2 μg of protein); C, pool of E2 activity isolated from DEAE-Sepharose column (2 μg of protein).

RESULTS

Properties of branched-chain oxo acid dehydrogenase from *P. aeruginosa* PAO

Specific activities of branched-chain oxo acid dehydrogenase preparations from *P. aeruginosa* PAO were 8–10 μmol of NAD^+ reduced/min per mg of protein, which is comparable with specific activities obtained with the purified complex from *P. putida* (Sokatch *et al.*, 1981*b*), bovine kidney (Pettit *et al.*, 1978), bovine liver (Heffelfinger *et al.*, 1983), rat kidney (Odessey, 1982) and rabbit liver (Paxton & Harris, 1982). Electrophoresis of the purified complex from *P. aeruginosa* PAO in denaturing gels revealed four polypeptides, of M_r 36000, 42000, 49000 and 50000 (Fig. 3) and a minor contaminant, of M_r 33000. For comparison, the subunit M_r values of branched-chain oxo acid dehydrogenase from bovine kidney are 35000, 46000, 52000 and 56000 (Pettit *et al.*, 1978; Heffelfinger *et al.*, 1983).

Branched-chain oxo acid dehydrogenase from *P. aeruginosa* PAO was completely dependent on CoA and NAD^+. Omission of thiamin pyrophosphate or $MgCl_2$ reduced the enzyme's activity by about 40%. *P. aeruginosa* PAO, like *P. putida*, produced two lipoamide dehydrogenases, LPD-glc and LPD-val. LPD-val from *P. aeruginosa* PAO was specifically required as the E3 subunit of the branched-chain oxo acid dehydrogenase complex. Somewhat surprisingly, LPD-val from *P. putida* had almost no ability to restore the activity of the complex from *P. aeruginosa* PAO. LPD-glc from either *P. putida* or *P. aeruginosa* PAO did not restore the activity of the complex. In the presence of L-valine, the K_m for 2-oxoisovalerate of the complex from *P. aeruginosa* was decreased from 0.304 to 0.034 mM, with no effect on $V_{max.}$. L-Valine had no effect on K_m values for NAD^+ or CoA. These results provide additional support for the concept that L-valine increases the affinity of the E1 subunit for the oxo acid substrates. The branched-chain oxo acids, 2-oxoisovalerate, 4-methyl-2-oxopentanoate ('2-oxoisocaproate') and 3-methyl-2-oxovalerate, as well as the straight-chain oxo acids, 2-oxobutyrate and 2-oxovalerate, acted as substrates in the standard assay described in the Experimental section. Pyruvate was slowly oxidized and 2-oxoglutarate was not oxidized, indicating lack of contamination with pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase.

E1 assay

The commonly used ferricyanide assay (Hager & Kornberg, 1961) did not work with branched-chain oxo acid dehydrogenase from either *P. putida* or *P. aeruginosa* PAO, so therefore the assay for E1 subunits of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase described by Sykes *et al.* (1985) was adapted to this purpose. The release of $^{14}CO_2$ from 2-oxo[1-^{14}C]isovalerate was proportional to the amount of enzyme up to 0.3 unit of branched-chain oxo acid dehydrogenase, and neither CoA nor NAD^+ was required. The E1 assay required $MgCl_2$, thiamin pyrophosphate and L-valine. L-Valine had the same effect in the E1 assay as was observed with the assay for the entire complex. The K_m for 2-oxoisovalerate in the absence of L-valine was decreased from 2.01 mM to 0.216 mM in the presence of L-valine, with no significant change in $V_{max.}$.

The substrate specificity of E1 was studied by addition of unlabelled oxo acids to the E1 assay in order to look for competition with 2-oxo[1-^{14}C]isovalerate for the binding site on E1 (Table 1). The unlabelled substrate was added at the same concentration as 2-oxo[^{14}C]isovalerate. In general, inhibition of CO_2 evolution paralleled the substrate specificity of the complete reaction. The one exception was pyruvate, which competed well with 2-oxoisovalerate for binding to E1 (Table 1), but was a poor substrate when the activity of the complex was measured.

Resolution of the complex

The complex was resolved by heat treatment, which destroyed E1, but released E2 and E3. The heat-treated

Table 1. Substrate specificity of E1 subunit of branched-chain oxo acid dehydrogenase from *P. aeruginosa* PAO

The concentration of unlabelled substrate in the E1 assay was 1 mM, and that of 2-oxo[1-^{14}C]isovalerate was 1 mM (sp. radioactivity 123000 c.p.m./μmol). The other reagents were as described in the Experimental section with 13 μg of enzyme.

Unlabelled substrate added to E1 assay	Inhibition (%)
4-Methyl-2-oxopentanoate	97
3-Methyl-2-oxovalerate	56
Pyruvate	46
2-Oxoglutarate	4
2-Oxobutyrate	96
2-Oxovalerate	99

complex was chromatographed over Sephacryl S-400, yielding a single peak which contained two proteins corresponding to the 49000- and 50000-M_r proteins. When the pool from the Sephacryl S-400 column was chromatographed over DEAE-Sepharose, E2 and E3 were cleanly separated (Fig. 2). The monomer M_r of the protein from the E2 pool was 49000, and that of the protein from the E3 pool was 50000 (Fig. 3). Attempts to determine the M_r of the native E2 subunit by gel filtration using h.p.l.c. (Delaney *et al.*, 1984) yielded a value in excess of 10^6, which indicates that the E2 subunit exists as a large complex. The E2 subunit isolated from bovine kidney branched-chain oxo acid dehydrogenase also had a high M_r (Cook *et al.*, 1985). The specific activity of the E2 subunit was 34 μmol of CoA released/5 min per mg of protein, with a trace of lipoamide dehydrogenase. The specific activity of LPD-val was 84 μmol of NADH oxidized/min per mg of protein, with no detectable E2 activity.

Properties of E2

E2 had a broad pH optimum between 6.9 and 7.7, but was routinely assayed at pH 7.0 because alkaline conditions favoured the hydrolysis of the substrate. In order to establish that the purified subunit catalysed transacylation rather than deacylation, 7.6 μg of purified E2 was incubated with 1 μmol of isobutyryl-CoA and 10 μmol of dihydrolipoamide. After 20 min, 0.78 μmol of free CoA was produced, with no change in isobutyryl residue as determined by hydroxamic acid reaction. The actual values for isobutyrohydroxamate in one typical experiment were 1.0 μmol at zero time and 1.12 μmol at 20 min. An increase of 5–10% in isobutyrohydroxamate was observed in several experiments.

E2 specifically required dihydrolipoamide as the acceptor of the isobutyryl residue (Table 2). Mercaptoethanol and GSH were without activity, whereas cysteine and dithiothreitol had slight activity. The kinetics of the reaction catalysed by E2 with isobutyryl-CoA, isovaleryl-CoA and propionyl-CoA were examined (Table 3). These acyl-CoA esters are oxidative-decarboxylation products of 2-oxoisovalerate, 4-methyl-2-oxopentanoate and 2-oxobutyrate respectively. Ratios of $k_{cat.}/K_m$ indicate that all three of these compounds are substrates, even though the K_m for propionyl-CoA is rather high. Acetyl-CoA was weakly active and succinyl-CoA was completely inactive, which further substantiates the lack of contamination by pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. L-Valine had no effect on K_m, which shows that the increased affinity of the complex for its oxo acid substrates in the presence of valine is specific for the E1 subunit. The K_m obtained by Chuang *et al.* (1984) for isobutyryl-CoA with E2 of bovine kidney branched-chain oxo acid dehydrogenase was 0.1 mM for isobutyryl-CoA and 0.05 mM for isovaleryl-CoA. It was not possible to obtain a realistic K_m for dihydrolipoamide, since, even at a concentration of 10 mM, activity as a function of dihydrolipoamide concentration was still increasing.

Table 2. Requirement for dihydrolipoamide in the E2 assay

All thiols were tested in the E2 assay at a final concentration of 20 mM with 2 μg of purified E2 subunit.

Thiol added to reaction	CoA formed (nmol/5 min)
Dihydrolipoamide	62
Mercaptoethanol	7
Glutathione	4
Cysteine	14
Dithiothreitol	18
None	0

Table 3. Kinetic constants for acyl-CoA substrates of the E2 subunits

Substrate	K_m (mM)	$K_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
Isobutyryl-CoA	0.518	4.83×10^4
Isovaleryl-CoA	0.624	6.00×10^4
Propionyl-CoA	4.64	3.34×10^4

Purification of LPD-glc and LPD-val

LPD-glc and LPD-val were purified from *P. aeruginosa* PAO as described previously for *P. putida* (Sokatch *et al.*, 1981*a*). The M_r values obtained from electrophoresis in denaturing gels were 54000 for LPD-glc and 50000 for LPD-val. The M_r values of the native proteins obtained by gel filtration using h.p.l.c. (Delaney *et al.*, 1984) were 120000 for LPD-glc and 96000 for LPD-val, which indicates that these flavoproteins are dimers, as are other lipoamide dehydrogenases (Williams, 1976). Spectra of oxidized LPD-glc and LPD-val were typical of flavoproteins and virtually identical with those obtained from *P. putida* PpG2 (Delaney *et al.*, 1984); LPD-glc of *P. aeruginosa* had a maximum at 455 nm, and LPD-val had a maximum at 460 nm.

It was decided to study the relationships of lipoamide

Table 4. Amino acid compositions of LPD-glc and LPD-val from *P. putida* PpG2 and *P. aeruginosa* PAO

References: [1]from Delaney *et al.* (1984); [2]from Stephens *et al.* (1983*c*).

	Content (residues/mol of enzyme)				
	LPD-glc		LPD-val		
	PpG2[1]	PAO	PpG2[1]	PAO	*E. coli*[2]
Asp	48	42	25	27	39
Thr	34	31	20	15	26
Ser	24	19	23	24	14
Glu	53	57	47	52	47
Pro	19	20	21	22	21
Gly	59	57	56	57	51
Ala	67	71	60	67	50
Val	57	58	49	53	45
Met	8	8	8	6	9
Ile	30	30	26	22	39
Leu	41	44	57	56	34
Tyr	8	8	3	7	8
Phe	14	16	9	6	14
Lys	35	38	20	12	39
His	10	12	15	17	13
Arg	17	18	23	31	15

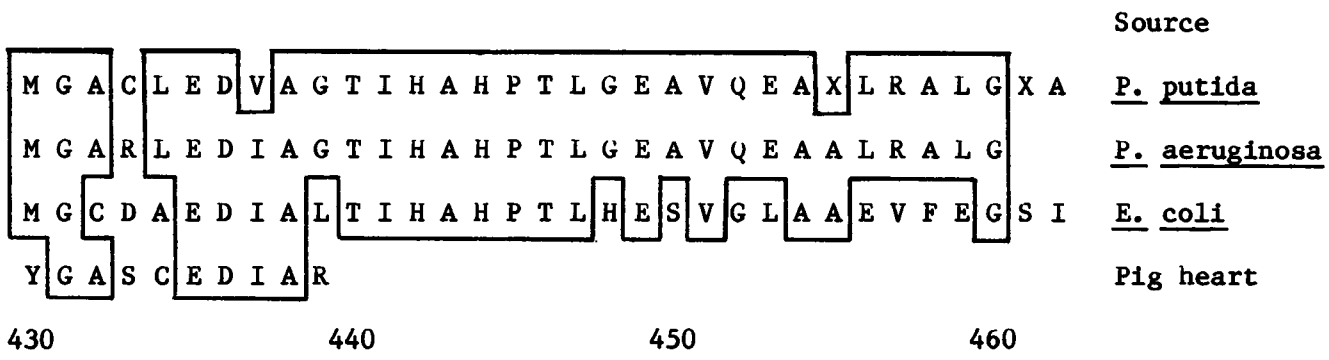


Fig. 4. Partial sequence of C-terminal peptides of LPD-val from *P. putida* and *P. aeruginosa*

Numbers refer to position of amino acids in lipoamide dehydrogenase of *E. coli* (Stephens *et al.*, 1983*c*). Sequences of *E. coli* and pig heart lipoamide dehydrogenases were taken from the same paper. Regions of homology are enclosed by boxes.

dehydrogenases from *P. aeruginosa* PAO to the corresponding proteins from *P. putida* PpG2. The amino acid compositions given in Table 4 for LPD-glc and LPD-val from the two pseudomonads are very similar. The amino acid composition of lipoamide dehydrogenase from *E. coli*, calculated from the DNA composition (Stephens *et al.*, 1983*c*), is included for comparison. There are some differences in the threonine and lysine contents of the two species of LPD-val, but no significant differences between amino acid compositions of the two species of LPD-glc. Comparison of amino acid compositions by the $S\Delta Q$ method (Marchalonis & Weltman, 1971) provided values of 6.74 for the two species of LPD-glc and 12.04 for the two species of LPD-val, which indicate highly significant relationships (Cornish-Bowden, 1983). $S\Delta Q$ values for the two species of LPD-glc compared with *E. coli* lipoamide dehydrogenase were approx. 21, which is also significant. However, $S\Delta Q$ values for the two species of LPD-val compared with *E. coli* lipoamide dehydrogenase were greater than 70, which is too large to be significant. For comparison, $S\Delta Q$ for *E. coli* and pig heart lipoamide dehydrogenases was 9.29 (Delaney *et al.*, 1984). These last two proteins have approx. 40% homology (Stephens *et al.*, 1983*c*). Antisera against LPD-glc and LPD-val from *P. putida* PpG2 caused substantial inhibition of activity of the homologous proteins from *P. aeruginosa* PAO.

Partial sequence of LPD-val

The sequences of *C*-terminal peptides from CNBr digests of LPD-val from *P. putida* and *P. aeruginosa* are shown in Fig. 4. Sequences of the two LPD-val CNBr digests were virtually identical, with only two amino acid substitutions at positions 4 and 8, both of which could have occurred by single base changes, leading to replacement of a cysteine by arginine and valine by isoleucine. Delaney *et al.* (1984) showed by analysis of amino acid compositions that LPD-val of *P. putida* was not closely related to either *E. coli* lipoamide dehydrogenase or LPD-glc. Therefore it was somewhat surprising to find that there was a distinct homology with a section of *E. coli* lipoamide dehydrogenase, beginning at amino acid 430, which is also at the *C*-terminal end and is in the interface region between the subunits of the dimer (Stephens *et al.*, 1983*c*). There is a segment of eight amino acids which are identical in all three proteins (TIHAHPTL), and over 50% homology between the *Pseudomonas* and *E. coli* lipoamide dehydrogenases. A small segment of pig heart lipoamide dehydrogenase which corresponds to this same region is available, and also appears to be homologous. A search of the Dayhoff protein sequence library failed to reveal any other proteins with similar homology. Human glutathione reductase did appear in the search, with a segment of 26 amino acids at the *C*-terminal end of the molecule with nine matches, for a 34.6% homology. Although this is not unusually high, the location at the end of the molecule suggests that this may be genuine homology.

DISCUSSION

The data presented in this paper established the identity of at least three of the four peptides of the purified branched-chain oxo acid dehydrogenase of *P. aeruginosa*. The purified 49000-M_r peptide of the *P. aeruginosa* complex catalysed the reactions expected of the E2 subunit. Although we were unable to isolate the E2 subunit from the *P. putida* complex, we were able to show (V. McCully, G. Burns & J. R. Sokatch, unpublished work) that the 46000-M_r protein of this complex was specifically labelled with *N*-ethyl[2,3-^{14}C]maleimide, which is presumed to label the dihydrolipoamide prosthetic group (Henderson *et al.*, 1979).

The 50000-M_r protein has been identified as the E3 subunit of branched-chain oxo acid dehydrogenase from *P. aeruginosa* and corresponds in every way to LPD-val of *P. putida* branched-chain oxo acid dehydrogenase (Sokatch *et al.*, 1981*a*). The homology between the *C*-terminal peptide of LPD-val and lipoamide dehydrogenase of *E. coli* is very interesting. The fact that no homology was found in any other of the amino acid sequences currently in the Dayhoff data bank suggests that this may be a highly conserved region of lipoamide dehydrogenase. This region of *E. coli* lipoamide dehydrogenase is the interface region between the dimeric subunits (Stephens *et al.*, 1983*c*). Both of the LPD-val proteins specifically functioned as the E3 subunit of branched-chain oxo acid dehydrogenase from their respective species. Both of the LPD-val proteins had absorption maxima at 460 nm, which is high for lipoamide dehydrogenase dehydrogenases but usual for other FAD-proteins, such as yeast and human glutathione reductases (Massey & Williams, 1965; Worthington & Rosemeyer, 1975). Mercuric reductase of

P. aeruginosa Tn501 has a maximum at 458 nm (Fox & Walsh, 1982). The amino acid sequence of the *C*-terminal peptides of LPD-val from *P. putida* and *P. aeruginosa*, the overall amino acid composition and the reactivity with antibody against LPD-val from *P. putida* suggests that these two proteins have considerable homology. In view of this, it is somewhat surprising that LPD-val from *P. putida* did not substitute better for LPD-val from *P. aeruginosa*.

Identification of the E2 and E3 subunits means that the 36000-M_r and/or 42000-M_r peptides must be E1. It is tempting to conclude that there are two E1 peptides, since this is the case with the mammalian complex and since the purified complex from *P. putida* also contains four peptides, two of which have been identified as E2 and E3. E1α of the mammalian enzyme is phosphorylated, and the function of E1β has not been established. Regulation of branched-chain oxo acid dehydrogenase from *Pseudomonas* takes place by means of the effector L-valine (Sokatch *et al.*, 1981*b*), which increases the affinity of E1 for branched-chain oxo acids. L-Valine had no effect on catalytic activity of either E2 or lipoamide dehydrogenase; thus there are potential roles for catalytic and regulatory subunits. When the cellular concentration of L-valine is low, such as when valine synthesis is taking place, the affinity of E1 for branched-chain oxo acids is low, preventing oxidation of these oxo acids, which are also precursors of branched-chain amino acids. When the concentration of L-valine is sufficiently high to serve as a carbon source, branched-chain oxo acid dehydrogenase is active, but there is also ample valine for protein synthesis.

The potential exists for studying the evolutionary origins of subunits of branched-chain oxo acid dehydrogenase. It is interesting, but it may be simply coincidental, that subunit M_r values of branched-chain oxo acid dehydrogenase from bovine kidney (Pettit *et al.*, 1978; Heffelfinger *et al.*, 1983) are similar to those of *P. putida* (Sokatch *et al.*, 1981*b*) and *P. aeruginosa*. Lowe *et al.* (1983) purified branched-chain oxo acid dehydrogenase of *Bacillus subtilis*, the function of which is to provide branched-chain fatty acids, which are required for membrane phospholipids (Kaneda, 1977). The *B. subtilis* complex appears to act as both pyruvate dehydrogenase and branched-chain oxo acid dehydrogenase, and raises the possibility that the latter may have evolved from the former. Mammalian branched-chain oxo acid dehydrogenases have significant activity with pyruvate (Pettit *et al.*, 1978; Cook *et al.*, 1985), which could be an indication of evolutionary origin or could be due to structural similarity of substrates. Branched-chain oxo acid dehydrogenase of *P. aeruginosa* binds pyruvate to E1 (Table 1), but pyruvate is not a good substrate for the complex, since acetyl-CoA is not a substrate for E2. The nucleotide sequence of the structural genes for E1 and E2 subunits of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase of *E. coli* have been determined, which revealed that the two E2 subunits shared regions of homology, but the E1 subunits did not (Stephens *et al.*, 1983*a*,*b*; Darlison *et al.*, 1984; Spencer *et al.*, 1984). This suggests that the respective E1 subunits evolved separately. It will be interesting to compare the structures of the E1 and E2 subunits of branched-chain oxo acid dehydrogenase with those of the subunits of *E. coli* oxo acid dehydrogenases.

Received 24 June 1985/18 September 1985; accepted 3 October 1985

This research was supported by U.S. Public Health Service grants AM 21737 and GM 30428 from the National Institutes of Health.

REFERENCES

Chuang, D. T., Hu, C.-W. C., Ku, L. S., Niu, W.-L., Myers, D. E. & Cox, R. P. (1984) J. Biol. Chem. **259**, 9277–9284
Cook, K. G., Bradford, A. P. & Yeaman, S. J. (1985) Biochem. J. **225**, 731–735
Cornish-Bowden, A. (1983) Methods Enzymol. **91**, 60–75
Crestfield, A. M., Moore, S. & Stein, W. H. (1963) J. Biol. Chem. **238**, 622–627
Darlison, M. B., Spencer, M. E. & Guest, J. R. (1984) Eur. J. Biochem. **141**, 351–359
Delaney, R., Burns, G. & Sokatch, J. R. (1984) FEBS Lett. **168**, 265–270
Fantania, H. R., Lau, K. S. & Randle, P. J. (1981) FEBS Lett. **132**, 285–288
Fox, B. & Walsh, C. T. (1982) J. Biol. Chem. **257**, 2498–2503
Hager, L. P. & Kornberg, H. L. (1961) Biochem. J. **78**, 194–198
Heffelfinger, S. C., Sewell, E. T. & Danner, D. J. (1983) Biochemistry **22**, 5519–5522
Henderson, C. E., Perham, R. N. & Finch, J. R. (1979) Cell **17**, 85–93
Kaneda, J. (1977) Bacteriol. Rev. **41**, 391–418
Laemmli, U. K. & Favre, M. (1973) J. Mol. Biol. **80**, 575–599
Lowe, P. N., Hodgson, J. A. & Perham, R. N. (1983) Biochem. J. **215**, 133–140
Marchalonis, J. J. & Weltman, J. C. (1971) Comp. Biochem. Physiol. B **38**, 609–625
Marshall, V. P. & Sokatch, J. R. (1972) J. Bacteriol. **110**, 1073–1081
Martin, R. R., Marshall, V. P., Sokatch, J. R. & Unger, L. (1973) J. Bacteriol. **115**, 198–204
Massey, V. & Williams, C. H. (1965) J. Biol. Chem. **240**, 4470–4480
Meister, A. (1957) Methods Enzymol. **3**, 404–414
Odessey, R. (1982) Biochem. J. **204**, 353–356
Patel, T. B. & Olson, M. S. (1982) Biochemistry **21**, 4259–4265
Paxton, R. & Harris, R. A. (1982) J. Biol. Chem. **257**, 14433–14439
Pettit, F. H., Yeaman, S. J. & Reed, L. J. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 4881–4885
Reed, L. J. (1974) Acc. Chem. Res. **7**, 40–46
Sokatch, J. R., McCully, V., Gebrosky, J. & Sokatch, D. J. (1981*a*) J. Bacteriol. **148**, 639–646
Sokatch, J. R., McCully, V. & Roberts, C. M. (1981*b*) J. Bacteriol. **148**, 647–652
Sokatch, J. R., McCully, V., Sahm, J. G. & Reyes-Maguire, M. (1983) J. Bacteriol. **153**, 969–975
Spencer, M. E., Darlison, M. G., Stephens, P. E., Duckenfield, I. K. & Guest, J. R. (1984) Eur. J. Biochem. **141**, 361–374
Stadtman, E. R. (1957) Methods Enzymol. **3**, 931–941
Steers, E., Craven, G. R., Anfinsen, C. B. & Bethune, J. L. (1965) J. Biol. Chem. **240**, 2478–2484
Stephens, P. E., Darlison, M. G., Lewis, H. M. & Guest, J. R. (1983*a*) Eur. J. Biochem. **133**, 155–162
Stephens, P. E., Darlison, M. G, Lewis, H. M. & Guest, J. R. (1983*b*) Eur. J. Biochem. **133**, 481–489
Stephens, P. E., Lewis, H. M., Darlison, M. G. & Guest, J. R. (1983*c*) Eur. J. Biochem. **135**, 519–527
Sykes, P. J., Menard, J., McCully, V. & Sokatch, J. R. (1985) J. Bacteriol. **162**, 203–208
Williams, C. H. (1976) Enzymes 3rd Ed. **13**, 89–173
Worthington, D. J. & Rosemeyer, M. A. (1975) Eur. J. Biochem. **60**, 459–466