Relationship of lipoamide dehydrogenases from *Pseudomonas putida* to other FAD-linked dehydrogenases

Robert Delaney, Gayle Burns[+] and John R. Sokatch[+]*

Department of Biochemistry and Molecular Biology and [+]Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, PO Box 26901, Oklahoma City, OK 73190, USA

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Pseudomonas putida produces two lipoamide dehydrogenases, LPD-glc and LPD-val. LPD-val is specifically required as the lipoamide dehydrogenase of branched-chain keto acid dehydrogenase and LPD-glc fulfills all other requirements for lipoamide dehydrogenase. Both proteins are dimers with one FAD per subunit. LPD-glc has an absorption maximum at 455 nm, but LPD-val has a maximum at 460 nm. Comparison of amino acid compositions revealed that LPD-glc was more closely related to *Escherichia coli* and pig heart lipoamide dehydrogenase than to LPD-val. LPD-val did not appear to be closely related to any of the proteins compared with the possible exception of mercuric reductase.

Lipoamide dehydrogenase *Branched-chain keto acid dehydrogenase* Pseudomonas putida

1. INTRODUCTION

Pseudomonas putida produces two lipoamide dehydrogenases which can be distinguished by their functions and monomer M_r values [1]. During growth on glucose, a single lipoamide dehydrogenase with an M_r 56000 designated LPD-glc is produced and is required for 2-ketoglutarate and probably pyruvate dehydrogenase and glycine oxidation [2]. When grown on valine, *P. putida* produces two lipoamide dehydrogenases, LPD-glc and LPD-val. LPD-val has a subunit M_r of 49000 and is the specific E3 subunit required for branched-chain keto acid dehydrogenase of *P. putida* [3]. Based on genetic data antigenic specificity and peptide maps, we have concluded that LPD-glc and LPD-val are products of separate structural genes [4]. The production of two lipoamide dehydrogenases is unique since other organisms produce only one [5]. The object of this study was to compare the chemical and physical properties of LPD-glc and LPD-val with those of FAD-linked dehydrogenases with disulfide active sites.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

Growth conditions for *P. putida* and preparation of LPD-glc and LPD-val were as in [1]. Protein was determined by the micro Bio-Rad method using instructions provided by the manufacturer. The absorption spectra of LPD-glc and LPD-val were read in a Gilford model 2600 spectrophotometer using the wavelength program. FAD was estimated at 455 nm for oxidized LPD-glc and 460 nm for LPD-val using the extinction coefficient of 11.3×10^3 cm^2/mmol [6]. M_r values were estimated by gel filtration using Sephadex G-200 [7]. M_r values were also determined by HPLC using a TSK-G3000 SW column (7.5 × 300 mm, LKB instruments Inc.) using a detector with a 254 nm filter. The buffer was 0.1 M potassium phosphate (pH 7.0) at a flow rate of 1.0 ml/min. M_r values were estimated by extrapolating from the peak retention time (fig.2). Conditions for acid hydrolysis of proteins and

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their amino acid determination have been described [8]. The loss of serine and threonine and the slow release of valine, isoleucine and leucine were corrected by extrapolation from 24, 48 and 72 h hydrolysates. Cystine and/or cysteine values were determined as cysteic acid after performic acid oxidation [9]. Cysteic acid was determined by HPLC analysis on an Altex Ultrasphere ODS column [10].

3. RESULTS

3.1. *Chemical composition*

The amino acid compositions of LPD-glc and LPD-val are given in table 1. Since LPD-val contains 57 leucine residues per subunit compared to 41 for LPD-glc, this is additional evidence for separate structural genes for LPD-glc and LPD-val since it would be impossible for LPD-val to arise by a post-translational modification of LPD-glc.

Because of the unique occurrence of LPD-val in *P. putida*, it was of interest to compare the amino acid compositions of LPD-glc and LPD-val with other lipoamide dehydrogenases and FAD-containing enzymes known to have similar active sites to determine if amino acid composition would suggest some degree of relationship (table 2). Proteins known to have similar active sites are lipoamide dehydrogenases of *E. coli* [11,12] and pig heart [13,14], glutathione reductases of yeast [15] and human red blood cells [16] and mercuric reductase of transposon Tn501 from *P. aeruginosa*

Table 1

Amino acid compositions of LPD-glc and LPD-val

	Residues per mol enzyme	
	LPD-glc	LPD-val
Cysteic acid	3	6
Aspartic acid	48	25
Threonine	34	20
Serine	24	23
Glutamic acid	53	47
Proline	19	21
Glycine	59	56
Alanine	67	60
Valine	57	49
Methionine	8	8
Isoleucine	30	26
Leucine	41	57
Tyrosine	8	3
Phenylalanine	14	9
Lysine	35	20
Histidine	10	15
Arginine	17	23

Table 2

$S\Delta Q$ values for FAD-containing proteins with a disulfide active site

	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1. LPD-glc	51.30	**20.35**	**21.46**	125.07	**26.53**	55.59	42.87	39.84
2. LPD-val		72.44	81.56	194.39	100.36	135.45	87.25	52.10
3. *E. coli* lpd			**9.29**	105.13	**21.99**	**37.15**	**32.73**	78.46
4. Pig heart lpd				110.01	**16.80**	**21.73**	**27.63**	87.13
5. Yeast lpd					99.52	101.27	138.52	143.51
6. *E. coli* GSH reductase						**32.39**	39.57	68.69
7. Yeast GSH reductase							37.93	137.52
8. Human GSH reductase								81.12
9. Mercuric reductase								

Column headings in the horizontal direction correspond to those in the vertical direction. GSH, glutathione; lpd, lipoamide dehydrogenase. Amino acid compositions for these calculations were taken from the literature: *E. coli* lipoamide dehydrogenase [21], pig heart lipoamide dehydrogenase [22], yeast lipoamide dehydrogenase [23], *E. coli* and yeast glutathione reductases [5], human glutathione reductase [19], and *P. aeruginosa* mercuric reductase (calculated from DNA sequence) [25]. Numbers in bold face meet the weak test of [19]

[17]. The calculations in table 2 are $S\Delta Q$ values [18] which are theoretically less sensitive to differences in length of protein. The author in [19] defined two levels of significance for calculations of this type. For a protein of 500 residues, the strong test is met if $S\Delta Q$ is less than 16.80; the weak test is satisfied if $S\Delta Q$ is less than 37.20. The calculations in table 2 were also used to construct a distance matrix ([20], fig.1).

Table 2 and fig.1 provided some surprising results. They show that LPD-glc and LPD-val appear to be only distantly related which helps explain our finding that antisera against these two proteins show very little cross-reactivity [4]. It is interesting that there is a high degree of relationship between *E. coli* and pig heart lipoamide dehydrogenase since pig heart lipoamide dehydrogenase can substitute for *E. coli* lipoamide dehydrogenase in pyruvate and 2-ketoglutarate

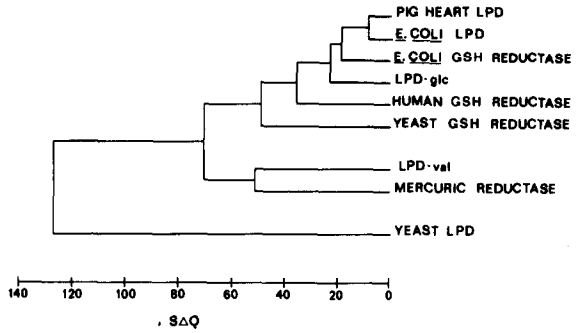

Fig.1. Evolutionary tree constructed from calculations in table 2.

dehydrogenases [26]. Glutathione reductases of *E. coli*, yeast and human erythrocytes are related to both pig heart and *E. coli* lipoamide dehydrogenases. The calculations in table 2 appear to fail with the relationship between mercuric reductase

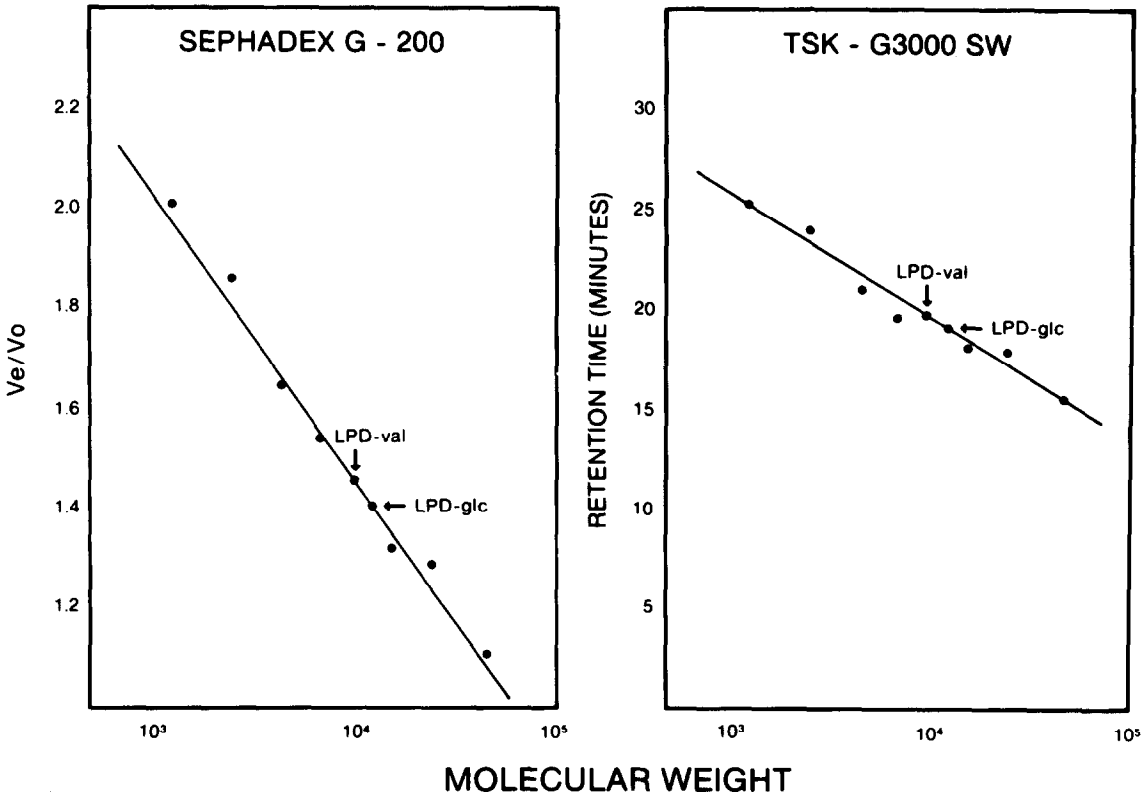

Fig.2. M_r determinations of LPD-glc and LPD-val by gel filtration using column chromatography with Sephadex G-200 and HPLC with a TSK-3000 SW column. The standards used and their M_r values were: cytochrome *c*, 12500; chymotrypsinogen A, 25000; ovalbumin, 45000; bovine serum albumin, 68000; aldolase, 158000; catalase, 240000 and ferritin, 450000.

and human glutathione reductase and *E. coli* lipoamide dehydrogenase all of which have been shown to be related by DNA sequence homology [25,27]. The reason for the discrepancy with mercuric reductase may be due to an unusually high alanine content and unusually low lysine content.

3.2. M_r values

The monomer M_r values of LPD-glc and LPD-val were determined to be 56000 and 49000, respectively [1]. The calibration curves for Sephadex G-200 and TSK-3000 SW are shown in fig.2. The M_r of LPD-glc by gel filtration with Sephadex G-200 was 125000, by HPLC it was 116000. The M_r of LPD-val by gel filtration with Sephadex G-200 was 104000, by HPLC it was 93000. It is clear that both LPD-glc and LPD-val are dimers in their native state.

3.3. Spectra

The spectra of LPD-glc and LPD-val are shown in fig.3. There was a small, but significant difference between the two spectra since LPD-glc exhibited a maximum at 455 nm with a shoulder at about 482 nm while LPD-val exhibited a maximum at 460 nm with a shoulder at 487 nm. The spectrum of LPD-glc is similar to that of other lipoamide dehydrogenases which have maxima at 455–456 nm [5]. However, yeast [28] and human glutathione reductases [29] have absorption maxima at 460 nm, while mercuric reductase has a maximum at 458 nm [30]. When LPD-glc and LPD-val were reduced with NADH, the maximum at 455 nm was reduced and shifted to a shorter wavelength (fig.3), which is typical of flavoproteins [5].

3.4. FAD content

Lipoamide dehydrogenases typically contain one FAD per subunit [5]. Our results show that LPD-glc contained 1.4 FAD per subunit and LPD-val contained 0.98 FAD/subunit. A sample of pig heart lipoamide dehydrogenase provided a result of 1.06 FAD per subunit. From these data, we concluded that LPD-glc and LPD-val contain one FAD per subunit.

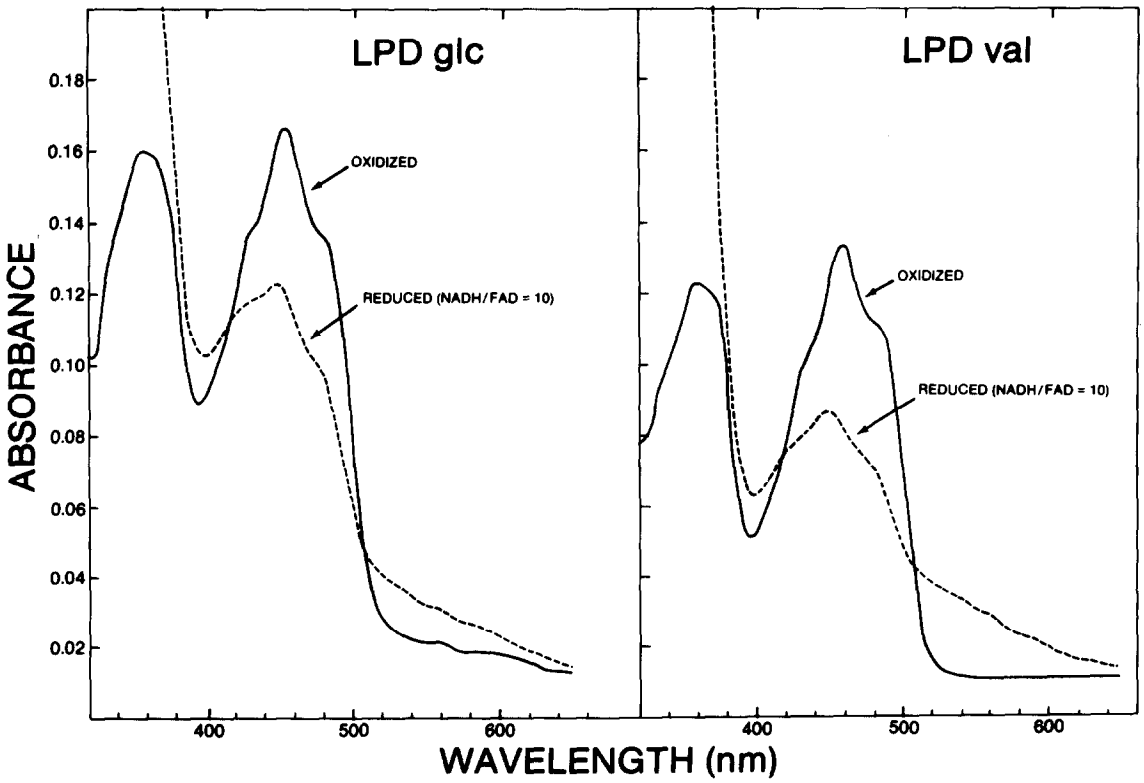

Fig.3. Spectra of oxidized and reduced LPD-glc and LPD-val. Spectra were read anaerobically using Thunberg tubes. The protein concentration was 0.5–1.0 mg/ml in 25 mM potassium phosphate, pH 7.0.

4. DISCUSSION

Our original hypothesis was that LPD-glc was used for pyruvate and 2-ketoglutarate dehydrogenases and would be equivalent to the lipoamide dehydrogenase of *E. coli* which serves these functions. The data in table 2 and fig.3 suggest that LPD-glc is indeed the equivalent of *E. coli* and pig heart lipoamide dehydrogenases. In this theory, LPD-val would be used for all other functions of lipoamide dehydrogenase. However, recent evidence from our laboratory shows that LPD-glc not LPD-val functions in the glycine oxidation by *P. putida* [4] which suggests that LPD-val is used solely as the lipoamide dehydrogenase of branched-chain keto acid dehydrogenase. Other evidence from our laboratory to be published at a later date will show that *P. aeruginosa* also forms two lipoamide dehydrogenases equivalent to LPD-glc and LPD-val. One objective of the current research was to discover why pseudomonads evolved two separate lipoamide dehydrogenases when most other organisms seem to function with just one. Fig.1 and the calculations in table 2 suggest that *E. coli* and pig heart lipoamide dehydrogenases, LPD-glc and the glutathione reductases represent the main evolutionary line with LPD-val and mercuric reductase branching off the main line at an earlier stage. Therefore, pseudomonads solved the problem of metabolism of branched-chain amino acids by evolving a separate lipoamide dehydrogenase and higher organisms made use of the existing lipoamide dehydrogenase for this purpose. Considering the anomalously low relationship of mercuric reductase to *E. coli* lipoamide dehydrogenase shown in table 2, it is possible that this protein may be even more closely related to LPD-glc and LPD-val than the calculations suggest.

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