Amino acid substitutions at glutamate-354 in dihydrolipoamide dehydrogenase of *Escherichia coli* lower the sensitivity of pyruvate dehydrogenase to NADH

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Pyruvate dehydrogenase (PDH) of *Escherichia coli* is inhibited by NADH. This inhibition is partially reversed by mutational alteration of the dihydrolipoamide dehydrogenase (LPD) component of the PDH complex (E354K or H322Y). Such a mutation in *lpd* led to a PDH complex that was functional in an anaerobic culture as seen by restoration of anaerobic growth of a *pflB*, *ldhA* double mutant of *E. coli* utilizing a PDH- and alcohol dehydrogenase-dependent homoethanol fermentation pathway. The glutamate at position 354 in LPD was systematically changed to all of the other natural amino acids to evaluate the physiological consequences. These amino acid replacements did not affect the PDH-dependent aerobic growth. With the exception of E354M, all changes also restored PDH-dependent anaerobic growth of and fermentation by an *ldhA*, *pflB* double mutant. The PDH complex with an LPD alteration E354G, E354P or E354W had an approximately 20-fold increase in the apparent K_i for NADH compared with the native complex. The apparent K_m for pyruvate or NAD⁺ for the mutated forms of PDH was not significantly different from that of the native enzyme. A structural model of LPD suggests that the amino acid at position 354 could influence movement of NADH from its binding site to the surface. These results indicate that glutamate at position 354 plays a structural role in establishing the NADH sensitivity of LPD and the PDH complex by restricting movement of the product/substrate NADH, although this amino acid is not directly associated with NAD(H) binding.

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INTRODUCTION

Pyruvate is at a pivotal point in the carbon and energy metabolism of *Escherichia coli*. During aerobic growth, pyruvate is oxidatively decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH) (EC 1.2.4.1) and further oxidized through the enzymes of the TCA cycle and respiratory chain. During anaerobic growth, due to the large decrease in PDH activity *in vivo*, pyruvate formate-lyase (PFL) serves as the primary route for synthesis of acetyl-CoA and carbon dioxide, primary metabolites required for cell growth (Fig. 1) (Bock & Sawers, 1996; Clark, 1989; Guest, 1995; Murarka *et al.*, 2010; Sawers &

Clark, 2004). Although PDH activity is very low during anaerobic growth, all genes encoding the PDH complex are transcribed and translated under these conditions (Cassey *et al.*, 1998; Hansen & Henning, 1966; Kaiser & Sawers, 1994; Kim *et al.*, 2008; Snoep *et al.*, 1993). The presence of a functional PDH complex in cell extracts of anaerobically grown *E. coli* suggests that the lack of PDH activity *in vivo* is due to regulation of the enzyme activity by intracellular metabolites. During fermentative growth, NADH pools are high in comparison to aerobic growth, and NADH has emerged as the primary inhibitor of PDH activity (de Graef *et al.*, 1999; Hansen & Henning, 1966; Kim *et al.*, 2008; Schmincke-Ott & Bisswanger, 1981; Shen & Atkinson, 1970). Inhibition of the PDH complex by NADH can be reduced by a single mutation in the dihydrolipoamide dehydrogenase (LPD; EC 1.8.1.4) subunit (either H322Y or E354K) of the PDH complex (Kim *et al.*, 2008). These mutations increased the apparent K_i for NADH, and thus

Abbreviations: LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PFL, pyruvate formate-lyase.

A set of supplementary methods, a supplementary figure and three supplementary tables are available with the online version of this paper.

Fig. 1. Fermentation pathways of *E. coli* leading to ethanol production in the wild-type and ethanologenic derivatives. In the wild-type, pyruvate is converted to acetyl-CoA by PFL. The reductant released during this oxidative decarboxylation is lost as H_2 through formate. Due to this loss, an equimolar amount of acetate and ethanol is produced to maintain redox balance. In the ethanologenic derivatives, PDH* is active during anaerobic growth and with active PDH*, the [H] released at this step is captured as NADH. The yield of two NADHs per acetyl-CoA produced through this pathway supports complete conversion of acetyl-CoA to ethanol to maintain redox balance. ACK, acetate kinase; ADH, alcohol dehydrogenase; FHL, formate hydrogen-lyase; PDH*, pyruvate dehydrogenase complex with a mutation in the dihydrolipoamide dehydrogenase component; PTA, phosphotransacetylase.

caused a reduction in the extent of inhibition. Glutamate 354 is located within the central domain of LPD and is not recognized as a constituent of the NAD^+ binding pocket. However, a mutation in human LPD at position 358 (the analogous position in *E. coli* LPD is 351) has been previously reported to alter NAD^+ binding (Brautigam *et al.*, 2005). Other mutations in the central domain of LPD have been reported but none were identified as influencing NADH inhibition (Benen *et al.*, 1991; Brautigam *et al.*, 2005; Cameron *et al.*, 2006; Hopkins & Williams, 1995).

A null mutant of *E. coli* lacking PDH activity requires acetate for aerobic growth (Russell & Guest, 1990) but had no detectable phenotype during anaerobic growth due to the presence of PFL (Fig. 1). During anaerobic growth, acetyl-CoA serves as a key intermediate for biosynthesis and as an electron acceptor to maintain redox balance (Bock & Sawers, 1996; Sawers & Clark, 2004). Mutant *E. coli* K-12 strains lacking both PFL and lactate dehydrogenase (LDH) activities are unable to grow anaerobically due to their inability to maintain redox balance (Clark, 1989; Kim *et al.*, 2007). Although native PDH complex is present in the cytoplasm of anaerobic *E. coli* (Hansen & Henning, 1966; Kaiser & Sawers, 1994; Murarka *et al.*, 2010; Snoep *et al.*, 1993), it is apparently inhibited by NADH, and the

residual activity was insufficient to support anaerobic growth (Kim *et al.*, 2007, 2008; Snoep *et al.*, 1993). PDH with an LPD alteration (H322Y or E354K) restored anaerobic growth of an *ldhA*, *pflB* double mutant by providing acetyl-CoA for redox balance (Kim *et al.*, 2008). This triple mutant produced ethanol by coupling PDH with alcohol dehydrogenase (Fig. 1).

Mutated PDH (NADH-insensitive) has potential industrial applications by doubling the NADH yield available for production of reduced fermentation products such as ethanol without the need for foreign genes. Although either the H322Y or E354K mutation supported anaerobic growth of *E. coli* K-12 strains, anaerobic growth rates of these mutants were lower than those of the wild-type strain (Kim *et al.*, 2007) and other mutations in LPD may be superior for biotechnological applications (Kim *et al.*, 2008). In this study, mutant forms of PDH were constructed in which glutamate at position 354 of LPD was systematically replaced by other amino acids. The resulting strains were evaluated for anaerobic growth, ethanol production and sensitivity to inhibition by NADH.

METHODS

Bacterial strains and plasmids. *E. coli* strains and plasmids used in this study are listed in Table S1 (available with the online version of this paper). All strains are derivatives of K-12.

Media and growth conditions. Luria broth (LB) and mineral salts medium were prepared as described previously (Lee *et al.*, 1985). Sugars were added to sterile medium at an initial concentration of 3 g l^{-1} for aerobic growth and 10 g l^{-1} for anaerobic growth. Aerobic cultures were grown in 10 ml medium in 125 ml Erlenmeyer flasks at 37 °C in a shaker (200 r.p.m.). Batch fermentation without pH control was performed in screw-cap tubes (13 × 100 mm) in which the appropriate medium was added to the top of the tube. Batch fermentation at pH 7.0 with pH control was carried out in 500 ml vessels containing 250 ml LB with 30 g glucose l^{-1} (Underwood *et al.*, 2002). Inoculum (1%, v/v) for the fermentations was grown aerobically at 37 °C, overnight. If needed, antibiotics were added at an initial concentration of 100 mg ampicillin l^{-1} or 50 mg kanamycin l^{-1} for plates and 20 mg kanamycin l^{-1} for liquid medium.

PCR-based site-directed mutagenesis of *lpdA* in plasmid pZS3.

The dihydrolipoamide dehydrogenase gene (*lpdA*) of *E. coli* strain W3110 was cloned into plasmid pBR322 (pZS3). For construction of plasmid pZS3, the complete *lpd* DNA (2.35 kbp) including the promoter and terminator region of the native gene was amplified by PCR from genomic DNA (see supplementary methods). The forward primer (5'-GCGAGCTAGCGCGGTTGCTTCACCATCTC-3') and the reverse primer (5'-GCAATGCCCGAGGCGTATTTCAGTCCGCA-TCAC-3') contained *NheI* and *AvaI* restriction endonuclease cleavage site (underlined), respectively. The PCR product and plasmid pBR322 were digested with restriction enzymes *NheI* and *AvaI*, and ligated together to construct plasmid pZS3 containing the native *lpd* gene. The *lpd* gene in plasmid pZS3 was mutagenized using PCR with two synthetic oligonucleotide primers containing the expected mutations [positions 1060–1062 of *lpd* representing glutamate in the native protein (A in the ATG codon of the *lpd* gene was defined as position 1)]. The forward primer contained the following sequence: 5'-CGCCTATACNNNNCCAGAAGTTG-3'. The underlined 'NNN' represent the codons for the 18 different amino acids except for

glutamic acid and lysine. The E354K mutation, *lpd101*, has been described previously (Kim *et al.*, 2008) and glutamate at position 354 is the natural amino acid. The reverse primer sequence was as follows: 5'-ATGGACGGGATAACTTTTCGGATC-3'. Using these different primer pairs, the entire plasmid pZS3 (5518 bp) was amplified. After purification, the PCR product was treated with T4 polynucleotide kinase, and circularized by T4 DNA ligase. The ligation product was transformed into *E. coli* Top10 chemical competent cells. The plasmid DNA isolated from these transformants was sequenced to confirm the specific introduced mutation in the *lpd* gene.

Construction of mutants. Construction of various mutant strains utilized standard techniques (Ausubel *et al.*, 1987; Davis *et al.*, 1980; Miller, 1972). Gene deletions in *E. coli* were constructed as described previously (Datsenko & Wanner, 2000) and verified by PCR. Two triple mutants of *E. coli* used in this study, strains YK100 and ZS2 [Δ (*focA-pflB*), Δ *ldhA* and Δ *lpd*]; Table S1], could not grow aerobically in glucose mineral salts medium and anaerobically in any of the media tested. These strains were used to evaluate the phenotypic properties conferred by various plasmids with different *lpd* mutations.

Transfer of the *lpd* mutation to the chromosome of *E. coli*. Three *lpd* alleles that supported anaerobic growth of strains YK100 and ZS2 (E354G, E354P and E354W) were amplified by PCR from appropriate plasmids (Table S1) and verified by sequencing. After purification, the linear PCR product was electroporated into *E. coli* strain ZS4 (strain ZS2 with pKD46) as described previously (Datsenko & Wanner, 2000). Transformants that grew in glucose mineral salts medium (aerobic) were selected and tested for kanamycin sensitivity and anaerobic growth. The chromosomal *lpd* gene from these derivatives was amplified by PCR and sequenced to confirm the presence of the desired *lpd* mutation.

Purification of PDH complex. The PDH complex was purified as described previously with minor modifications (Bisswanger, 1981; Kim *et al.*, 2008) and the details are listed in the Supplementary Methods. Protein purity was determined by SDS-PAGE (Laemmli, 1970) and enzyme activity was determined immediately using freshly prepared protein.

Purification of LPD. Purification of LPD followed the same procedure described above for purification of PDH up to the 150 000 g centrifugation step. After the centrifugation step at 150 000 g, the supernatant had significant LPD activity. This fraction was filtered through a 0.22 μ m filter and chromatographed through a hydroxyapatite column and gel filtration column as for PDH purification. The LPD-containing fractions were combined and concentrated. If needed, LPD was further purified using a Q-Sepharose column (2.0 $\times$ 20.0 cm; GE) and the protein was eluted with a linear NaCl gradient (0–500 mM in phosphate buffer, pH 7.5). Fractions with LPD activity were combined and dialysed against phosphate buffer. Protein purity was determined by SDS-PAGE (Laemmli, 1970).

Enzyme assays. LPD activity was measured in both the forward and reverse reaction as described by Patel & Hong (1998) and the reaction conditions are presented in the Supplementary Methods. One unit of enzyme activity was defined as the amount of enzyme required for oxidation of 1 μ mol NADH (or reduction of 1 μ mol NAD⁺) min⁻¹ (mg protein)⁻¹. Both reactions were performed at room temperature.

A standard assay for PDH complex was based on pyruvate-dependent reduction of NAD⁺ at 340 nm (ϵ =6220 M⁻¹ cm⁻¹) at room temperature, as described previously (Kim *et al.*, 2008). The reaction was started by addition of pyruvate. Enzyme activity is defined as μ mol NADH produced min⁻¹ (mg protein)⁻¹. The effect of NADH on enzyme activity was determined using the same reaction mixture with addition of various concentrations of NADH.

Homology modelling of *E. coli* LPD. Since a structure of *E. coli* LPD is not available, *Neisseria meningitidis* LPD X-ray structure (PDB-ID: 1OJT) (Li de la Sierra *et al.*, 1997) was used as a template for generation of an *E. coli* LPD model using the SWISS-MODEL program (Arnold *et al.*, 2006). Structures were superposed with each other for comparison using the COOT program (Emsley *et al.*, 2010). The *E. coli* LPD model was evaluated with the PROCHECK program (Laskowski *et al.*, 1993) to confirm the molecular geometry accuracy of the model. The figures were made and ray-traced with the PYMOL program (Delano, 2002).

Analytical methods. For determination of NAD⁺ and NADH pools, aerobic cultures were grown in LB medium (10 ml in 125 ml flask) on a shaker at 200 r.p.m. (37 °C). Anaerobic cultures were grown in LB + glucose (1%; w/v) in filled tubes without pH control. Aerobic cultures were inoculated (1%; v/v) from an aerobic mid-exponential-phase culture. Anaerobic cultures were inoculated to an initial OD₄₂₀ of 0.1 (measured with a Spectronic 20) from an anaerobic culture grown in the same medium. Cells were harvested at mid-exponential phase of growth, and NAD⁺ and NADH concentrations in cell extracts were determined essentially as described by Snoep *et al.* (1990) using an EnzyChrom NAD⁺/NADH assay kit (BioAssay Systems).

Glucose and fermentation products were determined by HPLC using an HP1090 chromatograph (Agilent Technologies) fitted with an Aminex HPX-87H column (Bio-Rad Laboratories) (Underwood *et al.*, 2002). Cell density was monitored at 420 nm. Protein concentration was determined by the Bradford method with BSA as the standard (Bradford, 1976).

RESULTS AND DISCUSSION

The anaerobic growth phenotype of strain SE2378, an *ldhA*, *pflB*, *lpd101* mutant of *E. coli*, is based on the *lpd101* mutation (E354K) that lowered the sensitivity of the PDH complex to NADH (Kim *et al.*, 2007, 2008). This mutant produced ethanol as the primary fermentation product, an indication of NADH-insensitive PDH*-supported anaerobic growth (Fig. 1). To evaluate the effect of other amino acids at position 354 of LPD on supporting anaerobic growth of an *ldhA*, *pflB* mutant, the 354-glutamate was changed to other natural amino acids and the effect of these changes on aerobic and anaerobic growth was determined.

Growth and fermentation properties of LPD mutants

E. coli strain ZS2 contains three mutations (*ldhA*, *pflB* and Δ *lpd*) and can be used to screen for a functional PDH complex (plasmid) under aerobic conditions and for an NADH-insensitive complex under anaerobic growth conditions. PDH mutations at this amino acid position of LPD were supplied to strain ZS2 from plasmids to compare their effectiveness for aerobic growth, anaerobic growth and ethanol production. All amino acid replacements of E354 in LPD supported aerobic growth in glucose mineral salts medium (Table S2). All amino acid replacements except methionine also restored anaerobic growth of strain ZS2, although the aerobic growth rate and cell yield of strain ZS2 carrying E354M were similar to that of the same

Table 1. Anaerobic growth and fermentation profiles of *E. coli* strains with different *lpd* mutations

Fermentations were conducted in LB with 30 g glucose l⁻¹ with pH control (pH 7.0) at 37 °C. UD, Undetectable below 0.5 mM. Data shown are means ± SD.

Strain	LPD alteration	Growth rate (h ⁻¹)	Cell yield (g l ⁻¹)	Glucose consumed (mM)	Product (mM)					Ethanol yield*
					Acetate	Formate	Lactate	Succinate	Ethanol	
W3110	Lpd +	1.09	1.66	159.3 ± 2.0	97.5 ± 1.0	209.3 ± 8.6	96.1 ± 6.8	14.7 ± 0.2	93.3 ± 3.3	29.2 ± 0.01
YK1	E354K	0.59	1.36	163.0 ± 2.5	10.4 ± 0.4	UD	11.6 ± 1.0	8.7 ± 1.4	305.4 ± 9.0	93.7 ± 0.03
ZS28	E354G	0.59	1.52	166.6 ± 2.3	11.4 ± 1.0	UD	13.0 ± 0.8	11.0 ± 0.4	288.6 ± 1.4	86.6 ± 0.01
ZS34	E354P	0.56	1.52	165.1 ± 0.8	14.9 ± 1.7	UD	13.5 ± 0.3	12.6 ± 0.7	287.7 ± 7.0	87.1 ± 0.02
ZS40	E354W	0.60	1.56	173.1 ± 1.9	18.4 ± 0.1	UD	17.2 ± 1.7	27.5 ± 1.4	246.7 ± 1.4	71.3 ± 0.01

*Ethanol yield is presented as a percentage of the theoretical maximum.

strain with a *lpd*⁺ derivative. This differential effect of E354M mutation suggests that this specific substitution did not eliminate the activity of the PDH complex and also did not alter its sensitivity to inhibition by NADH. This substitution was not further investigated. Modelling this substitution together with others could be used to delineate the boundaries of the change in structure needed to minimize NADH sensitivity of the PDH complex.

All other changes that replaced glutamate at 354 of LPD supported anaerobic growth and production of ethanol as the main fermentation product, as seen previously with the E354K mutation (Kim *et al.*, 2007, 2008). Although the culture medium from strain ZS2 with the LPD substitutions E354L, E354Q and E354T had a lower concentration of ethanol compared with others after the first 24 h (Table S2), the ethanol titre of these cultures increased to similar levels after 48 h of growth. These results suggest that only glutamate or methionine at position 354 of LPD renders the enzyme sensitive to NADH as seen by their inability to support anaerobic growth of strain ZS2 (Table S2).

Five of the mutations leading to the following changes, E354G, E354S, E354H, E354P and E354W, were selected based on the side chain of the substituted amino acid and tested in another triple mutant, strain YK100 that is also anaerobic growth minus (*ldhA*, *pflB*, *lpd*). PDH complexes containing these mutated forms of LPD supported

anaerobic growth of strain YK100 also suggesting potential NADH insensitivity of the PDH* complexes carrying these alterations. Three of the LPD alterations (E354G, E354P and E354W) were transferred to the chromosome and verified by PCR and sequencing.

Aerobic growth rate and cell yield of the chromosomal *lpd* mutants were similar to those of the wild-type strain W3110. In pH-controlled fermentations, *E. coli* wild-type strain W3110 grew at a specific growth rate of about 1.1 h⁻¹ while the three new LPD mutants (E354G, E354P and E354W) only supported anaerobic growth at about half of the growth rate of the wild-type (Table 1), a level that was similar to that of strain YK1 with the previously reported E354K mutation (Kim *et al.*, 2008). However, the cell yield of the wild-type and the mutants, determined at the end of growth phase, were similar. The primary fermentation product of these LPD mutants was ethanol except for strain W3110 that produced a mixture of products, as expected (Table 1, Fig. S1). The lower growth rate of the *lpd* mutants can be correlated with the lower rate of glucose consumption (about half that of the wild-type) since the yield of cells per glucose consumed was similar to that of the wild-type (Table S3). The lower rate of sugar consumption by the mutants could be a result of a low *in vivo* PDH activity that is limiting production of acetyl-CoA from pyruvate, a required electron acceptor, to maintain redox balance in these mutants. In spite of the

Table 2. Kinetic characteristics of the PDH complex isolated from *E. coli* strains with different LPD changes

Enzymes were purified from *E. coli* strains ZS3 (native protein), ZS28 [LPD (E354G)], ZS34 [LPD (E354P)] and ZS40 [LPD (E354W)].

LPD change	K _m (pyruvate) (mM)	K _m (NAD ⁺) (mM)	K _i (NADH) (μM)	V _{max} *	Specific activity*
Native	0.43	0.28	1.34	66.45	25.3
E354G	0.34	0.17	23.40	71.73	18.6
E354P	0.32	0.51	22.60	97.40	14.1
E354W	0.35	0.26	20.30	95.20	18.7

*μmol min⁻¹ (mg protein)⁻¹.

Fig. 2. Effect of LPD alteration on NADH sensitivity of PDH complex of *E. coli*. PDH activity was determined with varying concentrations of NADH at a fixed NAD^+ concentration of 0.5 mM. The specific activity of the native PDH complex without NADH was 25.32 U. The specific activities of the three PDH complexes with LPD alterations E354G, E354P and E354W without NADH were 18.63 U, 14.11 U and 18.66 U, respectively. The values presented are the fraction of the activity of each enzyme without added NADH.

plausible limitation at the PDH level, the three mutated forms of the PDH complex appear to be active during anaerobic growth, suggesting a reduced level of NADH sensitivity of the complex.

Biochemical properties of PDH from the LPD mutants

The PDH complex was purified from the wild-type strain and the three selected mutants with chromosomally integrated *lpd* mutations. Kinetic properties of these enzymes were determined, especially inhibition of activity by NADH. The native complex had the highest specific activity of 25.3 units under the reported assay condition while the specific activities of the three altered proteins

were slightly lower (Table 2). Among the three mutated forms of the PDH complex, the E354P form of the protein had the lowest specific activity, about 55 % of the activity of the native protein. However, the $V_{\max}$ of the three PDH complexes with a mutated form of LPD was similar to that of the native PDH or higher. The apparent K_m values for the substrates pyruvate and NAD^+ were not significantly different among the three mutated forms of the protein and the native enzyme. The apparent K_i for NADH for the native complex was determined to be 1.34 μM (Table 2, Fig. 2), a value similar to other reported values (Bisswanger & Henning, 1971; Kim *et al.*, 2008). In contrast, the apparent K_i for NADH for the three mutated forms of PDH was about 15-fold higher than that of the native PDH complex (Table 2). In agreement with the differences in apparent K_i , the PDH complex isolated from the mutant strains was also less sensitive to inhibition by NADH (Fig. 2). At an NADH concentration of 50 μM , native enzyme was inhibited by more than 90 %. Increasing the NAD^+ concentration to 1.0 mM and 2.0 mM decreased the extent of inhibition by 50 μM NADH to 71 and 46 %, respectively, in confirmation of competitive inhibition of the PDH complex by NADH. Irrespective of the NAD^+ and NADH concentration, an $[\text{NADH}]:[\text{NAD}^+]$ ratio of about 0.1 inhibited the native PDH complex by about 90 % (Fig. 2).

At the same $[\text{NADH}]:[\text{NAD}^+]$ ratio of 0.1, the three PDH complexes with the LPD alteration retained about 65 % of the control activity without added NADH. Although increasing the concentration of NADH in the assay did inhibit the PDH complexes from the mutants, this required significantly higher $[\text{NADH}]$. The observed increase in $V_{\max}$ for the PDH complex with the LPD alterations could arise from the lower affinity of the complex for NADH, the product and inhibitor of the reaction (Table 2). These results show a positive correlation between reduced

Table 3. $[\text{NADH}]:[\text{NAD}^+]$ ratio of wild-type *E. coli* and *lpd* mutants grown under aerobic and anaerobic conditions

Strain	Relevant genotype	$[\text{NADH}]:[\text{NAD}^+]$	
		Aerobic	Anaerobic
W3110	Wild-type	0.13	0.22
AH242	<i>ldhA</i> , <i>pflB</i>	0.22	0.76*
ZS2	<i>ldhA</i> , <i>pflB</i> , <i>lpd</i>	0.23	0.33*
YK1	<i>ldhA</i> , <i>pflB</i> , <i>lpd101</i>	0.23	0.36
ZS28	<i>ldhA</i> , <i>pflB</i> , <i>lpd109</i>	0.27	0.38
ZS34	<i>ldhA</i> , <i>pflB</i> , <i>lpd115</i>	0.16	0.41
ZS40	<i>ldhA</i> , <i>pflB</i> , <i>lpd121</i>	0.18	0.43

*Since strains AH242 and ZS2 are anaerobic-growth-negative, an aerobic culture at mid-exponential phase of growth was transferred to anaerobic conditions and cells were harvested after 2 h incubation at 37 °C. Presented results are the average of two independent experiments.

sensitivity of the PDH complex to NADH inhibition and anaerobic growth of appropriate *E. coli* mutants supported by the PDH complex.

[NADH]:[NAD⁺] ratio of LPD mutants

The results presented in Fig. 2 show that the native PDH is completely inhibited at an [NADH]:[NAD⁺] ratio of 0.2. *E. coli* grown anaerobically in mineral salts medium has been previously reported to have an [NADH]:[NAD⁺] ratio as high as 0.75 (de Graef *et al.*, 1999). Wild-type strain W3110 grown in rich medium anaerobically had an [NADH]:[NAD⁺] ratio of 0.22 (Table 3), a value that is higher than that needed for complete inhibition of PDH *in vitro* (Fig. 2). Strain AH242 lacking both LDH and PFL activities did not grow anaerobically, and when an aerobic culture of this strain was transferred to anaerobic conditions, the [NADH]:[NAD⁺] ratio increased to 0.76 within 2 h since this culture could not reoxidize NADH. Part of this increase is apparently due to native PDH activity since deleting LPD (strain ZS2) lowered this ratio by more than twofold. All four LPD* mutants had a higher [NADH]:[NAD⁺] ratio than the wild-type (Table 3) but a significantly lower ratio than that of strain AH242 with native PDH complex during anaerobic growth. The observed ratio of [NADH]:[NAD⁺] in these LPD* mutants is apparently due to a new redox equilibrium between NADH production by PDH* and NADH oxidation by alcohol dehydrogenase in ethanol production (Fig. 1). Anaerobic growth of these mutants even at this ratio (about 0.4) that is higher than that of the wild-type (about 0.2) is apparently due to the *lpd* mutation that lowered the affinity of the PDH complex for NADH (Table 2).

Biochemical properties of altered LPD

Since the mutation that lowered the sensitivity of PDH complex to NADH inhibition resides in the LPD component, the LPDs from each of the three mutants and the native enzyme were purified and their kinetic properties were determined. The apparent K_m for NAD⁺ for the native LPD was 0.14 mM in the forward reaction (Table 4). At seven times the apparent K_m value of NAD⁺ (1.0 mM), NADH at 0.1 mM inhibited 90 % of the LPD activity (Fig. 3a). Activity of the native LPD was completely inhibited by 0.14 mM NADH. As expected, increasing the NAD⁺ concentration partially reversed NADH inhibition as the ratio of NAD⁺ to NADH increased (data not presented). The 90 % inhibition of the LPD activity at an [NAD⁺]:[NADH] ratio of 10 was reduced to about 70 % inhibition by a twofold increase in this ratio at the same 0.1 mM NADH.

Although the apparent K_m for NAD⁺ for the three mutated forms of LPD was similar to that of the native protein, the apparent K_i for NADH was at least three times higher than that of the native protein (Table 4). The higher apparent K_i values for NADH led to a lower level of

Table 4. Kinetic characteristics of the LPD isolated from *E. coli* strains with different changes

Enzymes were purified from *E. coli* strains ZS3 (native protein), ZS28 [LPD (E354G)], ZS34 [LPD (E354P)] and ZS40 [LPD (E354W)]. The reported values are for the LPD forward reaction utilizing dihydrolipoic acid and NAD⁺ as substrates.

LPD change	K_m (NAD ⁺) (mM)	K_i (NADH) (μ M)
Native	0.14	2.20
E354G	0.15	7.36
E354P	0.19	6.74
E354W	0.14	6.57

inhibition of LPD activity by NADH (Fig. 3a). The three mutated forms of the enzyme had slightly different NADH inhibition profiles from the native enzyme. At an NADH concentration of 0.1 mM, the E354G form of the protein still retained about 60 % of its activity while the native protein lost about 90 % of its activity. The other two mutated forms of the protein had NADH inhibition profiles that were similar to that of E354G-LPD up to an NADH concentration of about 0.08 mM and differed significantly after that concentration (Fig. 3a).

In the reverse reaction, LPD is known to require NAD⁺ for activation (Kim *et al.*, 2008; Sahlman & Williams, 1989; Scouten & McManus, 1971) and a native LPD activity of about 10 units was increased by about 15-fold by 0.4 mM NAD⁺ (Fig. 3b). However, as seen previously with the E354K form of the enzyme (Kim *et al.*, 2008), the three mutated forms of LPD had higher activity in the absence of NAD⁺, and the amount of NAD⁺ required for full activation was also lower than the amount required for full activation of the native enzyme. Among the mutated forms studied, activity of the E354G form of the enzyme without NAD⁺ (128 units) was almost as high as that of the fully activated native enzyme (154 units). This activity of E354G-LPD was only increased by about 1.5-fold to 200 units by 0.1 mM NAD⁺. These results show that the E354G mutation almost eliminated the need for NAD⁺ for activation of the enzyme in the reverse reaction in which NADH serves as substrate.

Increasing the concentration of NADH, a substrate for the reverse reaction, increased the LPD activity until the highest activity was reached at about 30 μ M NADH for the native enzyme (Fig. 3c). NADH beyond this concentration inhibited the reverse reaction as reported previously (Sahlman & Williams, 1989), and at 0.3 mM NADH, the native enzyme only retained about 10 % of the highest observed activity. In these experiments, the concentration of NAD⁺ as an activator was 0.1 mM. Increasing the NAD⁺ concentration partially reversed the NADH inhibition of native LPD activity. The mutated forms of LPD required a higher concentration of NADH for maximal activity (about 100 μ M), indicating a higher apparent K_m for NADH that is in accordance with the observed higher

Fig. 3. Effect of changing glutamate at position of 354 on NADH sensitivity of *E. coli* LPD. (a) LPD forward reaction was assayed with 1 mM NAD⁺ as substrate and increasing concentrations of NADH as inhibitor. Specific activities of LPDs without added NADH were 410, 86.2, 31.7 and 81.9 units for the native LPD and the proteins with E354G, E354P and E354W changes, respectively. (b) NAD⁺ activation of LPD in the reverse reaction. Substrate NADH concentration was fixed at 0.1 mM. (c) LPD reverse reaction with 0.1 mM NAD⁺ as activator. The highest observed value for each mutant was set as 100% and these values were 118, 202.8, 120.9 and 115.8 units of activity for the E354E, E354G, E354P and E354W proteins, respectively.

apparent K_i for NADH in the forward reaction (Table 4). These mutated forms of the LPD were also less sensitive to NADH inhibition compared with the native enzyme in the reverse reaction. These results are in agreement with the observation that the amino acid changes at position 354 result in a protein with a lower affinity for NADH than the native enzyme.

Apparently, this difference in NADH sensitivity of the LPD and the resulting PDH complex is responsible for the observed PDH-dependent anaerobic growth of the mutants.

Homology modelling of *E. coli* LPD

In order to evaluate the potential structural changes in the LPD that led to the reduction in NADH sensitivity of the enzyme, a structural model of *E. coli* LPD was constructed. Since the *E. coli* LPD is yet to be defined structurally, the *N. meningitidis* LPD X-ray structure (PDB-ID, 1OJT) (Li de la Sierra *et al.*, 1997) was used as a template for this model construction. The 64% amino acid sequence identity between these two LPDs is expected to yield similar structural arrangements between them. Since detailed X-ray structures for the NAD(H)-bound form of the human LPD (PDB ID, 1ZMC and 1ZMD) (Brautigam *et al.*, 2005)

Fig. 4. Model of *E. coli* LPD monomer (coil diagram) depicting the surface-access to the NAD/NADH binding site and an inter-domain conduit from the NADH binding site to surface (dotted arrows). Amino acid E354 and its various mutational alterations (G, P, W and M) are included in different colours. NAD⁺ and NADH are depicted in stick form and coloured according to atom types. Inset: The conduit with E354 is expanded to indicate surrounding water molecules (spheres) through which E354 from one domain could interact with E64 and E65 from the other domain. Amino acids are in stick form. See text for details.

are available, these structures were also used as templates for generation of *E. coli* LPD homology structure with NAD(H). The three structures were superimposed with each other and showed a root-mean-square deviation (RMSD) within 1.0 Å (0.1 nm).

A structure corresponding to each of the substitutions of glutamate at position 354 (E354G, E354P, E354W and E354K) that yielded an LPD with altered NADH K_i was generated. The NAD⁺/NADH binding sites in *E. coli* LPD structure were identified by superimposition with the human LPD structure. In this structure, the binding site for the transiently bound NAD⁺ and NADH is at the end of a surface-accessible cleft created by two domains of the protein (Fig. 4). Both forms of the co-factor had the same adenine dinucleotide conformation but deviated in their nicotinamide mononucleotide conformation (Fig. 4). The glutamate at position 354 appears to act as a gate-keeper in this putative channel by its potential to hydrogen bond with amino acids E64 and E65 across the channel through water molecules [about 3–4 Å (0.3–0.4 nm) distance from E354] (Fig. 4, inset). Such an interaction between E354 and E64/E65 could restrict free movement of NADH to the surface with a putative decrease in the K_i of LPD to NADH (Table 4). Based on the LPD model, the methionine side chain also appears to protrude into the inter-domain channel and, as suggested for E354, methionine sulfur could hydrogen-bond across the putative conduit with E64/E65 from the other domain resulting in limited mobility of NADH from the binding pocket to the surface. Alteration of the glutamate to other amino acids, such as G, P, W or K, probably has a negligible effect on the rate of movement of NADH through the water-filled conduit by minimizing hydrogen bonds with neighbouring amino acids across this channel. If these amino acid changes increased the ability of NADH to travel through and out of the binding site, the kinetics of association/dissociation of the substrate/inhibitor with the protein may be altered. The higher apparent K_i for NADH observed with the mutated forms of the LPD (Table 4) are in agreement with such an alteration. These results suggest that one of the causes of reduced NADH sensitivity of LPD and PDH that led to PDH-dependent anaerobic growth and generation of homoethanol producing *E. coli* strains is an increase in NADH movement (leading to lower affinity) from the binding site in the LPD protein to the surface.

It is interesting to note that PDH has not evolved naturally towards an NADH-insensitive form that can also function anaerobically. Although all the substitutions at E-354 of LPD supported PDH activity, mutant strains with altered PDH (PDH*) grew at a significantly lower growth rate even under aerobic conditions compared with the wild-type with the native E354 form of LPD (Table S2). Even if the evolved PDH* in conjunction with ADH-E can help maintain redox balance during anaerobic growth through homoethanol production, it is at the expense of an additional net ATP per glucose that can be generated by conversion of pyruvate to acetyl-CoA through PFL and

further metabolism of acetyl-CoA to equimolar amounts of acetate and ethanol (Fig. 1). It is possible that the lower growth rate and reduced ATP yield per glucose during anaerobic growth diluted out spontaneously arising PDH* mutants in the population and yielded an evolutionary advantage to native PDH.

Conclusion

Substitution of glutamate at position 354 of LPD with any of the other natural amino acids, except methionine, led to a PDH complex that functions in an anaerobic *E. coli* *ldhA*, *pflB* double mutant producing ethanol as a fermentation product. In three of the substitutions, E354G, E354P and E354W, studied in detail, the alteration in LPD and the associated PDH complex lowered the affinity of the enzyme to NADH. Glutamate at position 354 is not known to interact with NAD⁺, NADH or FAD directly and the effect of the mutation in lowering the affinity for NADH is probably mediated through structural requirement of glutamate at this position as a potential gate-keeper in NAD(H) movement between the surface and the binding site in the protein.

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