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Altering the sensitivity of *Escherichia coli* pyruvate dehydrogenase complex to NADH inhibition by structure-guided design

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ABSTRACT

A sufficient supply of reducing equivalents is essential for obtaining the maximum yield of target products in anaerobic fermentation. The pyruvate dehydrogenase (PDH) complex controls the critical step in pyruvate conversion to acetyl-CoA and NADH. However, in anaerobic *Escherichia coli*, PDH residing in the dihydrolipoamide dehydrogenase (LPD) component is normally inactive due to inhibition by NADH. In this study, the protein engineering of LPD by structural analysis was explored to eliminate this inhibition. A novel IAA350/351/358VVV triple mutant was successfully verified to be more effective than other LPD mutants reported till date. Notably, PDH activity with the triple mutant at an [NADH]/[NAD^+] ratio of 0.15 was still higher than that of the wild-type without NADH addition. The altered enzyme of the PDH complex was also active in the presence of such high NADH levels. This is the first study concerning protein engineering of PDH by structure-guided design. The presence and functional activity of such an NADH-insensitive PDH complex provides a useful metabolic element for fermentation products and has potential for biotechnological application.

1. Introduction

Escherichia coli is a facultative heterotroph microorganism that grows well under both aerobic and anaerobic conditions. The mitochondrial pyruvate dehydrogenase (PDH) complex regulates the critical step in carbohydrate utilization, namely the conversion of pyruvate to acetyl-CoA and NADH [1,2]. Genes encoding the PDH complex have been characterized in *E. coli*. The three components of the complex are encoded by a single operon that includes a regulatory gene (*pdhR*) and the structural genes, *aceE* (pyruvate dehydrogenase, E1), *aceF* (dihydrolipoamide acetyltransferase, E2), and *lpd* (dihydrolipoamide dehydrogenase, E3) [3]. Although PDH is significant for aerobic growth of the bacteria, this activity can also be detected in cell extracts of *E. coli* when grown under anaerobic conditions. While, PDH activity is normally deficient *in vivo* of *E. coli* under anaerobic conditions. During aerobic growth, the NADH generated in glycolysis is ultimately oxidized by oxygen whereas the organic compounds generated from glycolysis act as electron acceptors to keep redox balance and maintain bacterial growth under anaerobic conditions. Therefore the intracellular [NADH]/[NAD^+] ratio (~ 0.75) of an anaerobic cell is much higher than that (~0.03) of an aerobic cell because of differences in electron acceptors between the two growth patterns [4]. In anaerobic *E. coli* cultures, PDH activity is either very low or undetectable due to inhibition by high level of NADH [5,6]. Further study has verified that NADH sensitivity of the PDH complex resides in the dihydrolipoamide dehydrogenase (LPD) component as only this enzyme interacts with NAD^+ as a substrate [7].

Industrial fermentation aims to produce valuable products from cheap feedstocks by utilizing diverse microbial functions through aerobic or anaerobic fermentation [8–10]. During anaerobic fermentation, the reducing power is mostly directed to product synthesis rather than being oxidized, resulting in a higher yield of target products [11]. For example, organic acids such as lactic acid and succinic acid are efficiently produced through anaerobic fermentation [12,13]. However, the maximum yield of succinic acid production through the anaerobic fermentative pathway is restricted by NADH supply. A sufficient supply of reducing equivalents is essential for obtaining the maximum yield of target fermentation products. PDH converts pyruvate

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Table 1
Primers used for site-directed mutagenesis of dihydrolipoamide dehydrogenase.

Primer[#]	Sequence (5′-3′)[§]
A358V +	CTATACCGAACCAGAAGTTGTTTGGGTGGGTCTGACTGAG
A358 V -	CTCAGTCAGACCCACCCAAACAACTTCTGGTTCGGTATAG
IA350/351 V V +	CGAAAGTTATCCCGTCCGTTGTTTATACCGAACCAGAA
IA350/351 V V -	TTCTGGTTCGGTATAAACAACGGACGGGATAACTTTCG
EA354/358KV +	GTCCATCGCCTATACCAAACCAGAAGTTGTTTGGGTGGGTCTGACTG
EA354/358KV -	CAGTCAGACCCACCCAAACAACTTCTGGTTTGGTATAGGCGATGGAC

[#] +, sense sequence; -, anti-sense sequence.
[§]The underlined codons indicate the respective mutation site.

into acetyl-CoA with NADH being produced; if PDH is activated, the NADH availability can be improved, leading to increased production yield when NADH is required in the pathway [14]. It has been reported that activating PDH by increasing *aceEF* gene expression has improved ethanol production in *E. coli* [15]. Activating PDH has also satisfied the reducing equivalent requirement to increase anaerobic butanol production [16]. In addition, the theoretical maximum yield of 0.75 mol/mol for anaerobic 1,3-propanediol production from glycerol can be achieved after activating PDH [17]. A high-succinate-producing strain was screened by metabolic evolution. It turned out that PDH was greatly activated and that the sensitivity of PDH complex to NADH inhibition was attenuated due to three nucleotide mutations in *lpd* [17]. PDH activity increased under anaerobic conditions, which improve NADH supply for succinate production. Therefore, this could be a potential strategy to improve NADH supply under anaerobic conditions by regulating PDH activity through modifying its E3 component, LPD.

Although there are few reports on LPD mutation, attempts to find more key sites that further increase PDH activity under anaerobic conditions are still highly desirable. Protein engineering for improving enzyme properties is playing increasingly important roles in chemical biotechnology, aiming to produce chemicals from biomass [18]. In this study, we first explored protein engineering of LPD by structural analysis to find more target sites and the IAA350/351/358VVV triple mutant was successfully verified to be the most effective at improving PDH activity. The mutated LPD attenuated sensitivity to NADH inhibition and also allowed the PDH complex to be more tolerant to NADH in anaerobic *E. coli* culture. The *lpd* mutation identified in this work may have wide applications in the production of reduced chemicals in *E. coli*, including succinate, ethanol, butanol, 1,3-propanediol and so on

2. Materials and methods

2.1. Strains, plasmids and media

The vector pMD19-T (TaKaRa, Dalian, China) was used as general cloning, and *E. coli* DH5α (Tiangen, Beijing, China) was used as the host strain for plasmid construction. Plasmid pET-28a(+) (Novagan, Lausanne, Switzerland) was used as protein expression vector and *E. coli* BL21(DE3) (Tiangen) was used as the host strain for target protein expression. *E. coli* cells were cultured aerobically at 37 °C in a rotatory shaker at 220 rpm in Luria-Bertani (LB) medium.

2.2. Strains and plasmids construction

The *lpd* gene from *E. coli* were amplified by polymerase chain reaction (PCR) using the following primers: forward primer (5′-GCGGG ATCCATGAGTACTGAAATCAAAACTC-3′) containing *Bam*HI restriction site and reverse primer (5′-ACACTCGAGTTACTTCTTCTTCGCTTT CGG-3′) containing *Xho*I restriction site. The amplified *lpd* fragments were first ligated into the vector pMD19-T for sequencing. After validating that the insert does not undergo mutations, the *lpd* fragments were then digested with *Bam*HI and *Xho*I and ligated into pET-28a(+) digested with the same enzymes to create pET-28a-LPD. The constructed pET-28a-LPD was then transformed into the competent cells of *E. coli* BL21 (DE3) for protein expression.

2.3. Site-directed mutagenesis

Site-directed PCR mutagenesis using *Pyrobest* DNA polymerase (TaKaRa, Dalian, China) was used to construct mutant strains. All the primers used for introducing the mutations were listed in Table 1. *Lpd* fragments with site-directed mutagenesis were amplified from pET-28a-LPD based on the standard procedures using specific primers. The restriction enzyme *Dpn*I (TaKaRa, Dalian, China) was used to eliminate the native plasmid. The *Dpn*I-digested product was then purified and transformed into the competent cells of *E. coli* BL21 (DE3). The cells were then plated on LB agar plates containing 30 μg/mL kanamycin. After overnight cultivation, single bacterial colonies were randomly selected for confirming by further gene sequencing.

2.4. Expression and purification of dihydrolipoamide dehydrogenase

E. coli BL21(DE3) harboring the pET-28a-LPD with or without mutation sites were cultured in LB media containing 30 μg/mL kanamycin at 37 °C in a rotatory shaker at 220 rpm. 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce appropriate expression of gene *lpd* when the cell growth reached an optical density (OD_{600}) of 0.6-0.8. Cells were harvested by centrifugation after induction at 25 °C for 8 h, resuspended in binding buffer composed of 10 mM imidazole (pH 7.8), 500 mM sodium chloride and 20 mM Tris-HCl. The cells were then disrupted by sonication. And the suspension was again centrifuged (12,000 × *g*, 10 min) so as to remove cell debris. LPD was purified by nickel affinity chromatography referring to the operating manual (Tiandz, Beijing, China). The purified LPD was eluted with the elution buffer composed of 300 mM imidazole (pH 7.8), 500 mM sodium chloride and 20 mM Tris-HCl. The effluent was then further purified with a filtration column HisTrap HP (GE Healthcare, Shanghai, China). The enzyme was eluted from the column with phosphate buffer (pH 7.0) at a 5 mL/min flow rate. The SDS-PAGE and the Bradford method were used to detect the purified enzyme and determine the total protein concentration, respectively.

2.5. Enzymatic activity assay

The method used for measuring PDH and LPD activity was achieved as described previously [4]. The crude extract in 100 mM Tris-HCl buffer (pH 7.5), 0.1 mM CoA, 0.2 mM thiamine pyrophosphate, 10 mM $MgCl_2$, 1 mM DTT, 2 mM NAD^+ consisted of 1 ml of reaction mixture. The pyruvate (5 mM) was added to initiate the enzymatic reaction. The generation of 1 μmol min^{-1} mg $protein^{-1}$ was regarded as one unit of enzyme activity. Various concentrations of NADH were added into the same reaction mixture to study the effect of NADH on enzyme activity.

The appropriate amount of enzyme, 1.5 mM EDTA, 3 mM NAD^+, 0.1 M KH_2PO_4 (pH 8.0), 3 mM NAD^+, 3 mM DL-dihydrolipoic acid consisted of 1 ml of forward reaction mixture. The generation of 1 mol