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Attempting to Remove the Substrate Inhibition of L-Lactate Dehydrogenase from *Bacillus stearothermophilus* by Site-Directed Mutagenesis

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Abstract

L-lactate dehydrogenase (LDH) catalyzes the interconversion of an oxo-acid (pyruvate) and hydroxy-acid (lactate) using the NADH/NAD^+ pair as a redox cofactor. The enzyme has a commercial significance, as it can be used to produce chiral building blocks for the synthesis of key pharmaceuticals and agrochemicals. However, the substrate inhibition which is due to an abortive NAD^+-pyruvate complex reducing the steady state concentration of functional LDH limits its use in industry. This substrate inhibition can be overcome by weaking the binding of NAD^+.

The conserved aspartic acid residue at position 38 was replaced by the longer basic arginine side chain (D38R) using PCR based overlap extension mutagenesis technique in the hope of weakening NAD^+-binding. The mutant gene was overexpressed in the *Escherichia coli* high-expression vector pKK223-3 in JM105 cells; then, the mutant protein was produced. Comparing the effect of substrate inhibition in the arginine-38 mutant with wild-type, substrate inhibition is decreased threefold.

Index Entries: NAD^+-dependent lactate dehydrogenase; substrate inhibition; protein engineering; biocatalysis; chiral hydroxyacids.

Introduction

The L-lactate dehydrogenase (L-LDH) enzyme from *Bacillus stearothermophilus* has been the subject of intensive research of its structure and catalytic properties. This has been prompted by its industrial potential in

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the production of chirally pure hydroxyl compounds used in the production of various pharmaceuticals and in medical diagnostics *(1–3)*. Experiments to synthesize 100% enantiomeric excess, chiral-substituted lactates using L-LDH from *B. stearothermophilus* both in its wild-type form and mutant versions have been carried out at Bristol University. LDH catalyses the interconversion of an oxo-acid (pyruvate) and hydroxy-acid (lactate) using the $NADH/NAD^+$ pair as a redox co-factor. The reaction in the pyruvate-to-lactate direction is achieved by reducing the carbonyl group of the oxo-acid substrate with a hydride ion (H^-) donated by NADH and a proton (H^+) from the protein. The production of chiral (S) α-hydroxyacids is now at the 10-ton scale in industry and any development facilitating the reduction of pyruvate at concentrations greater than 0.1 *M* would be profitable. However, in spite of having ability to produce chiral hydroxy acids (e.g., lactate) from their oxo acids with high stereochemical fidelity, *bs*LDH is not widely used in industry. The most common disadvantage of LDHs is their inhibition by the excess of substrate (pyruvate). This problem should be overcome in order to make LDH a more suitable enzyme for large-scale production of chiral (S)-hydroxyacids. Protein engineering techniques could be promising tools to create LDH with the desired properties.

As shown previously *(4)*, the mutation aspartic acid38glutamate (previously named aspartate52glutamate) in the LDH from *B. stearothermophilus* reduces the substrate inhibition by approximately twofold. The referred-to work demonstrates that the designed mutants would weaken the cofactor binding to relieve substrate inhibition. In this paper, we describe the construction of the arginine-38 mutant protein by PCR-based site-directed mutagenesis and compare the kinetic behavior of mutant protein with the wild type. This is significant, as threefold reduction of the substrate inhibition may lead to more practical and useful industrial applications.

Materials and Methods

Site-Directed Mutagenesis to Construct Asp38Arg Mutant bsLDH

Bacterial strain *Escherichia coli* JM105 [F′ traD36 $proA^+$ $proB^+$ laclq lacZΔM15/Δ (lac-pro) X111 thi rpsL (Strr) endA sbcB supE hsdR9] was used as a host to prepare all double-stranded DNA for mutagenesis and sequencing in the plasmid pKK223-3 (Pharmacia Biotech, Uppsala, Sweden). The plasmid pKK223-3 is a transcription expression vector and contains a tac promoter (IPTG-inducible) which drives expression of recombinant genes and ribosomal termination sequences as well as ampicilin resistance *(5)*. The same *E. coli* strain was also used as a host for transformation and expression of *bs*LDH proteins in pKK223–3. All PCRs were carried out in the presence of 2 μ*M* of each dNTPs (Fermentase), 20 pmol/1 μL of each primer, and 5 U *Taq* polymerase (Fermentase). Subsequently, oligonucleotides were used as PCRs primers (N-terminus; 5′CGGAATTCATGAAAAACAACGGTGGAGC-3′ and C-terminus; 5′AAAACTGCAGTCATCGCGTAAAAGCACG-3′) in order to amplify the

gene and construct a mutant gene (5′GTGCTCATC**CGC**GCGAATGAA-3′ and its complementary). Eight hundred-base pair (bp) and 150-bp fragments were obtained (initial denaturation at 94°C for 5 min, 94°C for 1.5 min, 55°C for 2 min, 72°C for 2 min, and a final extension at 72°C for 10 min, for 20 cycles). The oligonucleotides were joined by overlap extension (initial denaturation at 94°C for 5 min, 94°C for 2 min, 50°C for 1 min, 72°C for 4 min, and a final extension at 72°C for 10 min, for 7 cycles). After purification by 1% agarose gel, the gene product was amplified (initial denaturation at 94°C for 5 min, 94°C for 1.5 min, 55°C for 2.5 min, 72°C for 2 min, and a final extension at 72°C for 10 min, for 20 cycles). The mutant gene was first cloned into plasmid TA vector using QIAGEN PCR Cloning Kit. The gene product in TA vector was cut with restriction enzymes *Eco*RI and *Pst*I and ligated into similarly digested pKK223-2 expression vector. Ligation was electroporated into *E. coli* JM105 and selected on LB agar containing 100 µg/mL as described by Sambrook et al. *(6)*. Mutant double stranded DNA was sequenced to check for the correct amino acid change using the ABI Prism 3100 Avant automated sequencer at Molecular Biology and Genetics Dept., ITU.

Cell Culture and Enzyme Purification

The same protocols were used to purify both wild-type enzyme and D38R LDH from the bacterial cultures harboring the desired plasmid, which were grown on a rotary shaker in Lauria Broth containing ampicilin (100 µg/mL). Overnight cultures (3 L) were centrifuged at 6000*g* for 20 min**,** and the pelleted cells were resuspended in minimum 50 m*M*, pH 6.0 trithanolamine (TEA) volume. The cells were sonicated (five 30-s bursts) on wet ice, then centrifuged at 27,000*g* for 30 min. The protein in the supernatant was precipitated by adding 430 mg $(NH_4)_2SO_4$ per milliliter of supernatant and recovered by centrifugation (27,000*g*, 20 min). The resulting pellet was dissolved in a minimum volume of 50 m*M*, pH 6.0 TEA and dialyzed against same buffer overnight at 4°C. Fast protein liquid chromatography (FPLC; Biorad) system was used to purify *bs*LDH. After dialysis, the protein sample was loaded onto a Blue-Sepharose column (Amersham-Biorad), which had been washed with two column volumes of 50 m*M* TEA, pH 6.0. LDH was eluted by using a gradient from 0 *M* to 1 *M* NaCl in 10 m*M* TEA, pH 6.0.

Catalytic Kinetics Analysis

All kinetic parameters were measured in the absence and presence 5 m*M* fructose 1,6-bisphosphate (FBP), an enzyme activator *(7,8)* at 25°C in a reaction mixture containing, 50 m*M* TEA buffer at pH 6.0, 0.2 m*M* NADH, 5 n*M* enzyme, 0–100 m*M* pyruvate. Kinetic parameters were determined by observing the oxidation of the nicotinamide coenzymes at 340 nm in 10 mm, 1 mL quartz cuvets mounted in a Perkin Elmer Instruments Lambda 25 UV/VIS spectrometer. The data were calculated and fitted the nonlinear regression analysis programs within GRAFIT (Data Analysis and Graphics Program Version 3.01).

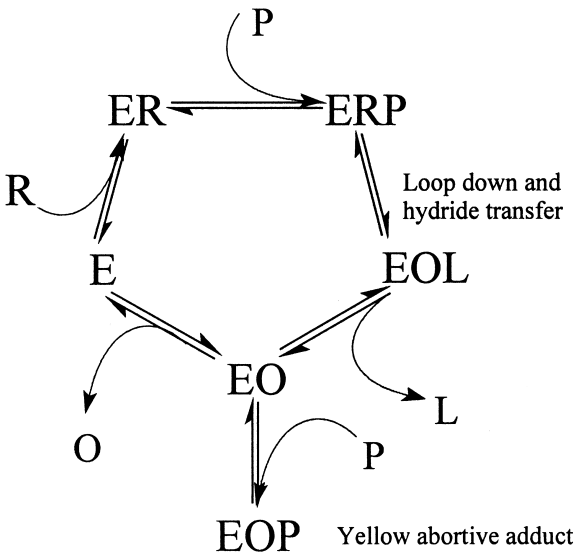

Fig. 1. The catalytic cycle of *Bacillus stearothermophilus* lactate dehydrogenase showing reversible formation of abortive ternary complex, E:NAD^+-Pyr. E, enzyme; R, NADH; O, NAD^+; P, pyruvate; L, lactate.

Results and Discussion

Both bacterial and mammalian LDHs show inhibition by high concentrations of pyruvate. It has been known for some time *(9–11)* that this inhibition is a consequence of the formation of LDH:NAD^+-pyruvate abortive ternary complex. The NAD (H)-dependent L-LDHs catalyze the reduction of pyruvate by an ordered catalytic mechanism. Figure 1 indicates all the steps of the catalytic cycle of LDH. Initially, the coenzyme NADH goes into reaction with the enzyme to form E:NADH binary complex. Then NADH transfers its hydride ion to substrate (pyruvate) to form lactate, which leaves the enzyme:NAD^+ binary complex. In the final step, the replacement of NADH with NAD^+ takes place. At high concentrations of substrate, before the release of oxidized cofactor NAD^+ from the enzyme, a problem arises from binding of pyruvate to the LDH:NAD^+ binary complex to form the inhibitory abortive adduct (E:NAD^+-Pyr). The main cause of this problem is that the rapid hydride transfer step from NADH to pyruvate is diminished by excess substrate. Furthermore, the characterization of the yellow inhibitory adduct is monitored spectrophotometrically at 327 nm, and the dissociation constant of pyruvate from LDH:NAD^+-pyruvate complex is similar to the inhibition constant for pyruvate.

The removal of the substrate inhibition using the protein engineering techniques is a challenge. For this, two strategies have been employed. One is to reduce the affinity of the LDH:NAD^+ complex for pyruvate. The second is to reduce the affinity of the enzyme for NAD^+. Previously, it was shown that the mutation on serine163leucine in the LDH from the

B. stearothermophilus (12), human muscle *(13)*, and human heart *(14)* isoform would remove the substrate inhibition using the first strategy. The second strategy was also tested by construction of mutation on aspartate38 glutamate in the LDH from *B. stearothermophilus (4)*. The referred-to work proves that the designed mutant would weaken the cofactor binding two-fold. This leads to production of more commercially significant mutants insertion of other large no acidic residues at the same position.

The binding site for NAD^+ is similar in all dehydrogenases. The NAD (H) coenzyme is in an open conformation and the adenine ribose ring of it is surrounded by Asp 38, Val 39, and Gly 99. Asp 38 and Gly 99 give some specificity to the adenine orientation. Asp 38 is an important residue in stabilizing NADH binding *(10)*. The adenine ribose is positioned by two hydrogen bonds to its hydroxyl. One of the two hydrogen bond acceptors, Asp 38, together with its neighboring residues moves 2 A° to provide sufficient room for the coenzyme. This interaction has been observed in all binary complexes in which the ribose is present *(15)*. In the wild-type enzyme, the aspartate residue forms a hydrogen bond with the 2′ hydroxyl of the adenine ribose of NADH *(15)*. As shown in ref. *16*, this aspartate residue in the LDH from *B. stearothermophilus* was replaced by serine. This mutant enhanced the catalytic efficiency of the enzyme with NADPH compared to the wild type. Although the mutant was not tested for the substrate inhibition, this aspartate was the key residue for weakening NAD(H) binding.

In this study, the conserved aspartic acid residue at position 38 has been replaced by the largest positively charged amino acid residue, arginine, to destabilize NAD(H) binding. The effect of this change on the kinetic behavior of enzyme was determined. Exchanging aspartic acid with argin-ine may cause several changes. One could be that the NAD^+-binding in the E:NAD^+-pyruvate complex is less favored, because of both pyruvate and NAD^+ substrates in the same oxidation state. In particular, such abortive complexes make the binding of E:NAD^+ weaker and offer fewer chances for the binding of pyruvate. In the wild type, the aspartic acid is a small, negatively charged molecule and binds NADH tightly. Because arginine is the longest basic amino acid, it is expected to disturb NADH binding, which would likely become weaker. That the mutant would have less substrate inhibition was initially the aim.

The mutant was constructed by using PCR-based site-directed mutagenesis. Despite the fact that many variants of PCR-based mutagenesis have been published *(17,18)*, the overlap extension PCR mutagenesis technique is the most common method as it is very fast, simple, and produces the 100% efficient results in the generation of mutant product *(19, 20)*. The sequencing results confirmed that the expected sequence was obtained for the D38R mutation.

Further experiments showed that the gene was overproducing protein without using any inducer and proved that this protein was correctly folded by the appearance of an intense band on a Coomassie Blue-stained sodium dodecyl sulfate (SDS) polyacrylamide gel (Fig. 2). Both the

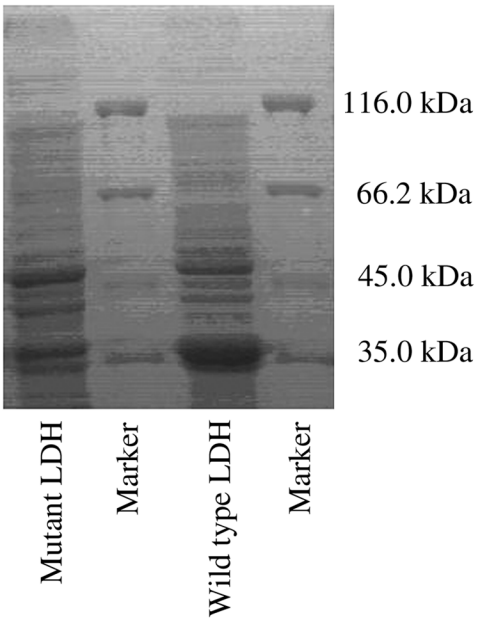

Fig. 2. The sodium dodecyl sulfat-polyacrylamide gel electrophoresis gel of the overexpressed D38R *Bacillus stearothermophilus* lactate dehydrogenase by Coomassie Blue.

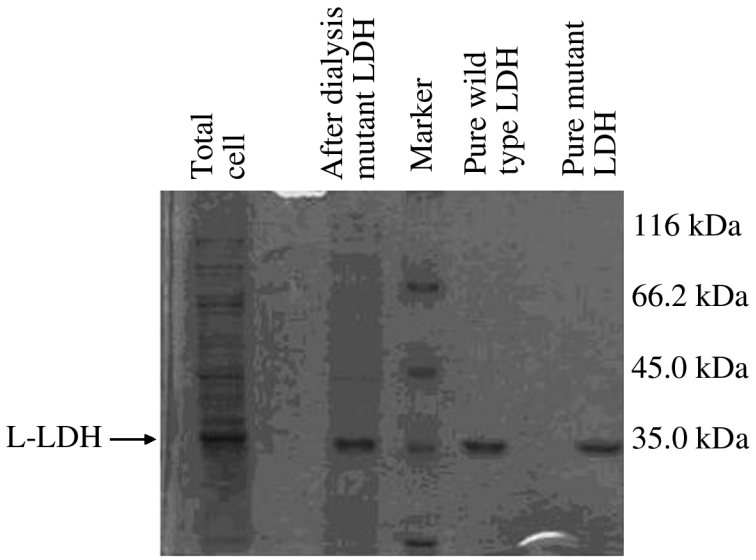

Fig. 3. The result of sodium dodecyl sulfat-polyacrylamide gel electrophoresis from the Q-Sepharose and Blue-Sepharose.

wild type and mutant proteins have been purified by using two different chromatography techniques, namely ion-exchange chromatography and affinity chromatography. Coomassie Brilliant Blue-stained SDS-polyacrylamide

Table 1
Kinetic Results for Both Wild-Type and Mutant *bs*LDH Proteins

	Wild-type LDH		Mutant LDH	
	+FBP	–FBP	+FBP	–FBP
V_{max} (abs/min)	0.0393	0.6106	0.3328	0.36
K_m (m*M*)	2.7617	32	3.7	19.3
K_i (m*M*)	10.9	–	31.1	–
k_{cat} (s^{-1})	21.06	327.22	178.35	194.85

LDH, lactate dehydrogenase.

gel electrophoresis (PAGE) showed that the purity of the proteins was over 95% after the Blue-Sepharose affinity chromatography (Fig. 3). Two pure proteins were kinetically characterized. The steady-state catalytic properties of both the wild type (Asp38) and the mutant (Arg38) with pyruvate in the presence (+FBP) and absence of FBP (–FBP) are shown in Table 1.

In conclusion, the D38R mutation in *bs*LDH decreases substrate inhibition threefold, whereas k_{cat} for pyruvate is only modestly reduced. This kinetic behavior is much better improved compared to that previously observed for the Asp38Glu mutation in *bs*LDH *(4)*. Although the threefold effect on substrate inhibition is not large, this work demonstrates the possibility of producing more commercially useful mutants by changing the conserved acidic aspartic acid residue to the largest basic arginine without losing the activity of the *bs*LDH.

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