Impact of Fructose 1,6-Bisphosphate on Enzyme Kinetics and Structure of Lactate Dehydrogenase

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ABSTRACT

The enzyme lactate dehydrogenase (LDH) is responsible for catalyzing lactic acid fermentation in the absence of oxygen. Fructose 1,6-bisphosphate is an allosteric modulator of LDH, known to impact its enzyme activity. To investigate if F16BP will activate or inhibit the enzyme, LDH is derived from *Geobacillus stearothermophilus*. As a result, F16-BP binds to LDH-GBS, causing a conformational change which increases the affinity on the enzyme's active site with the substrate. Furthermore, F16-BP is a positive allosteric effector as it enhances the enzyme's catalytic speed by lowering the detection limit and increasing the rate of conversion. Lastly, F16BP stabilizes the LDH-GBS in the tetramer form, allowing it to perform more efficiently compared to LDH-GBS not bound to F16BP.

INTRODUCTION

Lactate dehydrogenase (LDH) is an enzyme used in metabolic processes in prokaryotes and eukaryotes. Specifically, LDH is an oxidoreductase that catalyzes the reversible conversion of pyruvate to lactate in anaerobic conditions. Using nicotinamide adenine dinucleotide (NAD+/NADH) as a cofactor, pyruvate and NADH are converted to lactate and NAD+ (1). This allows the cell to recycle the supply of NAD+ for glycolysis, a critical metabolic pathway necessary for ATP production, when oxygen is not available.

As mentioned, LDH can be found in bacterial and vertebral cells, yet its activity is regulated differently (2). Since multicellular organisms are compartmentalized, LDH is regulated by substrate availability. Whereas, unicellular organisms use fructose 1,6-bisphosphate (F16BP) to function as an allosteric regulator for LDH. For this study, LDH is harvested from *Geobacillus stearothermophilus* (LDH-GBS), a gram-positive thermophile, because it serves as a suitable model due to stability, protein expression, and production of enantiopure α-hydroxy acids (2).

LDH is present as a tetramer in cells, or in its quaternary structure. Therefore, the addition of an allosteric regulator may result in a change of properties compared to the monomeric form of LDH. To study the change, Dithiobis succinimidyl propionate (DSP), a protein crosslinker, can be added to LDH-GBS. DSP contains amine-reactive NHS esters (N-hydroxysuccinimide ester) form stable amine bonds at pH 7-9. Amine bonds in a protein can be found in the lysine residues and the N-terminus of each polypeptide (3). The increased bond formation between multiple protein chains may cause a conformational change, which impacts enzyme behavior.

Enzyme kinetics refers to the rate at which an enzyme converts substrate into product—an important aspect of enzyme function. Kinetic parameters determine the efficiency of an enzyme in substrate conversion, maximum rate of enzyme saturation, and binding affinity. Therefore, the Michaelis-Menten model is used to define the catalytic parameters of LDH. Before application of the model, three assumptions are made (4). First, binding goes to equilibrium before catalysis can proceed. Second, at time = 0, there is no product so the catalysis reaction is not reversible. Third, the reaction is in a steady state. Based on these assumptions, the model states that the initial rate of an enzyme catalyzed reaction at any given concentration of substrate is related to V_{max} and K_M (4). The determination of those parameters

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will aid in determining k_{cat} and the specificity constant. Yet, these variables are subject to change if an allosteric modulator is added.

The objective of the study is to determine how fructose 1,6-bisphosphate allosterically regulates the quaternary structure and enzymatic activity of LDH-GBS.

RESULTS

Kinetic parameters

Table 1: Two technical replicates of LDH-GBS optimization with diluted concentrations of pyruvate (mM) to NAD+ (umol) concentrations at A340/min without F16BP

[Pyruvate] stock (mM)	[Pyruvate] in cuvette (mM)	dA340/min	umol NADH/min
100	50	0.5406	0.08691318328
50	25	0.3539	0.05689710611
20	10	0.3223	0.05181672026
10	5	0.4896	0.07871382637
5	2.5	0.4030	0.06479099678
2	1	0.2912	0.04681672026
1	0.5	0.3096	0.04977491961
0.5	0.25	0.0726	0.01167202572
100	50	0.5378	0.08646302251
50	25	0.4886	0.07855305466
20	10	0.4997	0.08033762058
10	5	0.5050	0.08118971061
5	2.5	0.3384	0.05440514469
2	1	0.3115	0.05008038585
1	0.5	0.2935	0.04718649518
0.5	0.25	0.0599	0.00963022508

Table 2: Two technical replicates of LDH-GBS optimization with diluted concentrations of pyruvate (mM) with F16BP added to NAD+ (umol) concentrations at A340/min.

[Pyruvate] stock (mM)	[Pyruvate] in cuvette (mM)	dA340/min	umol NADH/min
100	50	0.4215	0.06776527331
50	25	0.337	0.05418006431
20	10	0.2528	0.04064308682
10	5	0.2132	0.03427652733
5	2.5	0.1144	0.01839228296
2	1	0.0587	0.009437299035
1	0.5	0.0478	0.00768488746
0.5	0.25	0.0261	0.004196141479
100	50	0.3412	0.05485530547
50	25	0.3292	0.05292604502
20	10	0.2400	0.038585209
10	5	0.2066	0.03321543408
5	2.5	0.1058	0.0170096463
2	1	0.0503	0.00808681672
1	0.5	0.0432	0.006945337621
0.5	0.25	0.0202	0.003247588424

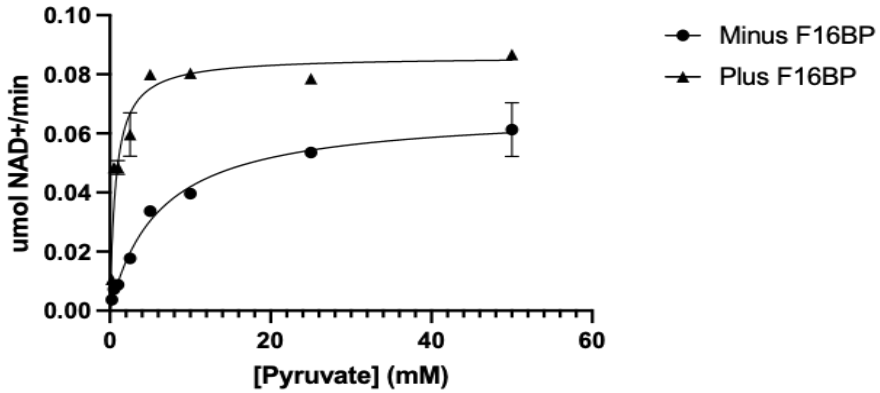

Figure 1: A nonlinear regression model was applied to the Michaelis-Menten curves to display the change in absorbance of NAD+/min at 340 nm as pyruvate concentration increases with and without fructose 1,6-bisphosphate. The R^2 value of -F16BP was 0.8970 and +F16BP was 0.9789.

The data from Tables 1 and 2 were plotted and the Michaelis-Menten curves in Figure 1 showed a visual representation of the decrease in K_M, as a lower concentration of NADH is required to initiate the reaction when F16BP is added. Other kinetic parameters, such as V_{max}, k_{cat}, and catalytic efficiency increased. The V_{max} of the +F16BP was greater than the V_{max} of the -F16BP by a scale of 1.3. The values of k_{cat} and catalytic efficiency increased by 3-fold and 15-fold respectively. Lastly, the nonlinear regression model yielded two R^2 values, which were 0.8970 (-F16BP) and 0.9789 (+F16BP). Although both values were positive, the higher R^2 value indicated that LDH-GBS is more sensitive to changes in concentration of pyruvate when F16BP is added compared to when it is not bound to LDH-GBS.

Table 3: Michaelis-Menten kinetic parameters of LDH-GBS with F16BP and without F16BP

	K_M (mM)	V_{max} (µmol/ml/s)	K_{cat} (s-1)	K_{cat}/K_M (s-1mM-1)
+5mM F16BP	0.7262	0.0860	11.03	15.1887
0mM F16BP	6.1360	0.0676	3.75	0.6114

Quaternary Structure Analysis:

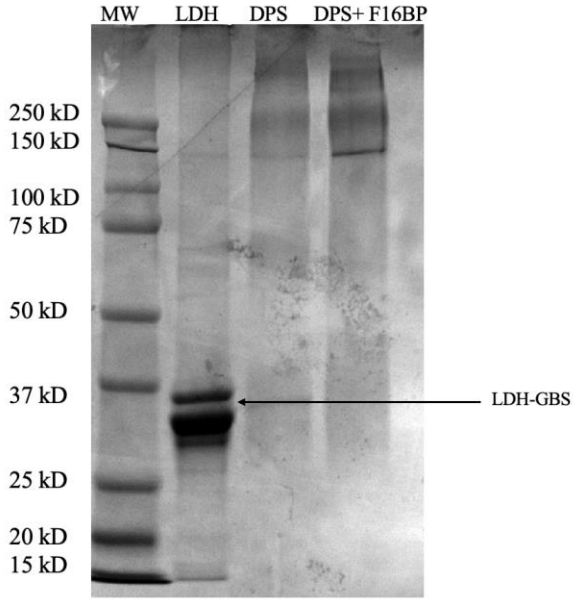

Figure 2: 10% SDS Page ran at 200V for 36 min. From left to right, the lanes include the molecular weight ladder (BioRad Precision Plus Protein Dual Color Standard), LDH-GBS, Dithiobis succinimidyl propionate (DSP) , and DSP with F16BP. LDH-GBS is approximately ~36.5 kD. A protein band was seen at ~150kD, which could be the tetramic form of LDH-GBS.

The monomeric form of LDH has a molecular weight of approximately 36.5 kD, as shown in the second lane from the left. DSP was added to the LDH-F16BP complex for chemical cross-linking and a protein band was seen at approximately 150 kD.

DISCUSSION

Fructose 1,6-bisphosphate was categorized as a positive allosteric regulator for LDH after kinetic parameter determination. The decrease in K_M indicated an increased binding affinity of LDH to its substrate. This was further supported by the values of V_{max} and k_{cat} in Table 3 that showed an increase in speed and concentration of substrate to

enzyme conversion. Lastly, the catalytic efficiency was analyzed using the ratio k_{cat}/K_M. A value greater than 1 meant Kcat was an indicator of the better substrate (5). This proved that fructose 1,6-bisphosphate increased the specificity of LDH, as more substrate molecules saturated the LDH enzyme molecules than if fructose 1,6-bisphosphate was not added.

Chemical cross-linking of LDH and F16BP using DSP was successful as shown in Figure 2. The monomeric form of LDH-GBS at 36.5 kD served as the control to confirm protein expression. When DSP was added, a protein band at approximately 146 kD was seen, but further immunoanalysis would need to confirm to determine if the tetramic form LDH-F16BP complex had expressed.

Methods

Continuous enzyme assay for kinetic parameter determination:

Eight 1:2 serial dilutions of pyruvate stock (100mM) with concentrations ranging from 0.25 mM to 50 mM were prepared. The 100mM pyruvate solution was transferred to a 1 mL cuvette and diluted with phosphate-buffered saline (PBS) to approximately 50mM. Before placing the cuvette in the Cary 60 UV-vis spectrophotometer, NADH (10 mM) and LDH-GBS (1 mg/ml) were added. The cuvette was quickly mixed and the absorbance was measured at 340 nm. This procedure was repeated in duplicate. The only modifications the second time were F16BP was added and LDH-GBS was diluted to 0.5 mg/mL.

Chemical Cross-Linking:

Three LDH-GBS (1 mg/mL) samples were prepared in microfuge tubes. The first only contained LDH-GBS with PBS to serve as control. The second tube was prepared with the cross-linker DSP using manufacturer's protocols (Thermo Scientific) and the third tube contained DSP, LDH-GBS, and F16BP. The samples were incubated at room temperature for 15 min and DSP was added to each sample, followed by a 30 min incubation period. Next, samples were quenched in Tris pH 7.5 (Tris base, deionized water, and HCl) and incubated for 15 min. SDS-PAGE sample buffer was added to each microfuge tube. The samples were heated (85°C, 10 min) and centrifuged for 1 min. Then, the SDS-PAGE was loaded with a molecular weight ladder (BioRad Protein Dual Standard Plus) and the three samples (LDH, DSP, DSP + F16BP) into their respective wells. The gel was run at 200V for approximately 1 hour. The gel was stained with Coomassie stain, incubated on a rocker for 15 min, and destained until ready for imaging.

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