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Impact of High Pyruvate Concentration on Kinetics of Rabbit Muscle Lactate Dehydrogenase

Matthew Warren Eggert · Mark E. Byrne · Robert P. Chambers

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Abstract In order to evaluate the effectiveness of L-lactate dehydrogenase (LDH) from rabbit muscle as a regenerative catalyst of the biologically important cofactor nicotinamide adenine dinucleotide (NAD), the kinetics over broad concentrations were studied to develop a suitable kinetic rate expression. Despite robust literature describing the intricate complexations, the mammalian rabbit muscle LDH lacks a quantitative kinetic rate expression accounting for simultaneous inhibition parameters, specifically at high pyruvate concentrations. Product inhibition by L-lactate was observed to reduce activity at concentrations greater than 25 mM, while expected substrate inhibition by pyruvate was significant above 4.3 mM concentration. The combined effect of ternary and binary complexes of pyruvate and the coenzymes led to experimental rates as little as a third of expected activity. The convenience of the statistical software package JMP allowed for effective determination of experimental kinetic constants and simplification to a suitable rate expression:

$$v = \frac{V_{max}(AB)}{K_{ia}K_b + K_bA + K_aB + AB + \frac{P}{K_{I-Lac}} + \frac{B^2A}{K_{I-Pyr}} + \frac{B^2Q}{K_{I-Pyr-NAD}}}$$

where the last three terms represent the inhibition complex terms for lactate, pyruvate, and pyruvate–NAD, respectively. The corresponding values of $K_{\text{I–Lac}}$, $K_{\text{I–Pyr}}$, and $K_{\text{I–Pyr–NAD}}$ for rabbit muscle LDH are 487.33 mM^{-1} and 29.91 mM and 97.47 mM at 22 °C and pH 7.8.

Keywords L-Lactate dehydrogenase · Enzyme kinetics · Pyruvate substrate inhibition · Rabbit muscle · Statistical software kinetic modeling

M. W. Eggert · R. P. Chambers (✉)
Department of Chemical Engineering, Auburn University, 240 Ross Hall, Auburn, AL 36849, USA
e-mail: chambro@auburn.edu

M. W. Eggert · M. E. Byrne
Biomimetic and Biohybrid Materials, Biomedical Devices, and Drug Delivery Laboratories,
Department of Chemical Engineering, Auburn University, Auburn, AL, USA

Introduction

The interconversion of pyruvate and lactate is an integral part of energy formation. During anaerobic respiration in muscle, the conversion of pyruvate to lactate by L-lactate dehydrogenase maintains energy production and regeneration of coenzyme nicotinamide adenine dinucleotide (NAD^+) from its reduced form (NADH). L-Lactate dehydrogenase (LDH) can participate in many multi-enzyme metabolic systems as an effective enzyme for regenerating NAD^+ from NADH. LDH is also potentially useful in the commercial production of poly(lactic acid). Kinetics play a critical role in enzyme activity, defining the concentrations of substrate and product at which binding processes occur and subsequently accounting for the speed of conversion. Specifically, a comprehensive analysis of L-lactate dehydrogenase kinetics is studied here to investigate viability in larger enzymatic processes. By determining all inhibition factors present, a kinetic rate expression can be utilized to describe and to understand concentration barriers that limit the overall reaction rate at high pyruvate concentrations.

The substrate inhibition of LDH, at pyruvate concentrations in excess of its Michaelis constant, is common characteristic in multiple sources and tissues. In the bi-substrate conversion of pyruvate and NADH to lactate and NAD, as pyruvate concentration surpasses a certain minimum, a simultaneous decline in rate occurs. This inhibition was initially suggested to be caused by formation of a dead-end, "ternary" complex involving enzyme, pyruvate, and the oxidized cofactor [1]. Subsequent studies indicated that inhibition is created when enzymatic coupling of the substrates leads to covalent joining of the C2 carbon of pyruvate with the C4 carbon on the oxidized nicotinamide ring, before it is released from the enzyme. The ternary formation has become better known as an E–NAD^+–Pyr adduct complex [2–5]. Additional work promoted the idea of simple enzyme–pyruvate binary complexes and other inhibiting ternary complexes involving only cofactors [6, 7]. Recently, the substrate binding has been examined with extremely small timescales to better understand the enzyme's intricate mechanics [8]. Furthermore, genetic engineering has led to mutant LDH with substrate inhibition knockouts that can endure higher pryuvate concentrations but sometimes diminish substrate binding and turnover rate [9]. In any instance, the presence of simple and complex substrate and product inhibitors necessitates the use of additional terms to a simple bi-substrate enzyme kinetic rate expression especially if large amounts of pyruvate are required for effective use of the enzyme. By creating a kinetic rate expression capable of accounting for each of the inhibiting complexes, our kinetic equation can be used to simulate systems that involve, and possibly require, the inhibiting pyruvate and/or lactate concentrations.

Methods

Chemicals and Enzymes

All chemicals were purchased at the purest grade available from Sigma-Aldrich (St. Louis, MO) and Fluka (Milwaukee, WI). Lyophilized enzyme L-lactate dehydrogenase (LDH, EC 1.1.1.27) from the rabbit's muscle was also obtained from Sigma-Aldrich (St. Louis, MO). Purity of the enzyme was listed on the label as having a specific activity of 891 U/mg (pH 7.5, 37 °C).

Enzyme Activity and Kinetics

L-Lactate dehydrogenase activity was assayed spectrophotometrically at 340 nm and 22 °C using a concentration of 2.3 mM pyruvate and 0.12 mM β-nicotinamide adenine dinucleotide, reduced disodium salt (NADH; ε_{340}=6.22 mM^{-1} cm^{-1}) as substrate and cofactor, respectively, in 200 mM sodium, 90 mM potassium, and 150 mM phosphate buffer, pH 7.8, which is a modification of a published procedure [10]. One unit of LDH activity was defined as the amount of enzyme converting 1.0 μmol of pyruvate per minute. The volumetric activity (mM min^{-1}) was defined as the enzyme activity per milliliter of enzyme solution for the free enzyme.

For kinetic determinations, a variety of substrate (pyruvate and NADH) and product (lactate and NAD) concentrations using the aforementioned buffer and temperature were investigated. All kinetic experiments were designed for the conversion of pyruvate to lactate. The kinetic experiments were divided into four sets wherein only two concentrations were varied per set while the initial concentration of the other components remained constant or zero. Pyruvate was varied against lactate to determine both lactate inhibition and pyruvate inhibition with lactate inhibition. Pyruvate was then also studied in the presence of different concentrations of reduced and oxidized cofactor to understand correlating effects: (1) Pyruvate and cofactor substrate NADH concentrations were varied; (2) NADH and NAD variants were tested at an non-inhibiting concentration of pyruvate; and (3) Pyruvate and cofactor product NAD were studied to investigate inhibition of the adduct. A total of 85 different kinetic experiments were produced and repeated several times to eliminate possible error. Pyruvate concentrations up to 40 mM and L-lactate concentrations as high as 100 mM were studied. Cofactor concentrations exceeding common biological amounts were also employed since higher concentrations are often more relevant for biotechnical catalysis. Concentrations of NADH ranged from 0.1 to 0.45 mM well above reported Michaelis–Menten constants of NADH from multiple LDH sources [11], while NAD concentrations ranged from 0 to 4 mM. The rates of oxidation of NADH were carried out for 5 min, and the linear portion of the reaction progress curve was used to analyze the kinetics. Typically, the rates reported are from the first 15 to 105 s, with concentrations from the median time (1 min) of the rate determination being used for kinetic analysis.

Development of Kinetic Rate Expression

In order to produce an accurate LDH kinetic rate expression for reacting pyruvate and NADH to lactate and NAD, kinetic parameters were necessary for all enzymatic steps over the concentrations of interest. For an enzymatic reaction mechanism involving two substrates, in which both interact with the enzyme before any product is released, a simple rate equation is obeyed [12]:

$$v = \frac{V_{\max}(AB)}{K_{ia}K_b + K_bA + K_aB + AB} \tag{1}$$

where A is the reduced cofactor concentration, B is the substrate concentration, and K_{ia}, K_a, and K_b are kinetic parameters, with K_{ia} denoting the dissociation of the substrate with enzyme. The mechanism of reaction also initially involves cofactor binding. The K_a and K_{ia} parameters were obtained from literature as kinetic experimental conditions were typically

above the cofactor saturating concentrations, i.e., nearly ten times the concentration of the binding constant. These values are 0.0107 and 0.00842 mM, respectively [13].

Kinetic Expression Terms and Constant Determination

Determination of kinetic terms was first approached by utilizing traditional plotting techniques, measuring inverse rates vs. substrate and/or inhibitory concentrations, in Microsoft Excel to verify linear or non-linear relationships. When possible, least square regressions of the linear plots were used to extract Michaelis–Menten constants or grouped terms that would fit into the general rate expression (detailed in later equations). Subsequently, multiple linear regressions were generated using JMP (version 8) statistical software from SAS Institute, Inc. (Cary, NC) using the rate data collected. Similarly, initial estimates of inhibition constants for terms were found by using the kinetic data at concentrations where that term had the most weighted significance in the kinetic rate expression.

Results and Discussion

Substrate Inhibition of Lactic Dehydrogenase by Pyruvate and Pyruvate–Coenzyme Ternary Complexes

Preliminary experiments in our laboratory indicated that the enzyme is inhibited markedly by large pyruvate concentrations. Therefore, we determined the effect of increasing concentrations of pyruvate on LDH. Experimental results indicate that the highest rate is at approximately 4.3 mM pyruvate. At concentrations significantly above 4.3 mM pyruvate, activity is less than 50% of the maximum (Fig. 1a). In addition, inhibition effects are observable above a 1-mM pyruvate concentration. The K_b value, without substrate inhibition effects, determined by double reciprocal plots of the initial reaction velocity against the concentration of pyruvate is 0.216 mM. The value matches well with literature [13] and is among the lowest pyruvate kinetic constant of various vertebrate sources [11, 14]. While the effect of pyruvate alone is apparent on the reaction rate, the inhibition is also dependent on the cofactor binding and formation of ternary complexes.

To investigate the effect of ternary complexes formed by the pyruvate substrate, L-lactate dehydrogenase, and both coenzymes, kinetic experiments at non-inhibiting pyruvate concentrations as well as higher inhibiting pyruvate concentrations were analyzed. NADH cofactor concentrations were studied against inhibiting pyruvate concentration up to 40 mM. Below saturating cofactor substrate concentrations, first order rate increases are expected to be observed for increasing NADH concentrations at lower pyruvate concentrations, 0.2 mM up to 4 mM pyruvate. However, at the experimental concentrations of NADH, the overall effect of NADH on reaction rate was minimal, given that NADH concentrations were ten times the concentration of K_a. Even with inhibiting pyruvate conditions above 4 mM, the effect of NADH concentration was minimal (Fig. 1b).

Complexity of the pyruvate substrate and NAD product inhibition was evaluated similarly. NAD concentration was varied from 0.25 mM to as high as 4.0 mM, while NADH concentration was held constant at 0.333 mM. Kinetic experiments at lower concentrations of pyruvate, 1.0 mM or less, experienced little change in kinetic rate as the initial NAD concentration increased. Despite the possibility that cofactor NAD presence can cause a reversal of reaction as L-lactate product builds up, this phenomenon does not occur at the lower pyruvate/high NAD initial conditions despite conversion of substrate to

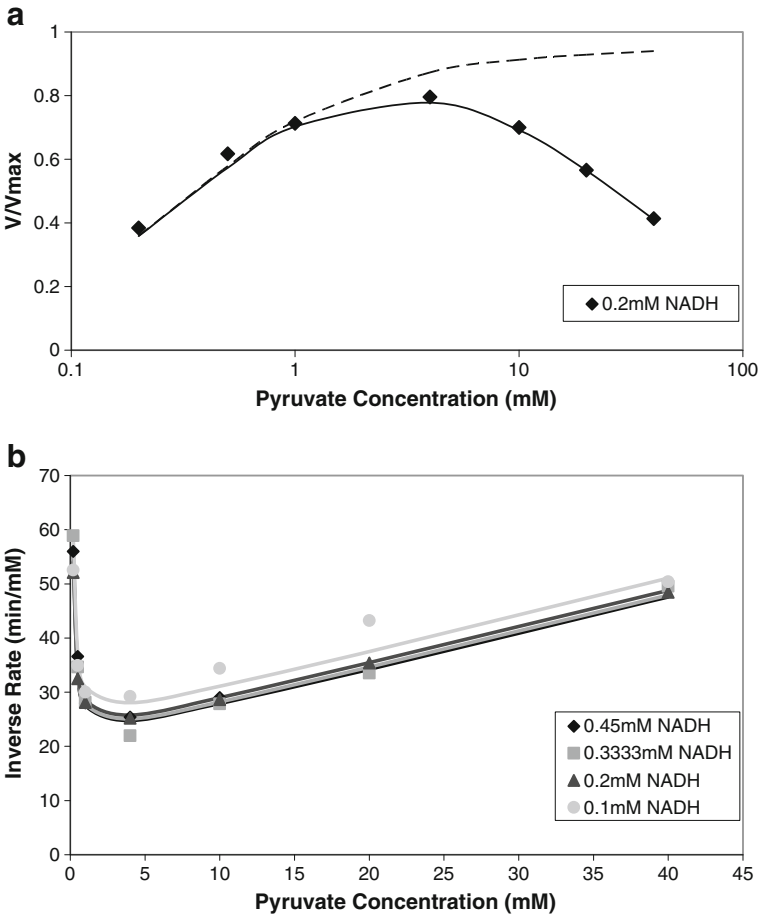

Fig. 1 **a** Classic example of relative velocity vs. pyruvate demonstrating substrate inhibition. **b** Reciprocal rate vs. pyruvate concentration plot demonstrating substrate inhibition. At high concentrations of pyruvate, a linear relationship exists in relation to the K_{I-Pyr} term. Enzyme assay volumetric activity: 0.0485 U/mL. *Lines of similar shading* to experimental points depict kinetic rate expression (Eq. 5) predictions. *Dashed line* in Fig. 1a depicts basic model (Eq. 1) predictions

near equimolar concentrations of pyruvate and lactate. This effect could be attributed to the saturation of the enzyme by NADH. Again, however, with inhibiting initial concentrations of pyruvate, the kinetic rate was affected significantly by the presence of high levels of NAD (Fig. 2a). The impact of the ternary complex, where NAD does not properly unbind from the enzyme and instead binds with a pyruvate intermediate, becomes elucidated by the hampered rates. While pyruvate alone (40 mM) as an inhibiting factor yields a relative rate around 46% of the maximum experimental rate, the result with 1 mM initial NAD is 33% of the maximum experimental rate and further declines to 14% at 4 mM NAD. The decline in catalytic rate with NAD can be directly observed against the higher pyruvate concentrations (Fig. 2b). Additionally, the initial cofactor ratio becomes of consequence to the reaction rate at higher NAD concentrations. Whereas, the saturating NADH does not notably affect the rate in relation to pyruvate, increasing NADH in relation to NAD decreases any onsetting inhibition (Fig. 2c).

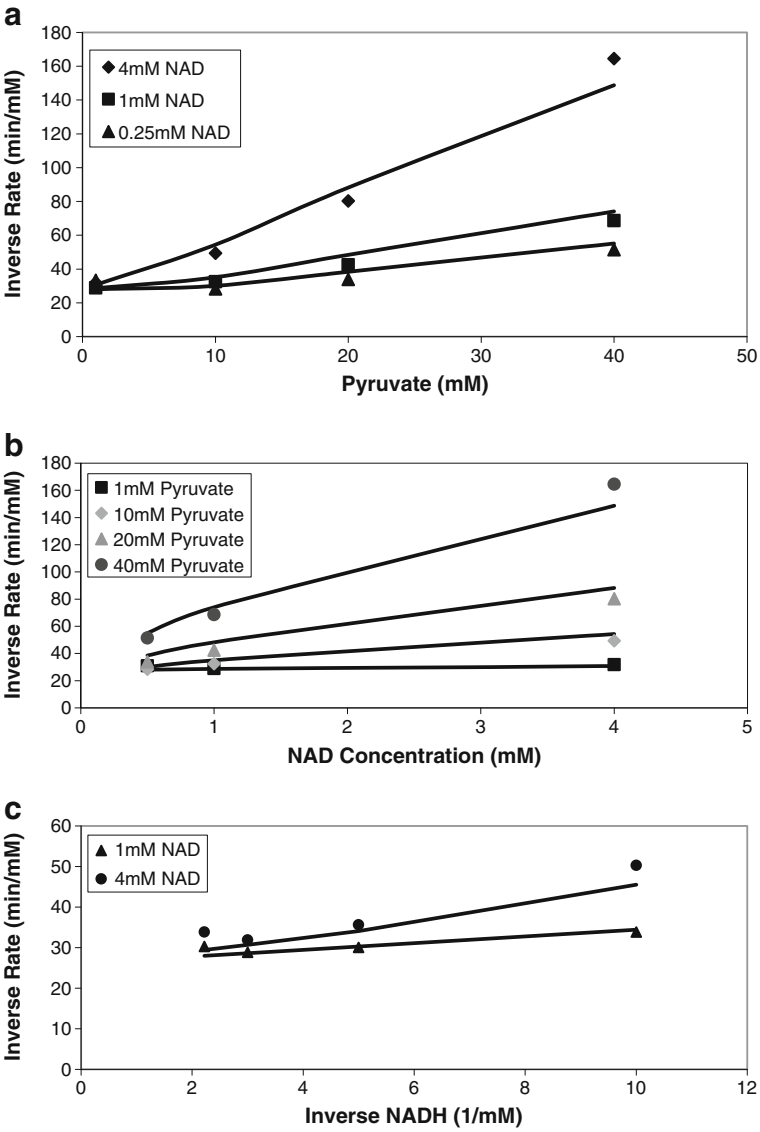

Fig. 2 **a** Inverse rate vs. concentration of pyruvate in the presence of product cofactor. **b** Replot of Fig. 2a depicting increasing NAD concentration. Initial NADH concentration: 0.35±0.013 mM. **c** Inverse rate vs. inverse NADH at elevated NAD concentrations. Initial pyruvate concentration: 1 mM. Enzyme assay volumetric activity: 0.0485 U/mL in each graph. *Solid lines* depict kinetic expression (Eq. 5) predictions

The inhibition effects noted illustrate the necessity for additional terms in the kinetic rate expression. Multiple linear modeling in the statistical software becomes a powerful tool for evaluating these new kinetic constants from the data. High concentrations of pyruvate have been shown to form a complex with the free enzyme, such that another kinetic parameter ($K_{I–Pyr}$) was added as a squared substrate term to the general equation (Eq. 1). Additionally,

the high pyruvate and high NAD inhibition effects suggest the presence of a ternary complex and the added kinetic parameter is $K_{\text{I–Pyr–NAD}}$.

$$v = \frac{V_{max}(AB)}{K_{ia}K_b + K_bA + K_aB + AB + \frac{B^2A}{K_{I-Pyr}} + \frac{B^2Q}{K_{I-Pyr-NAD}}} \tag{2}$$

The $K_{\text{I–Pyr}}$ has been used in analysis of LDH from other sources [11]; however, the $K_{\text{I–Pyr–NAD}}$ seen here is explored in detail.

Inversion of the rate expression allowed for a multiple linear regression against the assayed concentrations:

$$\frac{V_{max}}{v} = \frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1 + \frac{B}{K_{I-Pyr}} + \frac{BQ}{AK_{I-Pyr-NAD}} \tag{3}$$

The presence of saturating amount of NADH cofactor means the pyruvate binary complex effect is linear with the pyruvate concentration (Fig. 3a). The degree of enzyme–pyruvate–NAD^+ ternary complex, however, is demonstrated to be a combination of the pyruvate concentration and cofactor oxidized to reduced ratio. This may be accounted for by the competition of NADH and NAD for the binding site.

Because values of the first four terms were known or easily evaluated, the remaining factors were regressed upon, using the JMP statistical software package, initially at experimental concentrations where the accompanying term of the kinetic factor carried the greatest weight. The linear nature of the term expressing pyruvate–cofactor complex is demonstrated (Fig. 3b). Subsequent regressions utilizing the expanded data sets in JMP provided final kinetic constants of 0.301 mM for Kb, 29.91 mM for $K_{\text{I–Pyr}}$, and 97.47 mM for $K_{\text{I–Pyr–NAD}}$.

Product Inhibition of Lactic Dehydrogenase by L-Lactate in Response to Pyruvate Concentrations

The inverse rate vs. lactate product Dixon plots demonstrate rates that are 67% and 52% relative to the maximum experimental rate for concentrations of 50 and 100 mM L-lactate, respectively, at the lowest pyruvate concentration tested (Fig. 4). For similar lactate conditions involving high pyruvate substrate, the lactate effect is less apparent to the forward reaction giving rise to the flat lines. Lineweaver–Burk double reciprocal plots of the initial reaction velocity against the concentration of pyruvate for several concentrations of L-lactate indicated that the product inhibition is predominately noncompetitive. At concentrations on pyruvate conducive to $V_{\max}$, the slope of inverse velocity relative to lactate suggests a lactate product inhibition K_{i} estimate value of 126 mM for the rabbit muscle LDH enzyme.

Lactate also creates the potential for a reversible reaction. For reversible mechanisms such as that with LDH, an ordered bi–bi model with cofactor binding first and accounting for product inhibitions in each reaction direction can be generated [15] in correlation with the new terms:

$$v = \frac{V_{max}\left(AB - PQ/K_{eq}\right)}{\begin{array}{l} K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_bQ}{K_{iq}} + \frac{K_{iq}K_bK_aP}{K_{iq}K_p} \\ + \frac{K_{ia}K_bPQ}{K_pK_{iq}} + \frac{K_bK_qAP}{K_{iq}K_p} + \frac{K_pBQ}{K_{iq}} + \frac{ABP}{K_{ip}} + \frac{K_{ia}K_bBPQ}{K_pK_{iq}K_{ib}} + \frac{B^2A}{K_{I-Pyr}} + \frac{B^2Q}{K_{I-Pyr-NAD}} \end{array}} \tag{4}$$

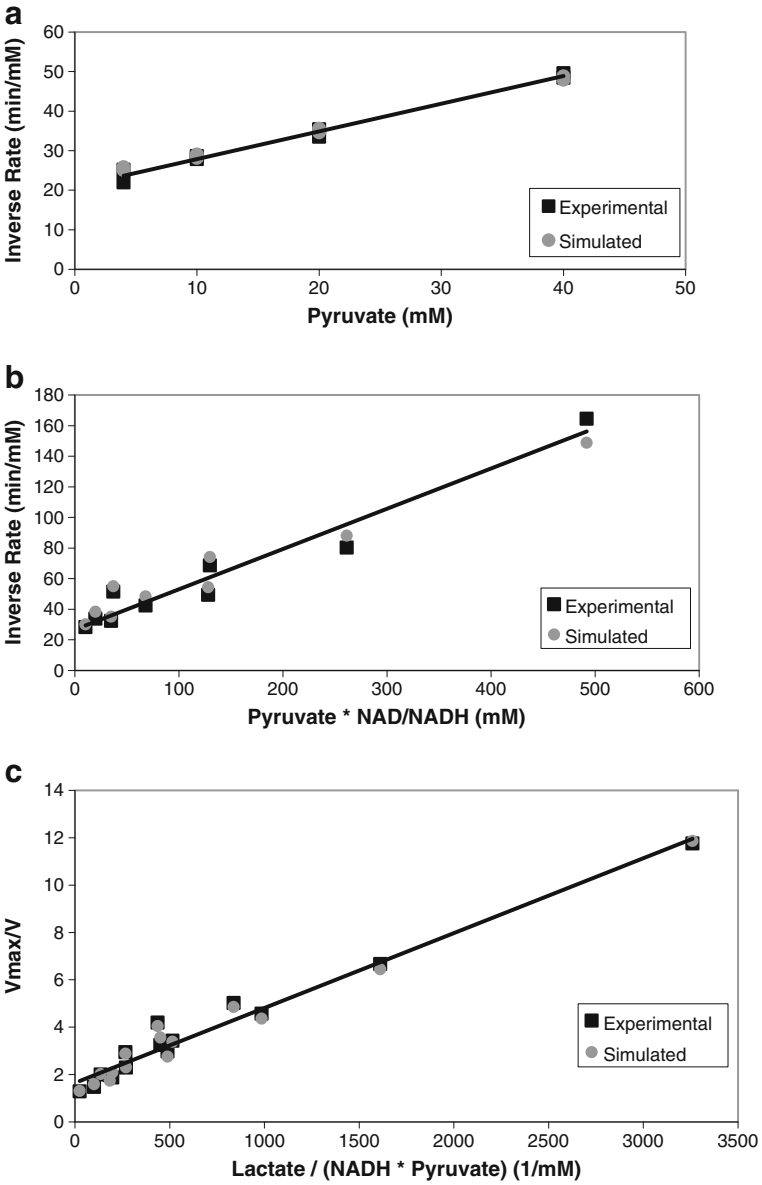

Fig. 3 Linearity of inverse rate with respect to concentrations of inhibition terms. Approximations of kinetic constants can be drawn from linear least squares regression for **a** $K_{I–Pyr}$ using data from Fig. 1, **b** $K_{I–Pyr–NAD}$ using data from Fig. 2, and **c** $K_{I–Lac}$ featuring data from Fig. 4a

where P indicates product concentration, and Q is the oxidized cofactor concentration. At the experimental reaction conditions, negligible reverse reaction occurred.

Initial modeling using Eq. 4 demonstrated little significance of several NAD terms that are evident at lower concentrations of NADH. The terms are important to the enzymatic reaction of lactate to pyruvate, but have little contribution at the conditions in this study. JMP analysis of the full Eq. 4 indicated that having multiple lactate terms is not statistically

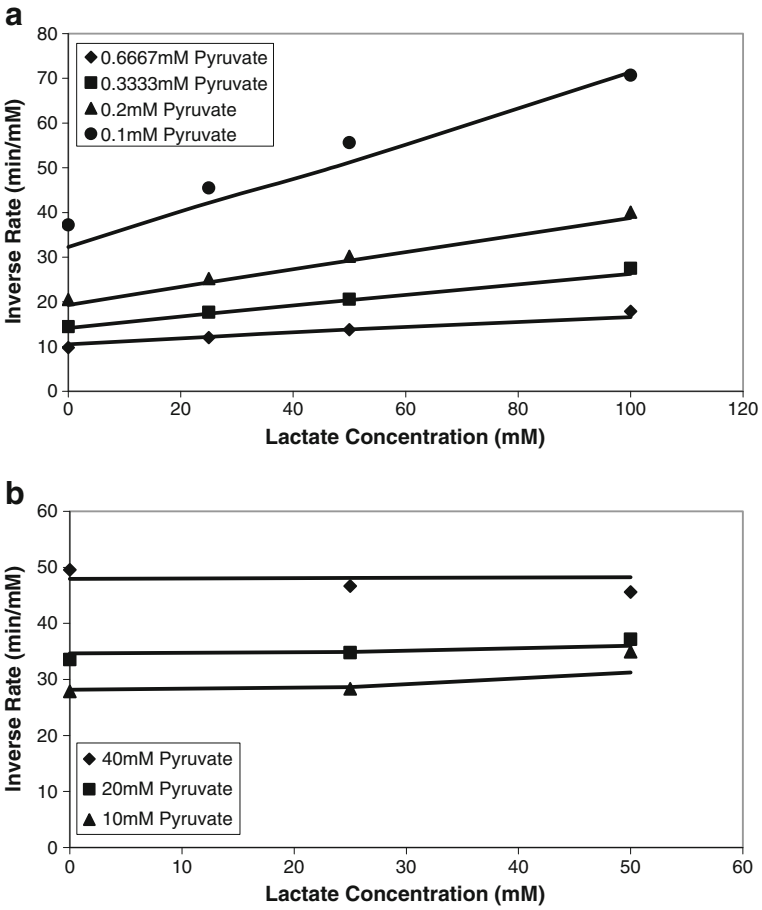

Fig. 4 Inverse velocity linearity to lactate concentrations. **a** At lower concentrations of pyruvate, lactate demonstrates inhibition at high concentration. Enzyme assay volumetric activity: 0.1663 U/mL. **b** In the presence of large pyruvate concentrations, lactate has a diminished effect. Enzyme assay volumetric activity: 0.0499 U/mL. Initial NADH concentration in both plots: 0.335±0.025 mM. *Solid lines* depict model (Eq. 5) predictions

significant. The final forward reaction rate including the remaining L-lactate product inhibition can be expressed inversely as:

$$\frac{V_{max}}{v} = \frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1 + \frac{P}{ABK_{I-Lac}} + \frac{B}{K_{I-Pyr}} + \frac{BQ}{AK_{I-Pyr-NAD}} \tag{5}$$

in which the remaining lactate terms simplify to one term. The linear nature of the term expressing lactate inhibition is provided, and the subsequent value for $K_{\text{I–Lac}}$ following all regressions is 487.33 mM^{-1} (Fig. 3c).

The final overall correlation coefficient between experimental and model kinetic expression rates (Eq. 5) is 97.5%. The correlation coefficient is 98.9% for experiments at higher enzyme concentration and is 91.5% for the larger data set at lower LDH protein concentrations (Fig. 5).

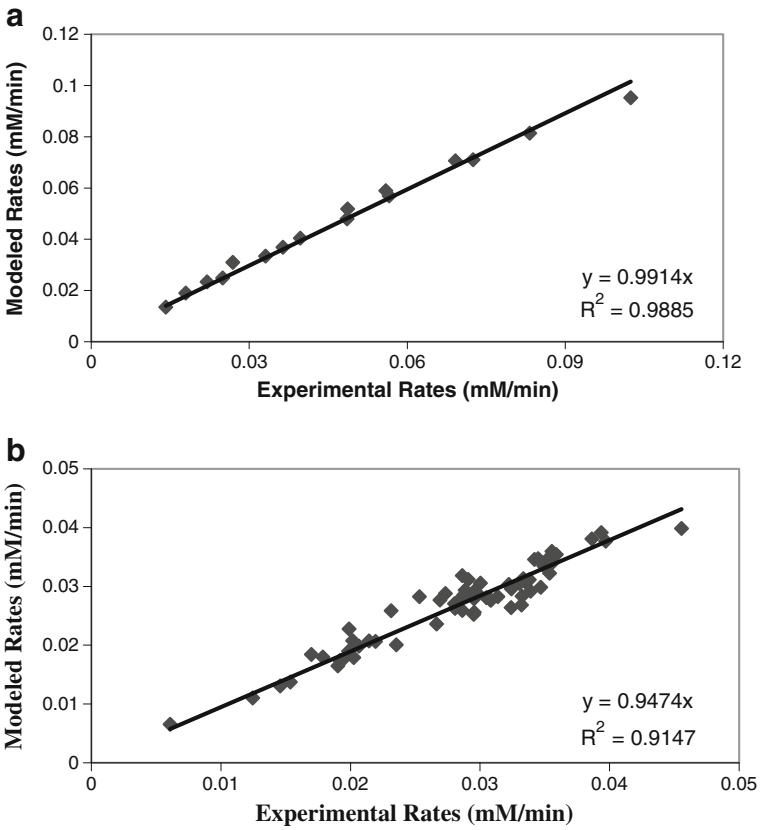

Fig. 5 Correlation of model and experimental values for all assays at **a** higher enzyme concentration (0.1663 U/mL) and **b** lower enzyme concentration (0.0405–0.0499 U/mL). Overall correlation for the entire data set is 97.5%

In 62 of the experimental assay experiments, the simulations exhibit less than 10% error of difference. In the instances of higher error, only six experimental conditions exceed 15% error. The regions of error occurred with the lowest levels of substrate where the binding constants were not fully investigated and literature values were utilized in their place. At these conditions, the simulation tended to produce slightly slower rates comparative to measurement. In nearly all the highest inhibition scenarios, the simulation provided excellent replication of the data.

Conclusions

Statistical software was a valuable tool in analyzing the three primary inhibiting factors effecting pyruvate to lactate conversion by rabbit skeletal muscle lactate dehydrogenase. For the first time, the substrate–cofactor product ternary complex is accounted for at high concentrations of pyruvate and NAD. The protein activity is seen to drop as much as 50% with pyruvate inhibition alone and, with ternary complexes involved, can further diminish to nearly a twelfth of optimal rate. The provided kinetic rate expression supplies a solid

basis to evaluate use of lactate dehydrogenase in a large-scale enzymatic system requiring cofactor regeneration or lactate production.

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