Kinetics of *Escherichia coli* B D-Lactate Dehydrogenase and Evidence for Pyruvate-controlled Change in Conformation*

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SUMMARY

Kinetic properties of D(-)-specific lactate dehydrogenase (D-lactate:diphosphopyridine nucleotide oxidoreductase, EC 1.1.1.28) from *Escherichia coli* B indicate the following.

1. The reaction catalyzed is essentially unidirectional, the oxidation of D(-)-lactate with diphosphopyridine nucleotide as coenzyme proceeding at only about 0.01% of the rate of pyruvate reduction.

2. The enzyme shows Michaelis-Menten kinetics for reduced diphosphopyridine nucleotide, but pyruvate activates it.

3. A lag in the rate of oxidation of the reduced diphosphopyridine nucleotide is eliminated by prior incubation with pyruvate. The duration of this lag is independent of enzyme concentration, suggesting that pyruvate causes a change in the conformation of the enzyme rather than in its state of aggregation. The identity of the molecular weight of the enzyme at a very low concentration in the absence and in the presence of the substrate confirms this finding.

4. Two lines of evidence indicate that there are two types of binding sites for pyruvate. The variation of Hill plots with pH is interpretable only in terms of two sites with different binding properties. Furthermore, the two sites bind different substrate analogues. Oxamate is an inhibitor of this enzyme, but does not substitute for pyruvate as an activator. On the other hand, α-ketobutyrate does not inhibit the catalysis but activates the enzyme, converting the substrate saturation curve from sigmoidal to hyperbolic.

5. The kinetic properties of the catalytic site appear to be similar to those of vertebrate L-lactate dehydrogenases, although the dissociation constants for both pyruvate and reduced diphosphopyridine nucleotide are considerably larger for the *E. coli* D-lactate dehydrogenase.

6. The kinetic properties of this enzyme are consistent with the course and regulation in vivo of pyruvate metabolism.

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that remained became constant (after about 1 hour), at which time the pyruvate was completely reduced. Samples were then assayed enzymatically with d-lactate dehydrogenase from L. mesenteroides and H4 l-lactate dehydrogenase from chicken heart for the formation of d-lactate and l-lactate, respectively (6).

**Kinetic Studies**—Most assays were performed on a Cary model 14 recording spectrophotometer. Rapid reactions were measured with a stop-flow apparatus (7), which consisted of two syringes containing the reactants connected in the cell of a Beckman DU spectrophotometer. The reactants were mixed rapidly in the spectrophotometric cell with an automatic plunger. The rate of reaction was recorded both on a Gilford recorder and on a Tektronix type 564 storage oscilloscope with a differential amplifier.

All assays were performed at room temperature, or about 23°. The ionic strength for all experiments was 0.24.

**Molecular Weights**—The molecular weight of the catalytic unit of the enzyme was determined by gel filtration (9) through Sephadex G-200, both in the absence and in the presence of pyruvate.

**Optical Rotatory Dispersion**—The optical rotatory characteristics of this lactate dehydrogenase were determined, both in the absence and in the presence of pyruvate, with a Cary 60 spectropolarimeter.

**RESULTS**

**Stereospecificity**—When this enzyme was used to catalyze the complete reduction of small amounts of pyruvate in the presence of excess DPNH, the pyruvate was stoichiometrically converted to the d(-) isomer of lactate. Results of a typical experiment are given in Table I.

The reduction of DPN+ by the enzyme was studied with a racemic mixture of lactate and pure d(-)- and l(+)-lactates. The assay mixture (1.0 ml) contained lactate (0.24 mM), DPN+ (0.7 mM), sodium pyrophosphate buffer (0.03 M), pH 8.9, and lactate dehydrogenase (45 units).

**Table I**

<table>
<thead>
<tr>
<th>Pyruvate concentration (M)</th>
<th>DPNH oxidized (mM)</th>
<th>d-Lactate (mM) formed</th>
<th>l-Lactate (mM) formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.05</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>0.88</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>0.55</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.94</td>
<td>0.95</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Determined from absorption at 340 nm.

**Table II**

<table>
<thead>
<tr>
<th>Isomer of lactate</th>
<th>Rate of reaction (mM/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL</td>
<td>0.037</td>
</tr>
<tr>
<td>D</td>
<td>0.13</td>
</tr>
<tr>
<td>L</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**EXPERIMENTAL PROCEDURE**

**Materials**

The purification of the n-lactate dehydrogenase from E. coli B is described in the preceding paper (2). For some of the kinetic studies, impure enzyme solutions were used. In no case were interfering activities present. Crystalline chicken H4 lactate dehydrogenase was prepared by the method of Pesce et al. (5). Leuconostoc mesenteroides n-lactate dehydrogenase was partly purified by Mrs. R. Garland. Egg white lysozyme was purchased from Worthington.

Coenzymes and adenine nucleotides were purchased from P-L Biochemicals, Milwaukee, Wisconsin, sodium pyruvate from Sigma, lithium n(-)-lactate and t(+)l-lactate from Miles Laboratory, Elkhart, Indiana, and a-ketobutyrate from K & K Laboratories, Inc., Brooklyn. Oxamic acid, purchased from Matheson Coleman and Bell (Division of the Matheson Company, Inc., East Rutherford, New Jersey), was neutralized with NaOH prior to use. Reagent solutions were always prepared each day and kept at 4°C.

Sephadex G 200 was purchased from Pharmacia, Uppsala.

An additional product between DPN+ and pyruvate, the reduced form of the binary complex, was prepared by Dr. R. H. Sarma of our laboratory.

**Methods**

**Determination of Stereospecificity**—The stereospecificity of this enzyme was determined with crude extracts and with 1% pure, 20% pure, and homogeneous preparations. Limiting amounts of pyruvate and excess DPNH were used, and DPNH oxidation was measured spectrophotometrically until the amount of DPNH

1 R. Garland and N. O. Kaplan, unpublished results.

2 R. H. Sarma and N. O. Kaplan, unpublished results.
Burk plot was about 20% higher than the rate observed under optimal conditions. This discrepancy is probably due to substrate inhibition.

The turnover number, at pH 7.5 and 0.3 mM DPNH, was $3.4 \times 10^4$ moles of DPNH oxidized per min per mole of lactate dehydrogenase.

**Hill Plots**—It is expected that a Hill plot for an enzyme with multiple, interacting binding sites will be sigmoidal, with the slope approaching 1.0 at the extremes of low and high substrate concentrations and being greater than 1 at intermediate concentrations. The Hill parameter, $n_H$, is defined as the slope of a tangent to the curve at half-maximal velocity, i.e. at $v/(V_{max} - v) = 1.0$ (9).

For *E. coli* lactate dehydrogenase, Hill plots for pyruvate are indeed sigmoidal, the slope approaching 1 at very low and at relatively high pyruvate concentrations. However, from the plot for pyruvate at pH 7.5, shown in Fig. 3, it was calculated that $n_H$ is 1.20. In other words, the slope approached 1 at a lower value of $v/(V_{max} - v)$ than expected from the theory of Hill plots, described by Wyman (9). Indeed, at the pyruvate concentration for which $v/(V_{max} - v)$ is about 0.7, the curve is already approaching a slope of unity. At lower values of $v/(V_{max} - v)$, the slope equals 1.9. A new parameter, $n'_H$, has been defined as the slope of the curve at $v/(V_{max} - v) = 0.5$, in order that the results obtained with this enzyme may be described numerically.

**Dependence of Rate upon pH**—The relation between rate and pyruvate concentration has been examined at several pH values. These results are summarized in Fig. 4 and Table III. Lineweaver-Burk plots indicate that the $K_m$ was relatively constant between pH 6.4 and pH 7.5, whereas the $V_{max}$ was a
It may be noted (Fig. 4) that it was not possible to determine meaningful values for $V_{\text{max}}$ and $K_{m}$ of this enzyme at pH 8.5, because the highest pyruvate concentration that could be used was 33 mM. Consequently, it was also impossible to make a Hill plot requiring the value of the $V_{\text{max}}$ in its construction. However, the sigmoidal character of this curve is readily apparent in Fig. 4. Also, the apparent $K_{m}$ for pyruvate is considerably greater than 7 mM since, at that concentration, the rate was only about 10% of that observed at 33 mM.

**Dependence of Rate upon DPNH Concentration**—The E. coli α-lactate dehydrogenase appears to follow Michaelis-Menten kinetics for DPNH at all pyruvate concentrations between 3 mM and 30 mM. Lineweaver-Burk plots for DPNH at several constant concentrations of pyruvate are shown in Fig. 6A.

The apparent $K_{m}$ for DPNH increases when the pyruvate concentration is reduced to levels below the apparent $K_{m}$ for pyruvate. Just as has been found for animal lactate dehydrogenase, the DPNH concentration at the common point for intersection is higher than any of the individual apparent $K_{m}$ values (10, 11). The $V_{\text{max}}$ at saturating levels of pyruvate was 322 μmoles of DPNH oxidized per min per mg of lactate dehydrogenase, in good agreement with the $V_{\text{max}}$ value obtained when pyruvate was varied at saturating levels of DPNH.

Lineweaver-Burk plots for pyruvate at different constant concentrations of DPNH (Fig. 6B) show that $K_{m} = 7$ mM at pH 7.5. The characteristics of Hill plots for pyruvate did not vary with changing DPNH concentrations in the range, 0.086 to 0.57 mM DPNH.

**Oxamate Inhibition**—Oxamate, an inhibitor of animal lactate dehydrogenases (12), also was found to be an inhibitor of the E. coli enzyme. The oxamate concentration was varied between 0 mM and 30 mM. The DPNH concentration was 0.33 mM; the buffer was 0.1 M potassium phosphate, pH 7.5. Experiments in which oxamate concentration was varied were done at a number of constant pyruvate concentrations between 0 and 30 mM, pH 6.4; Δ, pH 6.7; ◊, pH 7.5.

![Logarithm of Pyruvate Concentration](http://www.jbc.org/)

**Fig. 5.** Hill plots for pyruvate at several pH values. ○, pH 6.4; Δ, pH 6.7; ◊, pH 7.5.
The results are shown in Fig. 7, A and B. Fig. 7A is a plot of 1/v against oxamate concentration at different, constant pyruvate concentrations at different, constant levels of oxamate. The inhibition by oxamate was of the "mixed" type. The apparent $K_i$ for oxamate was about 18 mM. Hill plots for pyruvate at the different oxamate concentrations gave $n_V$ values of 1.5 to 1.9, with no indication that there was any regular change in $n_V$ as the oxamate concentration was increased.

With aspartate transcarbamylase, low concentrations of maleate activate the enzyme at low aspartate concentration, presumably by causing the same conformational change usually induced by the substrate, aspartate (13). No such activation by oxamate was found for E. coli lactate dehydrogenase at a low pyruvate concentration (Fig. 8).

\[ \alpha \text{-Ketobutyrate Inhibition} \] \[ \alpha \text{-Ketobutyrate is a very poor substrate for this enzyme; the rate of DPNH oxidation with 20 mM } \alpha \text{-ketobutyrate was about 1% of that with pyruvate as substrate. However, when 20 mM } \alpha \text{-ketobutyrate was added to the assay mixture and pyruvate was varied, the substrate saturation curve was hyperbolic, the Lineweaver-Burk plot was linear, and a Hill plot indicated that the reaction was first order in pyruvate (Fig. 9).} \]

\[ \text{ATP Inhibition—E. coli } \delta \text{-lactate dehydrogenase is inhibited by ATP, as are the } \delta \text{-lactate dehydrogenases of Butyribacterium rettgeri (14) and Lactobacillus plantarum (15) and the } \delta \text{-lactate dehydrogenase of the latter organism (15). Other adenine nucleotides do not appear to be inhibitors.} \]
ATP was a poor inhibitor at a concentration of 1 mM. At 4 mM, the triphosphate appeared to be competitive with DPNH (Fig. 10). Hill plots for pyruvate and for pyruvate with 4 mM ATP were virtually identical in shape although displaced from one another along the abscissa, indicating that ATP does not affect the interaction (or interactions) between pyruvate and the enzyme.

Product Inhibition—When the activity of a sample of lactate dehydrogenase is measured by the standard assay (2), the rate begins to decrease after about 0.15 μmole of DPNH has been oxidized. This decrease was thought to be caused either by product inhibition by DPN⁺ or n-lactate or, by analogy with the animal lactate dehydrogenases (16), by the formation of an abortive ternary complex among enzyme, pyruvate, and DPN⁺.

The enzyme was incubated for 2 min with DPN⁺ (0 to 0.67 mM), pyruvate (15 or 30 mM), and potassium phosphate buffer (0.1 M), pH 7.5. The reaction was initiated with 0.33 mM DPNH. No inhibition was observed when initial rates were measured.

n-lactate is virtually ineffective as an inhibitor of pyruvate reduction. The enzyme was incubated with n-lactate (0 to 30 mM), DPNH, and potassium phosphate buffer at the same concentrations as mentioned above. The assays were initiated after 2 min by the addition of 15 mM pyruvate. The apparent $K_i$ was about 0.25 M.

Inhibition by Reduced Binary Complex—As already mentioned, we believe that the substrate inhibition that we observed with vertebrate lactate dehydrogenases is caused by the formation of an abortive ternary complex (16-18). A binary complex of pyruvate and DPN⁺ has been synthesized that acts as an inhibitor of vertebrate lactate dehydrogenases and is presumed to mimic the observed substrate inhibition.

The purified reduced binary complex was shown to be an inhibitor of the E. coli n-lactate dehydrogenase. A plot of reciprocal rate against concentration of inhibitor is shown in Fig. 11. The enzyme was incubated for 5 min with complex (0 to 26 μM), pyruvate (30 mM), and potassium phosphate buffer (0.1 M), pH 7.5. The reaction was initiated with 0.33 mM DPNH. Initial rates of reaction were measured. Under these experimental conditions the apparent $K_i$ was 0.013 mM, which is lower than the apparent $K_m$ for DPNH.

Activation by Pyruvate—The suggestion that the conformation of the active site of the E. coli lactate dehydrogenase changes upon binding of pyruvate is derived from an examination of the effect of pyruvate upon the initial rate of its reaction with DPNH. We observed that there was a pronounced lag in the rate of reaction at low initial concentrations of pyruvate (Fig. 12). As the pyruvate concentration was increased, the duration of the lag decreased, until the lag could not be observed with a Cary model 14 recording spectrophotometer.

![Graph](image-url)
If this lag results from some conformational change in the lactate dehydrogenase upon binding of the substrate, giving a more active form of the enzyme, a lag should also be observed at a saturating concentration of pyruvate and should be eliminated by prior incubation with the substrate. This was observed with a stop-flow apparatus.

The results of three related experiments done with the stop-flow apparatus are summarized in Fig. 13 and Table IV.

In the first experiment, the lactate dehydrogenase was placed in one syringe and pyruvate and DPNH in the other prior to mixing. In this way, the enzyme was not exposed to either reactant until the reaction was initiated. As shown in Fig. 13, Curve A, a distinct lag was observed: 5 sec elapsed before a constant rate of oxidation of DPNH was observed. This constant rate amounted to 1.19 μmoles of DPNH oxidized per min, and was reproducible within about 1% in five trials.

In the next experiment, lactate dehydrogenase and DPNH were placed in one syringe and pyruvate in the other prior to mixing. In this case, the enzyme was incubated with coenzyme and the reaction was started by the addition of pyruvate upon mixing. A lag was observed, again lasting for about 5 sec. However, the rate calculated from the apparently linear portion of the curve was not reproducible but, rather, slowly fell as the time of incubation increased. The rate decreased 5% in 8 min; this decrease would not have been observed with a less reproducible method of assay. When the rate was extrapolated to zero time of incubation, it was 1.14 μmoles of DPNH oxidized...
Effects of substrate and coenzyme upon initial lag

Assays were performed with stop-flow apparatus as described in the text. The concentrations of the various reactants were lactate dehydrogenase, 4.5 μg per ml; pyruvate, 25 mM; and DPNH, 0.22 mM. Potassium phosphate buffer, 0.1 M, pH 7.5, was present in both syringes at a concentration of 0.1 M.

The rates appeared to be linear. The column was then calibrated to compensate for the pyruvate that was being added together with the enzyme.

The higher rate found in this experiment, in which there was no lag, probably is the “true” maximal rate of reaction. The lower rate found in the other two experiments may represent a portion of the curve, which appears to be linear, between the initial lag and the later slowing down of the reaction; this decrease in rate is presumably due to formation of the abortive ternary complex.

Further studies with the stop-flow apparatus showed that the duration of the lag did not vary as the enzyme concentration was changed between 1.8 and 9 μg per ml. The pseudo first order rate constants calculated for the activation depended on the concentration of pyruvate, below 20 mM. The observed dependence suggests that the change is second order in pyruvate. This is in agreement with the indication from Hill plots that the overall reaction is at least second order with respect to pyruvate.

Molecular Weight of Catalytic Unit—The observation that the half time of the lag was independent of the enzyme concentration suggested that there is no change in the state of aggregation of the subunits of the enzyme upon binding of pyruvate. This was confirmed by the determination of the molecular weight of a catalytic concentration of enzyme in the absence and in the presence of pyruvate.

The elution peak of the E. coli lactate dehydrogenase in the absence of pyruvate corresponds to a molecular weight of 130,000 ± 15,000 (Fig. 14). This value is in excellent agreement with that obtained by gel filtration with a higher concentration of enzyme (2). Furthermore, the elution pattern was the same in the presence of pyruvate, indicating that there is no change in the molecular weight of this enzyme upon binding of the substrate.

**Optical Rotatory Dispersion**—An attempt was made by optical rotatory dispersion to detect a change in the peptide Cotton effect upon binding of pyruvate. However, there were no differences apparent in the spectra between 225 and 350 μm with or without pyruvate present.

**DISCUSSION**

The simplest interpretation of the anomalous kinetics observed when pyruvate was varied is that there are two types of binding sites for the substrate on the enzyme. One type, with a relatively low dissociation constant, is associated with the activation of the enzyme by pyruvate. The second, with $K_d = 7$ mM at pH 7.5, is at the catalytic site of the enzyme.

If this hypothesis is correct, it would be expected that, at low pyruvate concentrations, the reaction would appear to be second order in pyruvate. However, at higher concentrations, the conformational, or allosteric, site would be wholly occupied, the enzyme would be fully activated, and the reaction would appear to be first order in the substrate. This hypothesis fits the observation that at low pyruvate concentrations the slope of the Hill plot for pyruvate is about 2, whereas at higher concentrations it is only 1.

If there are two distinct pyruvate-binding sites with different binding constants, the observation that the concentration range for which the slope of a Hill plot is 2 becomes smaller as the pH...
is lowered suggests that the $K_v$ for the allosteric site decreases more, or more rapidly, than does the $K_v$ for the catalytic site as the pH is lowered. Thus, $H^+$ would appear to be acting as an allosteric effector, by lowering the concentration of pyruvate at which the interactions are apparently abolished. It is noteworthy that Wittenberger and Fulco have recently published evidence suggesting that the D-lactate dehydrogenase from *Butyribacterium rettgeri* not only is activated by pyruvate but may also be activated by $H^+$ (14).

Since the enzyme appears to be fully activated at concentrations of pyruvate about 5 mM, it is possible to compare the kinetics observed with relatively high concentrations of pyruvate with what is known about vertebrate lactate dehydrogenases. The turnover numbers are similar (19). The $K_v$ for pyruvate (catalytic site) is affected similarly by a change in pH (20). Substrate inhibition decreases with pH for both enzymes (21). Oxamate inhibits both enzymes in a similar manner (12). The $K_v$ for DPNH is greater than any of the apparent $K_v$ values, and $K_v - K_a$ for pyruvate for both enzymes (11). Both enzymes are affected in the same way by the reduced binary complex. From these observations it would appear that the mechanism whereby pyruvate is reduced by the bacterial enzyme may be chemically similar to that which is operative in vertebrate lactate dehydrogenase. This similarity would not be surprising.

With regard to the activation by pyruvate, the lag in the initial rate of oxidation of DPNH is significant at saturating as well as at low concentrations of substrate. This lag is eliminated by prior incubation of the enzyme with the substrate, suggesting that the binding of pyruvate effects a change, either in the state of aggregation of the subunits or in the conformation of the enzyme. The former possibility has been ruled out by studies showing that the molecular weight of a very low concentration of enzyme is the same both in the absence and in the presence of pyruvate.

The conformational change probably is subtle, inasmuch as it is not detected by optical rotatory dispersion studies. This fact is not surprising, for x-ray crystallographic studies on hemoglobin have suggested that the Bohr effect is caused by a very small change in conformation, on the order of 7 A (22).

The studies with substrate analogues are interesting with regard to the hypothesis that there are two distinct pyruvate sites. Oxamate, an inhibitor, competes with pyruvate for the catalytic site on the enzyme; however, it does not activate the enzyme at low concentrations of pyruvate. Another analogue, $\alpha$-ketobutyrate, does not appear to bind to the catalytic site very well if at all; it is a very poor substrate and does not inhibit significantly the reaction of pyruvate at 20 mM. However, this analogue does act as an allosteric activator, changing the substrate saturation curve from sigmoidal to hyperbolic. This fact suggests that this compound may be replacing pyruvate at a second, allosteric site. This behavior of the *E. coli* lactate dehydrogenase with respect to $\alpha$-ketobutyrate is quite similar to that found by Wittenberger and Fulco for the D-lactate dehydrogenase from *B. rettgeri* with this analogue, except that it is an inhibitor of the *B. rettgeri* enzyme (14).

It is not surprising that the activity of the *E. coli* lactate dehydrogenase appears to be determined by the metabolic environment of the enzyme, since this organism is a facultative anaerobe and the fate of pyruvate differs markedly depending upon conditions of growth.

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*J. Everse and N. O. Kaplan, unpublished results.*
In E. coli it appears that the regulation of the pyruvate-lactate interconversion is effected by the presence of a totally distinct enzyme, d-lactate dehydrogenase, which acts essentially as a pyruvate reductase, and by flavin-linked lactate oxidases, which appear to be inducible enzymes (3). Hence, in E. coli, the regulation is controlled by both flavin and pyridine nucleotide-linked enzymes.

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REFERENCES

Kinetics of *Escherichia coli* B d-Lactate Dehydrogenase and Evidence for Pyruvate-controlled Change in Conformation
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