Studies on Rates of Abortive Ternary Complex Formation of Lactate Dehydrogenase Isozymes

(Received for publication, April 24, 1969)

THOMAS WUNTCHE,* ELLIOT S. VESELL,† AND RAYMOND F. CHEN

From the Section on Pharmacogenetics, Laboratory of Chemical Pharmacology, and Laboratory of Technical Development, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Rates of formation and dissociation of an abortive ternary complex between lactate dehydrogenase isozymes from various mammalian tissues, NAD⁺, and pyruvate were studied utilizing stopped-flow spectrophotofluorometry. Formation of the complex was accompanied by a decrease in protein fluorescence at 340 nm and a loss of lactate dehydrogenase activity. Rates of formation of an abortive lactate dehydrogenase-1 and lactate dehydrogenase-5 complex were compared at 25° and 40° over a range of pyruvate and enzyme concentrations, at two NAD⁺ concentrations, and with two coenzyme analogues. Dialysis against buffer produced an increase in protein fluorescence and recovery of lactate dehydrogenase activity that suggested dissociation of the abortive complex.

At pyruvate concentrations of 0.3 mM the abortive ternary complex formed twice as fast with lactate dehydrogenase-1 as with lactate dehydrogenase-5 and with 40.0 mM pyruvate it formed 5 times faster. The rate of complex formation increased with both isozymes when pyruvate concentrations were raised from 0.3 mM to 40.0 mM and when the temperature was elevated from 25° to 40°. However, the rates of abortive complex formation were inversely related to enzyme concentration. Compared to lactate dehydrogenase turnover numbers, the rates of formation of the abortive ternary complex were slow. Under physiological conditions of temperature (40°) and pyruvate concentration (0.3 to 1.0 mM), half the maximum fluorescence quenching required approximately 50 sec for partially purified lactate dehydrogenase-1. Changes in coenzyme concentration from 0.10 mM to 0.75 mM failed to alter the rates of abortive complex formation. With the 3-acetylpyridine analogue of NAD⁺, rates of abortive complex formation for both lactate dehydrogenase-1 and lactate dehydrogenase-5 were similar, whereas with deamino-NAD⁺ the abortive complex formed faster with lactate dehydrogenase-1 than with lactate dehydrogenase-5. Dialysis of the abortive complex resulted in recovery of enzyme activity; the rate of recovery was faster with lactate dehydrogenase-5 than with lactate dehydrogenase-1.

In 1961 Fromm (1) first demonstrated spectrophotometrically an abortive ternary complex composed of lactate dehydrogenase (L-lactate:NAD⁺ oxidoreductase, EC 1.1.1.27), NAD⁺, and pyruvate. Later he obtained kinetic (2) and spectrophotofluorometric (3) evidence for the formation of this abortive complex at high pyruvate concentrations. An analogous lactate dehydrogenase-NADH-lactate complex, however, was not detected. Zeve and Fromm (2) and Anderson, Florini, and Vestling (4) further showed that complex formation, whether in the case of an active or an abortive complex, followed a compulsory sequence, requiring first the binding of coenzyme and then the binding of substrate to the enzyme. Several other dehydrogenases have been shown by analysis of product inhibition to follow a similar ordered sequence mechanism (5-8).

Recently interest in the abortive lactate dehydrogenase-NAD⁺-pyruvate complex increased when Gutfreund et al. (9) demonstrated that the extent of pyruvate inhibition was correlated with the reversible formation of a complex between pyruvate and enzyme-bound NAD⁺. Formation of the abortive complex was measured by the increase in absorbance at 325 nm and by the decrease in protein fluorescence. In their studies with lactate dehydrogenase-1, Gutfreund et al. (9) observed that the rate of abortive complex formation was inversely related to enzyme concentration, directly related to pyruvate concentration, but independent of NAD⁺ concentrations above 0.2 mM.

Di Sabato (10) isolated and characterized an abortive ternary complex composed of lactate dehydrogenase from chicken heart, NAD⁺, and pyruvate. He demonstrated that the abortive complex possessed no enzymatic activity but that activity could be recovered by incubating the abortive complex in buffer. He suggested that the abortive complex may be relevant to the
phenomenon of lactate dehydrogenase inhibition by substrate at high pyruvate levels.

As emphasized by Srere (11), concentrations of enzyme commonly used in assays of enzymatic activity are generally much lower than the concentrations at which many enzymes exist in the cell. Hathaway and Criddle (12) have reported lactate dehydrogenase concentrations in rabbit skeletal muscle extracts of 2 to 3 mg per ml. Vesell (13) has suggested that concentrations of the enzyme in vivo several orders of magnitude greater than concentrations commonly used in assays may be a factor in determining to what extent substrate inhibits lactate dehydrogenase activity in vivo. Therefore, kinetic properties of lactate dehydrogenase isozymes, in particular the degree of substrate inhibition, should be examined at physiological enzyme concentrations. At these physiological concentrations of enzyme the abortive ternary complex forms slowest. The present experiments show that at high enzyme concentrations lactate dehydrogenase-1 is not inhibited by pyruvate concentrations up to 20.0 mM. These kinetic studies have been performed with a stopped-flow spectrophotofluorometric apparatus, which was also used to compare the rates of abortive ternary complex formation with lactate dehydrogenase-1 and lactate dehydrogenase-5. The two isozymes form and dissociate their abortive ternary complexes at markedly different rates.

**Materials and Methods**

**Chemicals**—Crystalline lactate dehydrogenase-1 from pig heart and lactate dehydrogenase-5 from rabbit skeletal muscle were purchased from Calbiochem. Other reagents were purchased from Sigma. Buffers were prepared from analytical grade chemicals.

**Electrophoresis**—Isozyme composition of various lactate dehydrogenase preparations was determined with a Millipore cellulose acetate (PhoroSlide) electrophoresis system (Millipore Corporation, Bedford, Massachusetts). All electrophoretic separations were performed at room temperature with a voltage gradient of 15.4 volts per cm applied for 15 min. Buffer for the electrode vessels was Tris-Cl, pH 7.4, 0.02 M. Sites of lactate dehydrogenase activity on the slides were visualized by the reaction with nitro blue tetrazolium formazan, in particular the degree of substrate inhibition, should be examined at physiological enzyme concentrations.

**Assay of Enzymes**—Activity of lactate dehydrogenase was assayed at 25° by measurement of the initial rate of NADH oxidation at 340 mp in a Beckman DB spectrophotometer (15). The 2.0-ml reaction mixture contained Tris-Cl buffer, pH 7.4, 0.02 M, appropriately diluted enzyme, and, in final concentrations, 1.25 x 10^{-4} M pyruvate and 2.5 x 10^{-4} M NADH. Qualitative comparisons of the activities of the complexed and free enzymes were made by diluting 0.025 mg of lactate dehydrogenase (either as the complex or as the free enzyme) in 2.0 ml of the nitro blue tetrazolium reaction mixture. Precipitation of the purple formazan was the criterion of lactate dehydrogenase activity.

The activity of crystalline lactate dehydrogenase-1 at concentrations as high as 0.5 mg per ml (3.5 x 10^{-4} mg) was determined over a range of pyruvate concentrations from 0.3 mM to 20.0 mM with the NADH concentration at 0.56 mM. Assays were made at 25° in an Amino-Bowan spectrophotofluorometer fitted with stopped-flow apparatus as described below under "Complex Formation." The reaction was initiated by mixing a solution of pyruvate and a solution containing lactate dehydrogenase and NADH. The final solution contained 0.1 M sodium phosphate buffer, pH 7.6, and the decrease in NADH fluorescence was followed at 460 mp (340 mp excitation). Although the initial fluorescence rises from both free and enzyme-bound NADH, the latter contributed negligible signal since the total NADH concentration was present in 50- to 3000-fold excess.

**Protein Determination**—Protein concentrations of crystalline lactate dehydrogenase solutions in 0.1 M sodium phosphate buffer, pH 7.0, were measured in a Beckman DB spectrophotometer. Values of 1.4d for E_{280} of a solution containing 1 mg of lactate dehydrogenase-1 per ml (9) and of 1.26 for a solution containing 1 mg of lactate dehydrogenase-5 per ml (16) were used. For the partially purified lactate dehydrogenase preparations, protein concentration was determined by the Warburg-Christian method (17). Concentrations were then adjusted so that the activities of lactate dehydrogenase-1 and lactate dehydrogenase-5 were identical.

**Purification of Lactate Dehydrogenase Isozymes**—Both lactate dehydrogenase-1 and lactate dehydrogenase-5 were purified from the kidneys of adult male Sprague-Dawley rats. The isozymes were extracted in Tris-Cl buffer, pH 7.4, 0.02 M, with a Waring Blender. After passage through cheesecloth and centrifugation at 10,000 x g for 20 min, the resultant supernatant was brought up to 50% saturation with (NH_{4})_{2}SO_{4}, and the enzyme was precipitated at 65% saturation.

For purification of lactate dehydrogenase-1, the precipitate was resuspended in 0.02 M Tris-Cl buffer, pH 6.5, and passed through a column of carboxymethyl Sephadex (Pharmacia) by means of the modified procedure of Hsieh and Vestling (18). A mixture of lactate dehydrogenase isozymes 1 through 4 was eluted with the pH 6.5 buffer, concentrated by dialysis against sucrose, and dialyzed against 0.02 M Tris-Cl buffer, pH 7.4. Lactate dehydrogenase-1 was separated from the isozyme mixture by chromatography on a DEAE-Sephadex column. Lactate dehydrogenase isozymes 4, 3, and 2 were eluted stepwise in 0.10 M, 0.14 M, and 0.18 M NaCl solutions, respectively. Pure lactate dehydrogenase-1 was eluted with 0.22 M salt, concentrated, and dialyzed against 0.1 M sodium phosphate buffer, pH 7.0.

For the purification of lactate dehydrogenase-5, the precipitate from 65% saturated (NH_{4})_{2}SO_{4} was resuspended in 0.02 M Tris-Cl buffer, pH 7.4, and chromatographed on a DEAE-Sephadex column (19). Pure lactate dehydrogenase-5 was eluted in 0.02 M Tris-Cl buffer, pH 7.4, concentrated, and dialyzed against 0.1 M sodium phosphate buffer, pH 7.0.

**Complex Formation**—Formation of the abortive ternary complex with lactate dehydrogenase, NAD\(^+\), and pyruvate is accompanied by the appearance of an absorption band at 325 mp and a quenching of protein fluorescence (9). It was followed by the decrease in protein fluorescence at 340 mp in an Amino-Bowan spectrophotofluorometer fitted with a stopped-flow accessory. Details of the operation of the stopped-flow apparatus have been described by Chen, Schechter, and Berger (19) and will be mentioned only briefly here. The air-driven mixer uses two 1.0-ml tuberculin syringes and achieves dead times of about 10 msec utilizing the laboratory compressed air line at 40 p.s.i. The signal was detected with an R136 phototube operated either with an Amino transistorized photomultiplier or with a 900-volt battery pack. Fast reactions were recorded on a Hewlett-Packard storage oscilloscope; slower reactions were con-
respectively, were utilized. All reagents were made up in 0.1 M sodium phosphate buffer, pH 7.0.

The abortive complex has been shown to be enzymatically inactive (10), and this observation was confirmed by the present experiments. When 0.1 ml of the complex was diluted into a reaction mixture containing 2.0 ml of nitro blue tetrazolium, no formazan precipitation occurred within a 3- to 5-min period. If an equivalent amount of free enzyme (0.25 mg per ml) against the phosphate buffer. An increase in protein fluorescence was taken to indicate dissociation of the complex, and lactate dehydrogenase activity was assayed by the nitro blue tetrazolium reaction.

RESULTS

Table I reveals that the abortive ternary complex formed several times faster with lactate dehydrogenase-1 than with lactate dehydrogenase-5. The $t_4$ values for the rate of abortive complex formation for lactate dehydrogenase-1 were 20 to 50% less than the values for lactate dehydrogenase-5. For both lactate dehydrogenase-1 and lactate dehydrogenase-5 a marked increase in the rate of abortive complex formation occurred when pyruvate concentrations were raised and when the temperature of the reaction was elevated from 25° to 40° (Table I).

The relation of pyruvate concentration to the rate of abortive complex formation at two coenzyme levels is shown in Fig. 1. Rates of abortive complex formation are expressed as the ratios of the $t_4$ values for lactate dehydrogenase-1 to the $t_4$ values for lactate dehydrogenase-5. With pyruvate concentrations up to 5.0 mM, the ratios remained constant at about 0.4, revealing an approximate 2-fold difference between lactate dehydrogenase-1 and lactate dehydrogenase-5. Variations in coenzyme concentrations from 0.1 mM to 0.75 mM failed to alter the $t_4$ values. Differences between lactate dehydrogenase-1 and lactate dehydrogenase-5 in rates of abortive complex formation were similar at 40° to those obtained at 25°. In confirmation of previously reported studies by Gutfreund et al. (9), we observed that rates of abortive complex formation were inversely related to the concentration of enzyme. With 5.0 mM pyruvate the $t_4$ values were linearly related to lactate dehydrogenase concentrations from 0.025 mg per ml to 0.375 mg per ml.

The abortive complex has been shown to be enzymatically inactive (10), and this observation was confirmed by the present experiments. When 0.1 ml of the complex was diluted into a reaction mixture containing 2.0 ml of nitro blue tetrazolium, no formazan precipitation occurred within a 3- to 5-min period. If an equivalent amount of free enzyme was treated similarly, the purple formazan precipitated almost immediately.

With two coenzyme analogues there were notable differences

---

**TABLE I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>25° with a final pyruvate concentration of</th>
<th>40° with a final pyruvate concentration of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3 mM</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>Lactate dehydrogenase-1 (rat kidney)</td>
<td>100</td>
<td>67</td>
</tr>
<tr>
<td>Lactate dehydrogenase-5 (rat kidney)</td>
<td>210</td>
<td>155</td>
</tr>
<tr>
<td>Lactate dehydrogenase-1 (bovine heart)</td>
<td>180</td>
<td>110</td>
</tr>
<tr>
<td>Lactate dehydrogenase-5 (rabbit muscle)</td>
<td>220</td>
<td>140</td>
</tr>
</tbody>
</table>

* Final enzyme concentrations were 0.25 mg per ml, and the final NAD+ concentration was 0.1 mM.

---

Fig. 1. Effect of pyruvate concentration on the relative rates of abortive complex formation at two levels of coenzyme. The conditions were the same as those given in Table I, from which these data are derived; crystalline bovine heart lactate dehydrogenase-1 (LDH-1) and rabbit muscle lactate dehydrogenase-5 (LDH-5) were used.

---

Complex Dissociation—Recovery of lactate dehydrogenase activity induced by dialysis of the abortive complex against a 300-fold excess of 0.1 M sodium phosphate buffer, pH 7.0, at 10° was followed with time. Correction was made for changes in protein concentration by similar dialysis of identical concentrations of free enzyme (0.25 mg per ml) against the phosphate buffer. An increase in protein fluorescence was taken to indicate dissociation of the complex, and lactate dehydrogenase activity was assayed by the nitro blue tetrazolium reaction.
TABLE II
Rates of abortive ternary complex formation with coenzyme analogues

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half time of</th>
<th>0.35 mM 3-Acetylpyridine NAD+ at final pyruvate concentrations of</th>
<th>0.32 mM Deamino-NAD+ at final pyruvate concentrations of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.3 mM</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>Lactate dehydrogenase-1 (bovine heart)</td>
<td>205</td>
<td>100</td>
<td>62</td>
</tr>
<tr>
<td>Lactate dehydrogenase-5 (rabbit muscle)</td>
<td>220</td>
<td>115</td>
<td>69</td>
</tr>
</tbody>
</table>

* Conditions were the same as those given in Table I for crystalline enzymes at 25°C, except for substitution of coenzyme analogues.

from results obtained with NAD+ in rates of abortive complex formation (Table II). With the 3-acetylpyridine NAD+ analogue, the rate of abortive complex formation was approximately similar for both lactate dehydrogenase-1 and lactate dehydrogenase-5. By contrast, with deamino-NAD+, greater differences occurred between lactate dehydrogenase-1 and lactate dehydrogenase-5 in rates of abortive ternary complex formation than when NAD+ was used. The abortive complex formed 3 to 4 times faster with lactate dehydrogenase-1 than with lactate dehydrogenase-5. However, for both isozymes the absolute rate of abortive complex formation was approximately half that obtained with NAD+ at comparable pyruvate levels. As with NAD+, the rate of abortive complex formation was greater at higher pyruvate concentrations with the analogues, and increasing the pyruvate concentration had negligible effect on the relative rates of abortive complex formation with these coenzyme analogues.

The data presented in Fig. 2 on the rates of recovery of protein fluorescence reveal further important differences between lactate dehydrogenase-1 and lactate dehydrogenase-5. In order to recover 50% of the fluorescence of the free enzyme, the abortive complex with lactate dehydrogenase-1 required over 90 min of dialysis, whereas abortive complex with lactate dehydrogenase-5 required only 30 min. On the basis of assays with the nitro blue tetrazolium reaction mixture, it was possible to recover about 80% of the lactate dehydrogenase activity.

Pyruvate inhibition of lactate dehydrogenase-1 did not occur when enzyme concentrations of 3.5 × 10⁻⁶ M (0.5 mg per ml) or 3.5 × 10⁻⁸ M (0.05 mg per ml) were used in the assay system. The substrate saturation curves for lactate dehydrogenase-1 at high concentrations presented in Fig. 3 revealed no loss of maximum enzyme activity under experimental conditions at pyruvate levels up to 20.0 mM. However, at enzyme concentrations of 1.8 × 10⁻⁷ M and lower, pyruvate inhibition was apparent and became more pronounced at lower enzyme concentrations.
Abortive Ternary Complex Formation with Lactate Dehydrogenase Isozymes

Vol. 244, No. 22

Maximum specific activity with 1.0 mm pyruvate remained constant over the range of enzyme concentrations examined at about 3.5 pmoles of NADH oxidized per sec per mg of lactate dehydrogenase.

Even at the highest lactate dehydrogenase concentration the initial velocities measured were calculated to involve more than one catalytic turnover. In the 0.2-ml reaction mixture, our highest lactate dehydrogenase concentration of $3.5 \times 10^{-6}$ M is equivalent to $7.0 \times 10^{-7}$ mmole of lactate dehydrogenase, based on a molecular weight of 140,000. This concentration of enzyme combines with $2.8 \times 10^{-4}$ mmole of NADH, since each lactate dehydrogenase molecule contains four binding sites for the coenzyme. At this enzyme concentration, the linear change in relative fluorescence intensity was 0.3 during the initial 0.1 sec of the reaction. This alteration corresponds to $3.4 \times 10^{-4}$ mmole of NADH oxidized and indicates that approximately 12 catalytic turnovers occurred during the measurement of activity at the highest lactate dehydrogenase concentration.

**DISCUSSION**

The present investigation is the first comparative study of rates of formation and dissociation of abortive ternary complexes composed of lactate dehydrogenase-1 or lactate dehydrogenase-5, NADH, and pyruvate. The abortive complex with lactate dehydrogenase-1 formed up to 5 times faster than the abortive complex with lactate dehydrogenase-5. This avidity of abortive complex formation with lactate dehydrogenase-1 suggests that it forms a stronger, more tightly bound abortive ternary complex. Slower dissociation of the lactate dehydrogenase-1 abortive complex further supports the conclusion that the abortive ternary complex more strongly binds lactate dehydrogenase-1 than lactate dehydrogenase-5.

Utilizing absorbance spectroscopy, Vestling and Künsch (20) studied the formation of abortive ternary complexes involving lactate dehydrogenase-5, NADH, and pyruvate or a-ketobutyrate. The very slow spectral changes accompanying abortive ternary complex formation lasted several hours and were interpreted as being associated with the allosteric binding of substrates to binary complexes of lactate dehydrogenase and NADH. These investigators proposed that the conformational adjustments accompanying substrate binding are slow and influence the electronic environment of the pyridine chromophore of the enzyme-bound NADH (20). Our data, based on quenching of tryptophan fluorescence of lactate dehydrogenase-1, are not entirely comparable to the spectrophotometric data of Vestling and Künsch (20) obtained at 341 nm. The present data do not exclude the possibility that we are indeed measuring the formation of two abortive ternary complexes and that the second complex forms by an unimolecular process.

Gutfreund et al. (9) showed that the abortive ternary complex formed more rapidly at low enzyme concentrations. Our results also reveal that the formation of the abortive ternary complex is inversely related to enzyme concentration. It seemed possible at the very low enzyme concentrations normally used in the assay that abortive ternary complex formation might be rapid enough to account for pyruvate inhibition (9). However, in the present experiments, at high enzyme concentrations approaching those that exist in vivo the formation of the lactate dehydrogenase NADH-pyruvate abortive complex is too slow to account for substrate inhibition. In fact, at enzyme concentrations approaching those existing in the cell, substrate inhibition cannot be shown. At lower enzyme concentrations, pyruvate inhibition may be due to abortive ternary complex formation, as well as to other factors such as the conformational changes in the enzyme described by Vestling and Künsch (20), Cridde, McMurray, and Gutfreund (21), and Fritz (16).

**Acknowledgment**—We wish to acknowledge the help and advice of Dr. Paul J. Fritz in the stepwise purification of lactate dehydrogenase isozymes from rat kidney.

**REFERENCES**


Studies on Rates of Abortive Ternary Complex Formation of Lactate Dehydrogenase Isozymes
Thomas Wuntch, Elliot S. Vesell and Raymond F. Chen

J. Biol. Chem. 1969, 244:6100-6104.

Access the most updated version of this article at http://www.jbc.org/content/244/22/6100

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/244/22/6100.full.html#ref-list-1