Evolution of Duplicated Lactate Dehydrogenase Isozymes in Salmon

ABORTIVE TERNARY COMPLEX FORMATION AND BREAKDOWN

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GEORGE S. BAILEY* AND SOO THYE LIM

From the Department of Biochemistry, Otago University, Dunedin, New Zealand

Salmon are known to have experienced extensive gene duplication and possess duplicate genes for H-type and for M-type lactate dehydrogenase (Bailey, G. S., Tsuyuki, H., and Wilson, A. C. (1976) J. Fish. Res. Board Can. 33, 760-767). The susceptibilities to abortive ternary complex formation and breakdown have now been examined at high levels of the purified isozymes, using varying nonequilibrium levels of substrates and coenzymes. One of the isozymes, Hα,, is shown to be somewhat intermediate between Hβ, and the Mα-Mβ, series in its potential for abortive ternary complex formation with pyruvate and NAD⁺, and in the susceptibility of the complex toward NADH-induced dissociation. These results support previous studies on isozyme tissue distribution, low enzyme level catalytic properties, and structural properties in suggesting that the Hα, isozyme has converged toward M, in salmon sufficiently to satisfy moderate demand for M-type catalytic activity in vivo.

However, we show here that abortive ternary complexes, including those of higher vertebrates, are highly susceptible to dissociation by NADH. Levels of NADH (1 to 3 µM) which are less than stoichiometric with respect to abortive ternary complex active sites (11.6 µM) induce immediate partial complex dissociation in vitro and limit possible complex formation. We interpret these results to suggest that abortive ternary complexes are unlikely to be important in vivo in regulating lactate dehydrogenase activity. We suggest instead that the roles of lactate dehydrogenase isozymes in vivo can be explained solely on the basis of their $K_m$ and $K_i$ parameters and their responses to substrate fluctuation, without the necessity to invoke abortive ternary complex formation or any other form of lactate dehydrogenase regulation.

The tissue distribution of lactate dehydrogenase (l-lactate:NAD oxidoreductase. EC 1.1.1.27) isozymes in higher vertebrates appears to reflect the aerobic nature of each particular cell type examined (1-3). Tissues such as breast muscle of nonmigratory birds with transitory requirements to support rapid anaerobic glycolysis have predominantly the M, isozyme of lactate dehydrogenase, whereas tissues such as cardiac muscle which are aerobic lactate-scavenging tissues have mostly the H, and H,M isozymes. However, the majority of tissues examined in a range of vertebrate species, including many species of pelagic fishes (4-6), contain varying but substantial levels of both M and H polypeptides in the form of the active tetramers M, M,H, M,H, and H,, apparently according to their requirements for lactate scavenging or for lactate production in different metabolic states. Very few cell types are observed which have only M, or H.

In contrast, the distribution of lactate dehydrogenase isozymes in salmonid fish tissues does not follow the above wide spread pattern (7, 8). The M, isozyme in salmon and trout is found in significant levels only in striated muscle, where it is the only type of lactate dehydrogenase present (7, 8). The metabolic requirements of all other cell types examined in salmon and trout are satisfied by tetrmeric combinations of two other polypeptides, Hα and Hβ (7-10), rather than by various combinations of H and M. The Hα and Hβ polypeptides have arisen as a result of gene duplication in salmonids and are closely related structurally (7-9). They may, however, have diverged significantly in function. It is very striking that such tissues as spleen, kidney, esophagus, brain, liver, heart, and pyloric caeca in salmon and trout are essentially devoid of M subunits, and contain only the Hα, Hα,Hβ, Hα,Hβ, HαHβ, and Hβ isozymes in varying levels. These same tissues in a wide range of other fresh water and salt water fish such as herring (Clupea harengus), Atlantic mackerel (Scomber scombrus), tiger barb (Barbus tetrazona), Atlantic cod (Gadus morhua), hickory shad (Alosa mediocris), longspine squirrelfish (Holocentrus rufus), and creek chub (Semotilus atromaculatus) all have roughly comparable levels of M, and H, activity (cf. Ref. 4).

The absence of significant levels of M-type polypeptide in most tissue types in salmonids must, therefore, imply either that salmonid fish are unusual in lactate/pyruvate metabo-
lism relative to other fishes, or that one of the special duplicated lactate dehydrogenase components in their tissues has evolved sufficient M-type catalytic behavior to support at least moderate demand for this function. Support for the latter proposal has been provided through recent studies on the catalytic properties at low enzyme levels of highly purified lactate dehydrogenases from salmon (Oncorhynchus tschawytscha) (7,8). The Hp4 isozyme was shown to have in vitro, low enzyme level catalytic properties typical of higher vertebrate H, enzymes, whereas the M, type enzyme from skeletal muscle exhibits typical M, isozyme kinetics. However, the Hp, enzyme appeared to be somewhat intermediate between Hp4 and the Ma,M,M series in most catalytic parameters measured, such as apparent Michaelis constants and resistance to product and substrate inhibition. These data, together with the unusual tissue distribution of isozymes, were taken to suggest that the Hp polypeptide of salmonid fish may have recently converged toward M in functional properties sufficiently to satisfy moderate demand for M-type lactate dehydrogenase activity in many cell types in these fish (7,8).

Recent studies by other workers on the regulation and metabolic significance of lactate dehydrogenase isozymes suggest that regulation of lactate dehydrogenase activity in vitro may be mediated via abortive ternary complex formation and breakdown, and this would be a major catalytic parameter of functional physiological importance which differentiates Hp, from M, type lactate dehydrogenase (for reviews, see Refs. 1 and 11). On this basis we extended our previous studies on the function of salmon lactate dehydrogenase isozymes to examine abortive ternary complex formation of the various highly purified isozymes at high enzyme levels under various equilibrium and nonequilibrium levels of coenzymes and substrates. The results presented here show how the Hp, isozyme is intermediate to Hp4 and the Ma,M,M series in response to parameters which influence formation and breakdown of abortive ternary complexes in vitro and provide further evidence that the Hp, isozyme in salmonid has undergone unique and rapid functional evolutionary change. The possible in vivo significance of abortive ternary complexes, however, appears from our results to be highly questionable.

**MATERIALS AND METHODS**

**Chemicals and Enzymes—** Sodium pyruvate, L(+)-lactic acid, NAD+, and NADH were purchased from Sigma. All other chemicals used were of reagent grade quality. Salmon H, H, and M, type lactate dehydrogenases were purified to homogeneity as previously described (7,8). Crystalline beef heart lactate dehydrogenase for standardization of L(+)-lactic acid was obtained from Sigma. Beef and chicken H, and M, isozymes were also partially purified to isozymic homogeneity by standard methods (7,8).

**Assay of Enzyme—** Lactate dehydrogenase activity was assayed by following the rate of oxidation of NADH as reported elsewhere (7). One unit of enzyme is defined as the amount of enzyme which causes an initial rate of oxidation of 1 μmol of NADH per min at 25°C.

The structure of the abortive ternary complex of NAD+, enolpyruvate, and lactate dehydrogenase has been shown to be a cova lent pyridine C,-substituted adduct of NAD+ and enolpyruvate bound to lactate dehydrogenase. Thus this abortive complex is in fact a binary and not a ternary complex (11). However, the term abortive ternary complex will be used here in order to avoid confusion with the present literature.

Previous studies of the catalytic properties of salmon lactate dehydrogenase isozymes at 15° and 25° have shown that, although certain parameters such as Michaelis constants show expected temperature dependence, the relative catalytic behavior of H, and M, isozymes at these temperatures is unchanged (9,12). The present studies were carried out at 25° because it is within the range of survival of salmon in oxygen-saturated water, and permits a valid comparison of the salmon enzymes with those from higher vertebrates, which have been extensively studied at 25°.

**RESULTS**

**Formation of Abortive Ternary Complexes between Lactate Dehydrogenase, Oxidized Coenzyme, and Pyruvate—** Salmon lactate dehydrogenase isozymes form abortive ternary complexes when incubated with NAD+ and pyruvate with absorbance maxima at 322 nm and 388 nm, similar to previous reports for abortive complex formation from mammalian enzymes (1,11,19-23). Fig. 1 shows the absorption spectrum of the formation of abortive ternary complexes of E NAD+pyruvate was initiated by adding a small volume of the respective substrate to a cuvette containing enzyme and either NAD+ or pyruvate in buffer. The reaction mixture was quickly stirred, and absorbance at 322 nm was followed as a function of time until equilibrium was reached.

A double tandem system of matched cuvettes was used in a Cary model 11B(C) recording spectrophotometer (cuvette volume 1.0 ml, light path 1.0 cm). All spectral measurements were made at 25°C.

**Stopped Flow Studies—** For the perturbation experiments, a Yan-Naco stop flow spectrophotometer model SPS-1 was used to initiate and follow the reactions. Throughout these studies the observation chamber and mixing syringes were thermostatically controlled at 25°C. The optical cell has a path length of 1.0 cm. For recording the optical change after mixing the two solutions a Hewlett-Packard model 1201A dual trace storage oscilloscope was connected to the photomultipliers. The oscillograms were recorded photographically. Optical changes were also recorded on a Tohoshin electronic chart recorder via a Riken-Denabi model TC290 transient time converter.

Syringe I contained an incubated mixture of the enzyme, NAD+, and pyruvate; Syringe I1 contained either NADH alone or NADH plus different concentrations of L(+)-lactate. Controls were carried out in which Syringe I contained enzyme alone in buffer and Syringe II contained a mixture of the four substrates.

All experiments were performed in 0.1 M potassium phosphate buffer, pH 7.5. The concentrations of the reagents in the observation chamber were one-half of those in the syringes, since equal volumes of the two solutions were mixed. All reagents were prepared freshly for each experiment except L(+)-lactate which was prepared from a stock solution. The L(+)-lactate was carefully standardized each time by the method of Hohorst (14). Pyruvate (15) and NAD+ (16) were also standardized by previously published procedures. NADH concentration was calculated from its known extinction coefficient of 6.22 (cm²/μmol) (15, 17). Lactate dehydrogenase concentrations were calculated, by means of standard assay conditions, from the molar catalytic activities (turnover numbers) and known molecular weights of the beef (18) and salmon (7,8) enzymes.

**PROTEIN DETERMINATION—** The protein content of solutions was routinely estimated by measuring their absorbance at 280 nm, assuming E1% = 1.35, or by the method of Lowry et al. (13).

**Abortive Ternary Complexes—** Before each experiment the enzymes were extensively dialyzed against 0.1 M potassium phosphate buffer, pH 7.5. This buffer was used for all the spectrophotometric determinations. At the end of the dialysis aliquots were taken and assayed. The formation of the abortive ternary complex of E NAD+pyruvate was initiated by adding a small volume of the respective substrate to a cuvette containing enzyme and either NAD+ or pyruvate in buffer. The reaction mixture was quickly stirred, and absorbance at 322 nm was followed as a function of time until equilibrium was reached.

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the abortive complex with the Hβ isozyme. Increasing the concentration of enzyme increases both the rate and amount of complex formed. The final ΔA_{232} value at fixed NAD^+ (1.0 mM) and pyruvate (0.25 mM) is proportional to enzyme concentration; a plot of ΔA_{232} versus enzyme concentration (not shown) is linear. Similar absorption spectra were also obtained for the M_1 and Hα isozymes.

The time course of ternary complex formation for the Hβ enzyme, as measured by increased absorbance at 322 nm, is shown in Fig. 2. The absorbance change reaches a plateau in 20 to 25 min. At the concentrations shown here (0.25 mM pyruvate, 1 mM NAD^+) ternary complex formation is complete within 25 min for all three isozymes. In the experiments described below, changes in A_{232} were monitored for up to 2 h to assure that complex formation was complete under the test conditions.

The various isozymes differ in their susceptibility to complex formation over a wide range of pyruvate and NAD^+ concentrations. Fig. 3 shows the effect of increasing pyruvate concentration on abortive ternary complex formation. Experiments were performed with 2.0 × 10^{-4} M salmon isozymes and 1.0 × 10^{-3} M NAD^+ in 0.1 M potassium phosphate buffer, pH 7.5, at 25°C. The "muscle" isozyme is purified salmon M_α-M_β.

Fig. 4 (right). Effect of NAD^+ concentration on abortive ternary complex formation. Experiments were performed with 7.0 × 10^{-6} M salmon isozymes and 2.5 × 10^{-5} M pyruvate in 0.1 M potassium phosphate buffer, pH 7.5, at 25°C.

### Table I

| Isozyme   | Dissociation constants of abortive ternary complexes |
|-----------|------------------------------------------------------|
| E^*NAD^+ + pyruvate. | D After Eisenthal and Cornish-Bowden (24). |
| Apparent dissociation constant of the ternary complex to | |
| Maximum extinction coefficient of the ternary complexes at 322 | |
| Maximum extinction coefficient of the ternary complexes at 322 | |
| Maximum extinction coefficient of the ternary complexes at 322 | |

Mechanism of Abortive Ternary Complex Formation — Previous proposals (23) have suggested that abortive ternary complex formation may proceed via dissociation of lactate dehydrogenase tetramers followed by formation of complexes between subunits, coenzyme, and substrate, and then reassociation of these subunit complexes into inhibited tetramers. This proposal was tested by incubating a mixture of isozymically pure chicken Hβ and chicken M_1 lactate dehydrogenases (2.0 μM each) with 1 mM pyruvate and 1 mM NAD^+ under the usual conditions for ternary complex formation.

Control samples of H_1 and M_1 were treated separately under the same conditions. To the degree that complex formation might involve dissociation and reassociation, one would expect the formation of H_2M_1, H_2M_β, and H_α-M_β heterotetramers in the mixed incubation following the reassociation process. However, starch gel electrophoresis of even vastly overloaded samples failed to reveal the presence of any heterotetramers following complex formation either in the mixed incubation, in single isozyme controls, or in control mixtures which were not subjected to abortive ternary complex formation (starch gels not shown). We conclude from this experiment that abortive ternary complex formation does not require randomized tetramer

Assuming the tissue concentration of lactate dehydrogenase in salmon fish to be similar to the approximations reported for several mammalian tissues (26), 2.0 × 10^{-4} M can be considered to be physiologically. The reported NAD^+ concentration in the soluble cell fraction is about 1.0 mM (25), and the concentration of pyruvate in resting muscles of the rainbow trout, by means of older methods, is estimated to be in the order of 0.39 mM (27).
dissociation, since under these conditions both isozymes were completely converted to the inhibited complex form, yet no evidence of dissociation was observed.

**Enzyme Inhibition by Abortive Ternary Complex Formation**—It is difficult to directly measure the degree of inhibition of catalytic activity due to abortive ternary complex formation, as any attempt at direct measurement of enzymatic activity involves some form of perturbation of the incubation mixture.

One method of estimating inhibition is to dilute the abortive complex mixture and assay its remaining enzymatic activity spectrophotometrically. The complex breaks down slowly upon dilution into buffer, with a resultant increase in enzymatic activity. Treatment of the data as a semilogarithmic decay of inhibition function and extrapolation to zero time after dilution (Fig. 5) gives an estimate of the degree of inhibition before dilution. For the Hβ enzyme incubated at 2 μM enzyme, 0.5 mM pyruvate, 1.0 mM NAD+, the degree of inhibition as measured by this method is 85 to 90%. A similar experiment with the Ma-Mβ preparation showed 39% initial inhibition with 0.5 mM pyruvate, and 54% inhibition with 2 mM pyruvate.

However, this value represents a minimum estimate of the degree of inhibition, as the enzyme assay mixture contains 0.14 mM NADH, which itself appears to induce partial rapid ternary complex breakdown. This was confirmed by inclusion of 0.14 mM NADH in the dilution medium prior to assay for the Hβ isozyme. In this case the measured "recovery" of enzymatic activity appeared at every time interval to be greater than when dilution was carried out in buffer alone (Fig. 5). Most importantly, the extrapolated "zero time" level of inhibition was only 75%, rather than the above 85 to 90%, although the samples were treated identically for abortive ternary complex formation, prior to dilution and assay. It is thus clear that the high level of NADH used in the enzyme assay mixtures in itself promotes further rapid dissociation of approximately 15% of the diluted abortive complex, and that a more accurate assessment of the degree of inhibition by this method would be provided after correction for this NADH effect.

Dissociation constants for the breakdown of abortive complex upon dilution were also derived from these experiments. Plots of log (fraction inhibition) versus time give dissociation constants of 0.237 min⁻¹ for Ma-MBα, 0.107 min⁻¹ for Hβ, and 0.204 min⁻¹ for Hβ, diluted in buffer plus 0.14 mM NADH. These results reinforce those in Fig. 3 and Table I in showing that the abortive ternary complex formed by Ma-MBα is less readily formed and more unstable when extensively diluted than is the Hβ complex, and also show that NADH enhances complex breakdown.

The initial rate of inhibition of abortive ternary complexes was also assessed by stopped flow spectrophotometry, a procedure which is sensitive and allows the recording of very rapid activation events. The three purified salmon lactate dehydrogenases were incubated for up to 2 h to permit complex formation (2 μM enzyme, 0.5 mM pyruvate, and 1 mM NAD⁺) and then perturbed in the stopped flow spectrophotometer by addition of NADH (final concentration 0.05 mM). Fig. 6A shows the rate of NADH oxidation for the Hβ isozyme abortive ternary complex. Following the addition of NADH there is a significant lag period of at least 100 ms during which the final NADH concentration remains static; this is followed by a rate of NADH oxidation which gradually increases to the control value. The fact that the curve is initially horizontal implies that the enzyme is essentially completely inhibited at the start of the perturbation period. Without prior incubation to allow abortive ternary complex formation, oxidation of NADH occurs immediately upon its addition, without any observable lag. These results support the dilution recovery experiments in demonstrating that the Hβ isozyme is largely or fully inactivated upon incubation with 0.5 mM pyruvate and 1 mM NAD⁺. They also demonstrate that abortive ternary complexes could not exist in vivo should the steady state level of free NADH approach 50 μM.

Fig. 6B shows a similar experiment with the Ma-MBα isozyme preparation. In this case the lag period is virtually eliminated, and reaction ensues almost immediately on addition of NADH. From the dilution recovery experiments we would have expected approximately 40% initial inhibition of enzymatic activity and thus a reduced initial slope relative to the control. There is in fact a small but reproducible lag period, implying some degree of initial inhibition. However, due to the enhanced susceptibility of the Ma-MBα complex to NADH-induced dissociation, the lag is insufficient to permit accurate estimation of the degree of inhibition.

The inclusion of lactate enhances the effect of NADH at inducing abortive ternary complex breakdown (Fig. 6A); at 1 mM lactate the lag period before NADH oxidation commences is reduced to 40 ms, and at 25 mM lactate (not shown) NADH oxidation begins virtually immediately. However, lactate alone, in the absence of NADH, has no effect on the stability of the complex. Abortive ternary complexes formed either at
A very rapid drop in plex dissociation could not be accurately determined in the these conditions abortive complex re-forms. The level of NADH in the resulting equilibrium mixture is unphysiologically low (lo-').

Controls including perturbation with buffer or with lactate as high as 6 mM show no such drop. Note that in this experiment the concentration of NADH used (2.5 μM) is less than the concentration of tetrameric subunits (11.6 μM). Hence in this experimental system, where initially the enzyme is essentially fully inhibited, addition of 2.5 μM NADH produces a nonequilibrium and physiologically reasonable mixture of substrates where production of lactate is favored. This system comes very close to mimicking the putative (1) nonequilibrium conditions in aerobic tissues where abortive complex is proposed to be required to prevent the reduction of pyruvate to lactate. Yet under these conditions it is in fact seen that abortive complex is rapidly and substantially dissociated, and lactate is produced following the reactivation of lactate dehydrogenase activity. The level of NADH in the resulting equilibrium mixture of substrates is unphysiologically low (10^-6 M), and under these conditions abortive complex re-forms.

Unfortunately the maximum degree of NADH-induced complex dissociation could not be accurately determined in the above experiment since A_{342} monitoring was of necessity discontinuous, although from the shape of the recovery curve, dissociation would clearly exceed 25%. This experiment, therefore, provides only a semiquantitative assessment of the amount of abortive complex which could exist at low, steady state NADH concentrations. We have, therefore, attempted to quantify the possible effects of steady state levels of NADH in the 1 to 3 μM range in a different way. Initial perturbation of pre-formed abortive complex with sufficiently high NADH assures that the equilibrium mixture formed upon dissociation and enzyme activation will still contain micromolar levels of NADH. As shown in Fig. 8, perturbation of abortive complex (2.9 μM enzyme, 3 μM pyruvate, 1 mM NAD^+) with 2.6 mM NADH induces breakdown and produces an equilibrium mixture containing 2.5 μM NADH, 0.38 mM pyruvate, 3.8 mM NAD^+, and 2.6 mM lactate. (Final values are calculated assuming an apparent equilibrium constant for the lactate dehydrogenase reaction (31) at pH 7.5, 25°C, of 1.1 × 10^4.) The levels of pyruvate, and in particular, NAD^+ in this final equilibrium mixture were perhaps 3-fold above normal physiological (95) concentrations; this was necessary to achieve a mixture which was reasonably physiological and yet still contained 2.5 μM NADH. Note, however, that the effect of elevated pyruvate and NAD^+ is to maximize the possible degree of abortive complex formed, yet even under these conditions, re-formation of the dissociated complex was severely limited (final A_{342} = 0.011) when compared to the amount of complex formed by the control in the presence of enzyme, pyruvate, and NADH alone (final A_{342} = 0.054). Hence in a steady state situation where the levels of substrates constitute an equilibrium mixture of reasonably physiological proportions, lactate dehydrogenase would be approximately 75% active.

The inhibitory effect of NADH on the initial formation of abortive complex was also examined directly, without prior

Effects of NADH on Abortive Ternary Complex Stability — Attempts to quantitate the effects of very low steady state levels of NADH (1 to 5 μM) on abortive ternary complex dissociation by direct enzyme assay gave unsatisfactory results. Even slight dissociation, or residual enzymatic activity, would immediately oxidize this small amount of NADH, and the kinetics of this event could not be accurately followed at 340 nm due to the small change in absorbance. However, perturbation of abortive complexes by low levels of NADH can be shown to cause substantial complex breakdown by directly measuring changes in complex A_{342} absorption upon addition of NADH. As shown in Fig. 7, perturbation of abortive ternary complexes by even very low levels of NADH (2.5 μM) results in a very rapid drop in A_{342}, followed by a slow re-formation of complex after the small amount of added NADH is oxidized.

FIG. 6. Perturbation of abortive ternary complexes by NADH and lactate. Mixture of 2 μM enzyme (Hα4, Hβ4, or Mα3,Mβ4), 1 mM NAD^+, and pyruvate (0.5 mM) in 0.1 M potassium phosphate buffer, pH 7.5, were incubated up to 3 h at 25°C and subsequently transferred to Syringe I of the stopped flow apparatus. Syringe II contained physiological or saturating levels of pyruvate (0.5 mM or 5 mM, respectively) are stable in the presence of 6 mM lactate for periods of up to 4 h, as measured by A_{342}.
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Complex perturbation. An equilibrium mixture of substrates similar to the final mixture produced above was incubated with fresh enzyme, and the time course of complex formation was monitored at 322 nm. A parallel control contained enzyme, pyruvate, and NAD+ alone. As shown in Fig. 9, NADH reduced the extent of complex formed to approximately 30% of the control value at all times measured. The extent to which the enzyme was catalytically inhibited was also directly examined by enzyme assay by means of the dilution recovery technique. After 2 h incubation of the above mixtures, inhibition had reached its final value; the enzyme in the presence of pyruvate and NAD+ alone was 80% inhibited, but only 21% inhibited if NADH (and lactate) were also present.

Similar effects were observed with lower levels of NADH. Incubation of enzyme with an equilibrium mixture of 1 μM NADH, 0.7 mM pyruvate, 3.3 mM NAD+, and 2.3 mM lactate again resulted in reduced complex formation (final increase in $A_{322} = 0.024$) compared to the control with enzyme, NAD+, and pyruvate alone (final $A_{322} = 0.075$).

These effects were seen at a ratio of NADH:pyruvate dehydrogenase active sites of 1:1.9, but are in fact independent of enzyme concentration within a reasonable physiological range. The above substrate equilibrium mixtures were incubated with beef H4 enzyme ranging from 52 to 174 units/ml (1.0 to 3.5 μM). The percentage of inhibition due to abortive complex formation by lactate dehydrogenase, pyruvate, and NAD+ remained constant, as did the reduction in this inhibition due to the added presence of 1 μM NADH. The ratio of NADH to active sites in these experiments ranged from 1.04:0.0 to 1.0:14, so the effect of NADH is not a stoichiometric one. Due to the rapid rate of NADH-induced dissociation relative to complex re-formation even a low rate of NADH:active sites will assure the presence of a large and significant percentage of enzymatically active lactate dehydrogenase. From the results at 2.5 μM and 1 μM NADH, it would appear that steady state concentrations of NADH above 4 μM would totally prevent abortive complex formation.

Relationship between $A_{322}$ Changes and Enzyme Inhibition – An important assumption of the above experiment is that changes in complex concentration as monitored by absorbance changes at 322 nm should directly reflect changes in the degree of enzyme inhibition. The results in Fig. 10 demonstrate that there is indeed a direct correlation between enzyme inhibition as measured by dilution recovery assay and the degree of abortive complex formed as judged by $A_{322}$ changes.

It is important in this context to emphasize that formation of abortive complexes involves the enol tautomer of pyruvate (11, 22), which may vary between lots of pyruvate. Therefore, even
though different lots of pyruvate are standardized for total pyruvate content, the ability of these lots to elicit abortive complex may vary due to variable keto:enol ratios. For example the results of Figs. 1 to 4 cannot be directly compared to those in Figs. 5 to 10, since two different lots of pyruvate were used, possibly differing in enol content. For our studies this factor was unimportant since we were interested in comparing the relative behavior of different isozymes. Determination of the rate and of the absolute potential of a given lactate dehydrogenase for abortive complex formation would of course require that the initial enol pyruvate levels be known accurately, and that the rate of approach to tautomeric equilibrium be known in the system.

**DISCUSSION**

**Properties of Duplicated Salmon Enzymes** — The results presented here support our earlier suggestion (7, 8) that one of the duplicated H-type lactate dehydrogenases in salmonid fish, Ha+, is converging toward M, in functional properties. This enzyme was shown previously to be somewhat intermediate to the salmon Hβ and the M-type isozymes in all low enzyme level, *in vitro* catalytic properties examined, including apparent Michaelis constants for pyruvate and lactate, substrate optima, substrate inhibition, and product inhibition. In the present study, the formation and stabilities of abortive ternary complexes for all three enzymes were examined at concentrations of enzyme, coenzyme, and substrate chosen to approximate physiological levels. At all levels of pyruvate and NAD+ likely to be physiologically relevant, the extent of abortive ternary complex formed by the Ha+ isozyme was found to be somewhat intermediate to that of Hβ and the M+ isozymes. Similarly, the complex of Ha+-pyruvate-NAD+, once formed, was intermediate in its susceptibility to NADH-induced dissociation. Whether the *in vitro* catalytic properties examined here in fact provide direct, or only indirect, assessments of the true *in vivo* behavior in the cell. Recent arguments suggest that the lactate dehydrogenase reaction in aerobic cells always lies at, or very near, equilibrium (33, 34) and that, because of the position of the equilibrium constant and of the measured concentrations of lactate and pyruvate, the ratio of free, unbound NAD+:NADH will always be very high, approximately $10^5$ in rat liver. Similar values have been derived from measurements made with a number of aerobic tissues, including cardiac muscle (35), mammary gland (36), renal cortex (37), and adipose tissue (38), indicating that the oxidation-reduction states of the pyridine nucleotides in these tissues are all similar. Since the level of NAD+ has been found to range from 0.2 to 1.0 mM in such tissues (25) it follows that the concentration of free NADH must be very low, approximately 1 μM under normal metabolic conditions for aerobic cells. This concentration is 30- to 50-fold lower than the NAD+ optima or apparent Michaelis constants for NADH of typical vertebrate lactate dehydrogenase (39, 40), and as long as pyruvate levels are maintained at a low level (by pyruvate dehydrogenase (41-43)) the lactate dehydrogenase isozyme would be operating far from equilibrium.

We further question on the basis of thermodynamic and kinetic considerations whether abortive ternary complex formation, or any other form or reversible *in vitro* inhibition, need even be invoked as an essential means of controlling H+ enzyme behavior in the cell. Recent arguments suggest that the lactate dehydrogenase reaction in aerobic cells always lies at, or very near, equilibrium (33, 34) and that, because of the position of the equilibrium constant and of the measured concentrations of lactate and pyruvate, the ratio of free, unbound NAD+:NADH will always be very high, approximately $10^5$ in rat liver. Similar values have been derived from measurements made with a number of aerobic tissues, including cardiac muscle (35), mammary gland (36), renal cortex (37), and adipose tissue (38), indicating that the oxidation-reduction states of the pyridine nucleotides in these tissues are all similar. Since the level of NAD+ has been found to range from 0.2 to 1.0 mM in such tissues (25) it follows that the concentration of free NADH must be very low, approximately 1 μM under normal metabolic conditions for aerobic cells. This concentration is 30- to 50-fold lower than the NAD+ optima or apparent Michaelis constants for NADH of typical vertebrate lactate dehydrogenase (39, 40), and as long as pyruvate levels are maintained at a low level (by pyruvate dehydrogenase (41-43)) the lactate dehydrogenase isozyme would be operating far below optimum. Hence, unless there were excessively high levels of enzyme in these tissues, lactate formation could proceed at best only very slowly under such conditions. In contrast, the levels of NAD+ and lactate which exist during cardiac lactate utilization are in fact very near or even above the $K_v$ values for these substrates, and lactate to pyruvate conversion could occur much more efficiently and rapidly than the reverse reaction. One is, therefore, led to question the need for abortive ternary complex formation in order to prevent pyruvate reduction by lactate dehydrogenase under aerobic conditions; the rates of reaction in either direction may reach the appropriate levels simply as determined by $K_v$ values and substrate/product levels, as defined by Haldane (44, 45). Only under anaerobic conditions where pyruvate and NADH levels climb, such as occurs in skeletal muscle during vigorous exercise or in cardiac muscle during myocardial infarction or experimentally induced ischemia, could either lactate dehydrogenase isozyme efficiently catalyze pyruvate reduction. Under such conditions the M+ isozyme continues to function efficiently due to its resistance to product inhibition by lactate (46).
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It is our belief that measurement of the potential for formation and breakdown of abortive ternary complexes in vitro isa very useful parameter for comparison of lactate dehydrogenase isozymes but that such studies provide only indirect information on the catalytic properties of importance to isozyme function in vivo. There appears to be no necessity for invoking abortive ternary complex formation as a means of regulation of H1 isozyme activity in vivo. It remains to be demonstrated that such complexes occur in vivo, or that their formation is in fact required for preventing lactate formation, or indeed that the equilibrium for the lactate dehydrogenase reaction ever favors net lactate formation in aerobic cells in vivo. To the contrary, we provide evidence that extremely low levels of NADH in the physiological range rapidly dissociate abortive complexes in vitro, and would thus limit their formation in vivo.

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