Studies on the Kinetic Mechanism of Lipoamide Dehydrogenase from Rat Liver Mitochondria*

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SUMMARY

The kinetic mechanism of lipoamide dehydrogenase has been studied at pH 8.0, 37°C, using the enzyme from rat liver mitochondria. Initial velocity patterns obtained for both the forward and reverse reactions were a series of parallel lines. Michaelis constants for the reactants are: NAD, 0.52 mM; dihydrolipoamide, 0.49 mM; NADH, 0.062 mM; lipoamide, 0.84 mM.

Isotopic exchange between NAD and NADH was also studied. The exchange rate in the absence of lipoic derivatives was not significantly increased by the presence of all of the reactants. These data, as well as the results from product inhibition studies, are consistent with a Bi Bi Ping Pong mechanism for this enzyme as originally proposed by Massey et al. (MASSEY, V., GIBSON, Q. H., AND VEERG, C. (1960) Biochem. J. 77, 341). In addition, the formation of kinetically significant abortive complexes between the enzyme and NADH or lipoamide is shown.

Lipoamide dehydrogenase (reduced NAD: lipoamide oxidoreductase, EC 1.6.4.3) is the flavoprotein component of the α-keto acid dehydrogenase complexes catalyzing the terminal pyridine nucleotide-linked oxidation of the protein-bound dihydrolipoamide moieties in the overall oxidation of pyruvate and α-keto glutarate. Although in this role the lipoamide dehydrogenase is presumably bound in the multienzyme complex, it can be isolated free from the other enzyme components and shown to catalyze the reversible NAD-dependent oxidation of added dihydrolipoamide and other reduced lipoic acid derivatives.

Dihydrolipoamide + NAD⁺ ⇌ lipoamide + NADH + H⁺ (1)

Because of its function in α-keto acid oxidation, it is not surprising that lipoamide dehydrogenase is very widely distributed in nature. Indeed, the enzyme has been isolated from a number of mammalian, bacterial, and plant sources, including pig heart (1), beef liver (2), human liver (3), pig brain (4), Escherichia coli (5), Saccharomyces cerevisiae (6), and spinach leaves (7). Reports on some of the physical properties of the enzyme indicate that there is very little species variation, at least with respect to the molecular weight, sedimentation coefficient, fluorescence properties, visible absorption spectra, flavin content, and the nature of the flavin prosthetic group. In all cases, the molecular weight appears to be around 100,000 and the enzyme contains 2 moles of FAD per mole of enzyme.

The kinetic mechanism of lipoamide dehydrogenase was first studied by Massey and his co-workers using the enzyme from pig heart (1) and bakers' yeast (6). From studies of the spectral properties of the various oxidized and reduced forms of the enzyme and from results of initial velocity experiments and stopped flow studies, they proposed a ping-pong or binary complex type mechanism in which the oxidized enzyme is first reduced by dihydrolipoamide, yielding a stable enzyme intermediate which is then reoxidized by NAD.

However, more recently, this mechanism has been questioned by Visser et al. (8). They reported that their initial velocity and product inhibition studies indicated rather a sequential mechanism at least for the pig heart enzyme, involving the formation of a ternary complex with the oxidized pyridine nucleotide and reduced lipoic acid derivatives. Further work has not been reported to resolve this discrepancy.

This paper reports on initial velocity and product inhibition studies as well as preliminary experiments on the isotopic exchange in one of the partial reactions (NAD ⇌ NADH) of lipoamide dehydrogenase from rat liver mitochondria. These studies do not intend to represent a comprehensive investigation of the reaction mechanism. Instead, they are an attempt to clarify some of the discrepancies reported recently in the literature and to elucidate some of the anomalous behavior of this enzyme that has recently come to light.

MATERIALS AND METHODS

Enzyme Preparation—Lipoamide dehydrogenase was purified from rat liver mitochondria by the method described by Massey (9) for the enzyme from pig heart.

Mitochondria were prepared from frozen rat liver (10), lyophilized, and stored frozen until use. The lyophilized powder was extracted with 0.05 M potassium phosphate buffer, pH 7.0, + 0.1 mM EDTA as described by McClure et al. (11). The extract was precipitated at 0.35 saturation with solid (NH₄)₂SO₄, and

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the precipitate removed by centrifugation. The clear amber
colored supernatant fraction was used as the starting material
for the preparation of lipoamide dehydrogenase.

The subsequent steps followed the procedure of Massey (9),
including (NH₄)₂SO₄ fractionation between 0.50 and 0.80 satu-
rating, heating to 55° for 5 min, a calcium phosphate gel-cellulose
column chromatography step followed by a second (NH₄)₂SO₄
fractionation, between 0.40 and 0.75 saturation, and a final heat
step, 72° for 6 min.

The enzyme was then dialyzed against 0.01 M potassium
phosphate, pH 7.4, + 0.3 mM EDTA and applied to a DEAE-
cellulose column equilibrated with the same buffer. Approxi-
mately 12% of the total enzyme activity was eluted with the
wash. The remaining activity was tightly bound and was subse-
sequently eluted with 0.3 M potassium phosphate, pH 7.4, + 0.3
mM EDTA as originally described by Lusty (2). This fraction
was concentrated and dialyzed against 0.1 M potassium phos-
phate, pH 7.4, + 0.3 mM EDTA and used in all of the experi-
ments described below.

Substrates—Dihydrolipoamide was prepared by reducing
lipoamide with sodium borohydride as described by Reed et al.
(12) and recrystallized twice before use from benzene:n-hexane.
Lipoamide was also recrystallized before use. The mixed \textit{z}
forms of lipoamide and dihydrolipoamide were used in all ex-
periments. Stock solutions of dihydrolipoamide were made in
5 mM EDTA to prevent aerobic oxidation of the sulfhydryl
groups. Both lipoamide and dihydrolipoamide were added as
dilute ethanolic solutions and made up just prior to use.

Lipoamide was obtained from Sigma Chemical Co., NAD and
NADH chromatopure were obtained from P-L Biochemicals
Inc., and [carbonyl-¹⁴C]NAD was purchased from Amersham/
Searle Corp. The pyridine nucleotides were used without fur-
ther purification. Other chemicals used were of reagent grade.

Kinetic Stoichiometry—Initial velocity experiments were carried out
measuring the reaction in both the forward direction, NAD re-
duction by dihydrolipoamide and, in the reverse direction, using
lipoamide as the electron acceptor. In all cases, the reaction
medium contained, in a 3-ml volume, 0.1 M potassium phos-
phate, pH 8.0, 1.0 mM EDTA, and reactants at concentrations specified.

After appropriate temperature equilibration, the reaction was
initiated by the addition of enzyme and the rate monitored at
340 nm using a recording Gilford spectrophotometer equipped with a constant temperature control attachment. Initial reac-
tion velocities are expressed as micromoles of NADH oxidized
(or NAD reduced)·min⁻¹. Corrections were made for the ab-
sorption contribution at 340 nm from changes in lipoamide con-
centrations.

\[ \text{NAD} = \text{NADH Exchange} \]

In the isotopic exchange experiments, the reaction medium contained 0.1 M potassium phos-
phate, pH 8.0, 1.0 mM EDTA, and reactants as specified in a
final volume of 0.3 ml. After a 2-min incubation at 37°, in-
cluding lipoamide dehydrogenase and reactants, the exchange
was initiated by the addition of 5 μl of [¹⁴C]NAD (1.56 × 10⁶
cpm per ml) in tracer quantities. At specified times, aliquots
were transferred to tubes and placed in a boiling water bath for
15 s to terminate the reaction. Ten-microliter aliquots were
spotted on DEAE-cellulose paper (Whatman, DE-81) spotted
beforehand with carrier NAD and NADH. The pyridine nu-
cleotides were separated by ascending chromatography for 1 hr,
50 min in 0.15 M ammonium bicarbonate (13). The papers
were dried and the pyridine nucleotide spots located under an
ultraviolet lamp. The sections containing nucleotides were cut
out, transferred to vials containing toluene, 2,5-diphenyloxazole
(POPOP) (4 g per liter), 1,4-bis[2-(5-phenyloxazolyl)]benzene
(POPOP) (100 mg per liter) and counted using a Packard Tri-
Carb liquid scintillation counter.

The radioactivity of the [¹⁴C]NAD was calculated by determin-
ing the percentage of [¹⁴C]NAD converted to [¹⁴C]NADH by al-
cohol dehydrogenase and excess alcohol as suggested by Silver-
stein and Boyer (14).

The results described in the exchange experiments are expressed
as nanomoles of [¹⁴C]NADH found per total reaction volume,
and are corrected for background radioactivity found in the
NADH areas determined using denatured lipoamide dehydro-
genase.

Treatment of Data—The data obtained from the initial velocity
experiments for both the forward and reverse reactions were
fitted to the general equation for a Bi Bi Ping Pong mechanism:

\[
 v = \frac{V_{AB}}{K_v + K_{f}A + AB}
\]
 using the FORTRAN program kindly provided by W. W. Cleland,
Department of Biochemistry, University of Wisconsin. Michael-

a's constants for all of the reactants and the maximum veloc-
ities for the forward and reverse reactions are expressed as
weight averaged from several experiments.

The data from the product inhibition studies were not ap-
p lied to a computer analysis. Rather, each line was fitted using a
least squares analysis and inhibition constants were determined
by replotting slopes or intercepts.

In the analysis of the kinetic mechanism, the nomenclature
used is that described by Cleland (15, 16).

Results

Initial Velocity Experiments—In the forward reaction, when
dihydrolipoamide was varied at fixed levels of NAD, double
reciprocal plots obtained at 25° were concave down. At 37°,
however, linear patterns were always obtained. Because of the
difficulties in interpreting kinetic mechanisms from nonlinear
initial velocity data, we chose to carry out all of the studies at
37°. This anomalous effect of temperature will be discussed in
more detail below.

Initial velocity patterns obtained when NAD or dihydrollo-
amide was varied at fixed levels of the second substrate were a
series of parallel lines (Fig. 1A). Similarly, in the reverse reac-
tion, varying NADH or lipoamide also yielded parallel double
reciprocal plots (Fig. 1B). These patterns are consistent with a
ping-pong mechanism for this enzyme, and the kinetic param-
eters were calculated assuming this mechanism. When NAD
and dihydrolipoamide were varied at a fixed ratio, the resulting
reciprocal plot, as shown in Fig. 2, was linear. Analogous re-
sults were obtained for the reverse reaction, varying NADH,
and lipoamide at a fixed constant ratio. These results are again
consistent with the ping-pong mechanism; in a sequential mech-
anism, such plots would be expected to be parabolic (17).

Table I tabulates the Michaelis constants and the maximum
velocities for both the forward and reverse reactions. The ex-
perimental value for \( V_f \) and \( V_r \) were best estimated from experi-
ments as illustrated in Fig. 2. \( V_f \) was corrected for subrate
inhibition by NADH and lipoamide as described by Garces and
Cleland (18). It should be pointed out that no significant sub-
strate inhibition by NADH or lipoamide is seen in experiments,
as illustrated in Fig. 1B, since both substrates were varied only
at relatively low concentrations.

The experimentally determined value for the equilibrium
FIG. 1. Initial velocity patterns for the forward and reverse reactions. A, NAD is varied at fixed concentrations of dihydrolipoamide as indicated; 0.90 µg of enzyme protein. B, NADH is varied at fixed concentrations of lipoamide. Lipoamide concentrations from top to bottom are: 0.36 mM, 0.54 mM, 0.81 mM, and 1.62 mM; 0.87 µg of enzyme protein. Reaction conditions: 37°C; 0.1 M potassium phosphate, pH 8.0; 1.0 mM EDTA.

FIG. 2. Double reciprocal plots varying substrates at a fixed ratio. NADH varied with lipoamide/NADH = 10.33; 0.77 µg of enzyme protein. NAD varied with dihydrolipoamide/NAD = 1.07; 0.20 µg of enzyme protein. Reaction conditions are as in Fig. 1. Initial velocity is expressed as micromoles of substrate converted min⁻¹ µg protein⁻¹.

constant for Reaction 1 was 1.20 and can be compared to the Haldane expression defined in terms of Michaelis constants and maximum velocities. For a ping-pong mechanism with two substrates and two products, this particular Haldane expression (19) is given by

\[ K_{oe} = \frac{V_1}{V_2} \frac{K_2K_3}{K_3K_4} \]

The calculated \( K_{oe} \) from this expression is 1.55. The agreement between the calculated and experimentally determined values for the equilibrium constant is quite reasonable if one considers the experimental errors in determining all of these kinetic parameters.

Product Inhibition Experiments—The product inhibition studies to be described here were carried out measuring the reaction in the forward direction using NADH and lipoamide as product inhibitors.

At fixed high levels of NAD (4.2 mM or approximately 8 times the Michaelis constant for this substrate), the inhibition by NADH appeared to be competitive with dihydrolipoamide (Fig. 3). However, at lower fixed levels of NAD (0.52 mM) this inhibition pattern became noncompetitive (Fig. 4). Essentially similar patterns were obtained when NAD was the varied substrate and dihydrolipoamide was held constant. At 5.0 mM dihydrolipoamide, the inhibition by NADH was competitive versus NAD (Fig. 5); at 0.5 mM dihydrolipoamide, the inhibition was noncompetitive (Fig. 6). Again, the intercept effect was dependent on the concentration of the fixed substrate.

The inhibition patterns obtained using lipoamide as the product inhibitor were entirely analogous to those found using NADH. Competitive inhibition patterns were observed only when the concentration of the fixed substrate was relatively high. At concentrations near the Michaelis constants, noncompetitive inhibition patterns were observed.

\[ NAD \rightleftharpoons NADH Exchange \]—Since isotopic exchange between substrate-product pairs in the absence of other reactants should be observed in a Bi Bi Ping Pong mechanism, lipoamide dehydrogenase would be expected to catalyze the exchange between NAD and NADH in the absence of lipoamide and dihydrolipoamide. Fig. 7 shows the results obtained when the formation of \(^{14}C\)NADH from \(^{14}C\)NAD was measured both in the absence of other reactants and under equilibrium conditions in the presence of lipoamide and dihydrolipoamide. In this latter experiment, reactants were added at equilibrium concentrations and parallel experiments indicated that there was no change in the concentrations of the reactants during the time course of the isotopic exchange measurements. From Fig. 7 it is apparent that there is no stimulation of the NAD \rightleftharpoons NADH exchange rate by the addition of the other reactants.

Effect of Temperature—As reported above, when dihydrolipoamide was varied at fixed NAD concentrations, the Lineweaver-Burk plots were concave down at 25°C, while at 37°C no apparent deviation from linearity was observed. The initial velocity data were plotted according to the Hill equation

\[ \log \frac{v}{V - v} = n \log A - \log K \]

where A in this case is dihydrolipoamide. From Fig. 8, the Hill parameters for lipoamide dehydrogenase from rat liver mitochondria at 37°C, pH 8

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Michaelis constant ( K_m )</th>
<th>Velocity ( V_max )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Forward reaction</td>
<td>( K_{dihydrolipoamide} ) : 0.49 ± 0.01</td>
<td>4.14 ± 0.18 \times 10^4</td>
</tr>
<tr>
<td></td>
<td>( K_{NAD} ) : 0.52 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Maximum velocity (( V_{max} ))</td>
<td>3.02</td>
<td>1.49 ± 0.07 \times 10^4</td>
</tr>
<tr>
<td>2. Reverse reaction</td>
<td>( K_{NADH} ) : 0.062 ± 0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( K_{dihydrolipoamide} ) : 0.84 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Maximum velocity (( V_{max} ))</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

* Assuming a molecular weight of 100,000.
I/DIHYDROLIPOAMIDE, CmM)-l

I/DIHYDROLIPOAMIDE, CmM)-l

FIG. 3 (left). Product inhibition by NADH. Dihydrolipoamide was the varied substrate. NAD was held constant at 4.2 mM. Other conditions are as in Fig. 1, with 0.63 μg of enzyme protein. Inset shows the replot of the slopes versus NADH concentrations.

FIG. 4 (left center). Product inhibition by NADH. Dihydrolipoamide was the varied substrate. NAD was held constant at 0.52 mM. Other conditions are as in Fig. 1, with 1.02 μg of enzyme protein. Inset shows the replot of the slopes and intercepts versus NADH concentrations.

FIG. 5 (right center). Product inhibition by NADH. NAD was the varied substrate. Dihydrolipoamide was held constant at 5.0 mM. Other conditions are as in Fig. 1, with 0.92 μg of enzyme protein. Inset shows the replot of the slopes versus NADH concentrations.

FIG. 6 (right). Product inhibition by NADH. NAD was the varied substrate. Dihydrolipoamide was held constant at 0.50 mM. Other conditions are as in Fig. 1, with 0.92 μg of enzyme protein.

coefficient, n, was calculated to be 0.6 at 25°C, a value significantly less than unity. At 37°C, n was approximately 1, as expected from classical Michaelis-Menten kinetics. Corresponding studies at 10°C gave values of n near 0.5.

We have investigated the possibility that this effect of temperature on lipoamide dehydrogenase resulted from some temperature-dependent protein aggregation phenomenon, by measuring the sedimentation velocity of the active enzyme species using the method described by Cohen and Mire (20). These experiments indicated that there was no significant change in the sedimentation coefficient of the active enzyme at temperatures varying from 6°C to 35°C at similar protein concentrations.

Furthermore, when we measured the variation of the maximum velocity with temperature to determine the Arrhenius activation energy, we found no apparent break in the plot at least between 9.5°C and 39°C. Indeed, breaks in Arrhenius plots are often observed with enzymes that undergo substantial temperature-dependent conformational changes characterized by
relatively large changes in enthalpy (21). The Arrhenius activation energy for the forward reaction for lipoamide dehydrogenase was found to be 9.6 Cal mole−1.

Effect of pH on Forward Reaction—Fig. 9 shows a plot of the variation of the maximum velocity with pH. The reaction has a maximum velocity around pH 8 and decreases substantially at more acid pH. A group with an apparent pK of approximately 7 appears to be involved in the rate limiting step of the reaction.

DISCUSSION

In the initial velocity studies with rat liver lipoamide dehydrogenase, parallel Lineweaver-Burk plots were obtained at 37° varying substrates in both the forward and reverse directions. Such patterns are characteristic of ping-pong or binary complex mechanisms where the first product is released from the enzyme before the addition of the second substrate. The reaction can be written as

\[
dihydrolipoamide + E \rightarrow \frac{k_3}{k_4} \text{(dihydrolipoamide } - E \rightarrow \text{ lipoamide } - F)\]

\[
NAD + F \rightarrow \frac{k_6}{k_8} \text{(NAD } - F \rightarrow \text{ NADH } - E) \equiv \text{ NADH } + E (4)
\]

where \( E \) and \( F \) denote the stable oxidized and reduced states of the enzyme, respectively.

Although parallel initial velocity patterns are often indicative of ping-pong mechanisms, they are by no means conclusive evidence. Indeed, there are a number of reports in the literature suggesting ping-pong type mechanisms on the basis of parallel initial velocity patterns, when in fact further experimental evidence has indicated that the enzymes studied actually catalyze sequential type reactions. It is therefore important to substantiate the apparent reaction order from other lines of evidence. Some of the experiments that can be carried out in this respect include product inhibition studies and isotopic exchange experiments.

The isotopic exchange studies between NAD and NADH in this partial reaction of lipoamide dehydrogenase can be used as further evidence to support the proposed ping pong mechanism for this enzyme. In a sequential mechanism, isotopic exchange between the pyridine nucleotide pair would not be expected without the further addition of the other reactants. This is clearly not the case here. In fact, the exchange rate in the absence of the other reactants is at least as fast as the equilibrium exchange rate observed under nearly comparable conditions.

In a normal Bi Bi Ping Pong mechanism, in the absence of "dead end" inhibition, product inhibition by NADH would be expected to be competitive with dihydrolipoamide and non-competitive with respect to NAD. Furthermore, inhibition versus NAD would be expected to be overcome at saturating concentrations of dihydrolipoamide (16). Obviously, the inhibition patterns observed experimentally were not those predicted.

However, if NADH were also a dead end inhibitor, combining with the same stable enzyme form as NAD, namely \( F \) in Equation 4, and if the product and dead end inhibition constants were nearly comparable in magnitude, then the inhibition patterns obtained would depend largely on the concentration of the fixed unvaried substrate. Inhibition by NADH would be competitive with dihydrolipoamide only at elevated concentrations of NAD and competitive with NAD at near saturating levels of dihydrolipoamide. In addition, at lower concentrations of the fixed substrate, noncompetitive inhibition patterns would be expected varying either NAD or dihydrolipoamide. These predicted patterns were, in fact, observed experimentally.

An analogous argument can be made to account for the inhibition patterns observed using lipoamide.

The inhibition patterns observed for lipoamide dehydrogenase can be readily shown theoretically by examining the rate equation for this kinetic mechanism.

The rate equation for a Bi Bi Ping Pong mechanism in the absence of dead end inhibition has already been derived (15) and can be written in the form:

\[
v = \frac{V_i (A B - P Q / K_{eq})}{K_{s A} + A B + \frac{K_{s A} P}{K_{eq}} + \frac{K_{s A} Q}{K_{eq}}} \frac{K_{s A} Q}{K_{eq}} + \frac{K_{s A} P}{K_{eq}} (5)
\]

where \( A, B, P \) and \( Q \) are concentrations of dihydrolipoamide, NAD, lipoamide, and NADH, respectively; \( K_{s A}, K_{s B}, K_{s P} \) and \( K_{s Q} \) are Michaelis constants; \( K_{e A}, K_{e B}, K_{e P} \) and \( K_{e Q} \) are the inhibition constants for the reactants; \( K_{eq} \) is the equilibrium constant, and \( V_i \) is the maximum velocity of the reaction in the direction of NADH formation.

In the presence of added dead end inhibition by NADH and lipoamide, the rate equation will contain the terms \( (1 + P / K_{e P}) \) and \( (1 + Q / K_{e Q}) \), where \( K_{e P} \) and \( K_{e Q} \) are the dissociation constants for the dead end complexes. The rate equation, including these terms and substituting the appropriate Haldane relationship (15) for the equilibrium constant, can be rewritten in reciprocal form as follows, where NADH (Q) is the product inhibitor:

(a) varying dihydrolipoamide (A)—

\[
1 \frac{v}{V_i} = \frac{K_{s A}}{1 + \frac{Q}{K_{e Q}} \left(1 + \frac{K_{s A}}{K_{e A}} \right) \frac{1}{K_{s A} K_{e A} B} \left(1 + \frac{Q}{K_{e Q}} \right)} + \frac{1}{V_i} \left[1 + \frac{K_{s A}}{K_{e A}} \left(1 + \frac{Q}{K_{e Q}} \right) \right] (6)
\]

(b) varying NAD (B)—

\[
1 \frac{v}{V_i} = \frac{K_{s B}}{1 + \frac{Q}{K_{e Q}} \left(1 + \frac{K_{s B}}{K_{e B}} + \frac{K_{s B} K_{e B}}{K_{e B} K_{e A}} \right) \frac{1}{K_{s B} K_{e B} A} \left(1 + \frac{Q}{K_{e Q}} \right)} + \frac{1}{V_i} \left[1 + \frac{K_{s B}}{A} \left(1 + \frac{Q}{K_{e Q}} \right) \right] (7)
\]

An inspection of those equations will show that although noncompetitive inhibition patterns would be expected varying either dihydrolipoamide or NAD, these patterns would only be observed experimentally when the terms \( K_{e A} / B \) (in Equation 6) or \( K_{e A} / A \) (in Equation 7) are of significant magnitude. When these terms become very small the effect of varying concentrations of product, NADH, on the vertical intercept becomes negligible and apparently competitive inhibition patterns will always be observed.

Although, theoretically, slope replots of the double reciprocal plots should be parabolic, apparently linear replots were observed experimentally, as seen in the insets of Figs. 3 to 6. These results can be explained by the fact that the shape of the parabola will, of course, depend on the numerical values of the correspond-
ing coefficients of the appropriate equations and segments of the parabola can appear to be linear.

The product inhibition constants, $K_{ip}$ and $K_{ip}^*$, and the dead end dissociation constants, $K_{ip}$ and $K_{ip}^*$, were calculated from the corresponding intercept replots. The inhibition constants for lipoamide $K_{ip}$ and $K_{ip}$ were 7.6 mM and 4.7 mM, respectively, for NADH, $K_{ip}$ and $K_{ip}^*$ 0.19 mM and 0.29 mM, respectively.

The formation of kinetically significant abortive or dead end complexes between reactants and various enzyme forms (22) has been demonstrated for a number of enzymes. In fact, this often restricts the usefulness of product inhibition studies to demonstrate unambiguously the reaction order of the enzymes studied. In the case of lipoamide dehydrogenase, dead end inhibition by NADH and lipoamide was not entirely unexpected. It is likely that the enzyme contains a single pocket for the absorption of reactants and that this site is structurally quite similar in both the oxidized and reduced states of the enzyme.

The evidence cited above using rat liver lipoamide dehydrogenase supports the ping-pong or binary complex mechanism originally proposed by Massey et al. (1) for the enzyme from pig heart. From initial velocity studies, varying substrates in the forward direction, parallel initial velocity patterns were also reported for the pig heart enzyme. In stopped flow studies, Massey and his co-workers were able to demonstrate the reduction of the oxidized enzyme by either NADH or dihydrolipoamide in the absence of the other reactants. Furthermore, these reduction rates were sufficiently fast to account for the maximum velocity observed in the over-all reaction from steady state kinetics.

Although we cannot adequately account for the discrepancy between our results and those reported by Visser et al. (8), it is worth noting that their kinetic studies were carried out at 25° where nonlinear initial velocity patterns were observed, making unambiguous interpretation of the kinetic mechanism exceedingly difficult.

Although there are a number of explanations to account for the nonlinear initial velocity patterns observed at 25°, one explanation can be made in terms of the negative cooperativity model formulated by Koshland and his co-workers (23) based on ligand-induced conformational changes. Lipoamide dehydrogenase appears to contain two active sites per molecule (24) which may not be entirely independent, such that the binding of dihydrolipoamide at one site may affect the binding at the second site. From Fig. 8, the Hill coefficient was 0.6 at 25° in keeping with this model (25). The loss of cooperativity at elevated temperatures, 37°, may result from some temperature-dependent conformational change, possibly associated with the flavin-protein interaction at the active site.

At present, little is known concerning the over-all reaction mechanism involved in the transfer of electrons from NADH or dihydrolipoamide to the acceptor molecules, although the participation of an active center disulfide-dithiol redox couple in catalysis in addition to the FAD prosthetic group appears to be well established (24, 26, 27). The results from experiments on the variation of the reaction velocity with pH as shown in Fig. 9, may indicate the participation of a second group, with an apparent pK of approximately 7. Further studies with modified lipoamide dehydrogenase should be helpful in elucidating the nature of this species.

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