Saccharopine Dehydrogenase

SUBSTRATE INHIBITION STUDIES

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In the direction of reductive condensation of α-ketoglutarate and lysine, saccharopine dehydrogenase (N^+-(glutar-2-yl)-L-lysine:NAD oxidoreductase (lysine-forming)) is inhibited by high concentrations of α-ketoglutarate and lysine, but not by NADH. NAD^+ and saccharopine show no substrate inhibition in the reverse direction. Substrate inhibition by α-ketoglutarate and lysine is linear uncompetitive versus NADH. However, when the inhibition is examined with α-ketoglutarate or lysine as the variable substrate, the double reciprocal plots show a family of curves concave up. The curvature is more pronounced with increasing concentrations of the inhibitory substrate, suggesting an interaction of variable substrate with the enzyme form carrying the inhibitory substrate. These inhibition patterns, the lack of interaction of structural analogs of lysine such as ornithine and norleucine with the E-NAD^+ complex (Fujioka, M., and Nakatani, Y. (1972) Eur. J. Biochem. 25, 301-307), the identity of values of inhibition constants of α-ketoglutarate and lysine obtained with either one as the substrate inhibitor, and the substrate inhibition data in the presence of a reaction product, NAD^+, are consistent with the mechanism that substrate inhibition results from the formation of a dead-end E-NAD^+-α-ketoglutarate complex followed by the addition of lysine to this abortive complex.

A number of pyridine nucleotide-linked dehydrogenases have been shown to be inhibited by high concentrations of the substrates. The substrate inhibition has generally been considered as arising from the formation of a complex of a substrate and an enzyme form which is not supposed to react with. In pyridine nucleotide dehydrogenases, many examples are known in which substrate inhibition is caused by the formation of an abortive enzyme-oxidized coenzyme-oxidized substrate or enzyme-reduced coenzyme-reduced substrate complex (1-5). Recently substrate inhibition resulting from the combination of a substrate with central complexes has also been reported (6, 7).

It was previously shown that saccharopine dehydrogenase (N^+-(glutar-2-yl)-L-lysine:NAD oxidoreductase (lysine-forming)) which catalyzed a reversible reaction

Saccharopine + NAD^+ + H_2O <=> lysine
+ α-ketoglutarate + NADH + H^+

according to Equation 1 was subject to substrate inhibition by α-ketoglutarate in the reverse direction (8). Subsequently, it was found that lysine was also inhibitory at very high concentrations. The results of further studies, described herein, are consistent with the mechanism that the inhibition by these two substrates arises from the formation of a dead-end enzyme-NAD^+-α-ketoglutarate complex to which lysine adds subsequently, thus inhibiting the release of NAD^+.

EXPERIMENTAL PROCEDURE

Materials—NAD^+ and NADH (disodium salt) were obtained from Oriental Yeast Co., Osaka, and α-ketoglutarate from Sigma Chemical Co. Saccharopine was prepared as described previously (9). Other chemicals were obtained locally and were of the highest grade available. Saccharopine dehydrogenase was purified from autolyzates of bakers' yeast (Oriental Yeast Co., Osaka) as described elsewhere (9).

Rate Measurements—The saccharopine dehydrogenase reaction was followed by measuring the appearance or disappearance of NADH at 340 nm (Reaction 1) with a Hitachi model 124 double beam spectrophotometer equipped with a recorder model 556. The rate measurements were made in 2.0 ml of 0.1 M potassium phosphate buffer, pH 6.8, at 20°C. The concentrations of reactants are given in legends to figures. The reaction was started by the addition of 0.01 ml of an appropriately diluted enzyme solution (0%) to the mixture at 20°C which contained all other reaction components, and linear portions of the recorder tracings were taken as initial velocities. Dilution of a stock enzyme solution was made with 0.1 M potassium phosphate buffer, pH 6.8, containing 0.1% crystalline bovine serum albumin to minimize the loss of activity during experiments (9).

Noncompetitive substrate inhibition data (Fig. 5) were fitted to the equation

\[ v = \frac{V_{\text{max}}}{1 + K_i + K_{\text{in}} + K_i K_{\text{in}}} + \left( K_i + K_{\text{in}} \right) \]

and the values of apparent inhibition constants for slope (K_i) and intercept (K_{\text{in}}) were obtained by the least squares method using a FORTRAN program of Cleland (10). Data processing was done on a digital computer, NEAC-2200 model 700, by courtesy of the Computation Center, Osaka University.

The points drawn in the figures represent experimentally obtained values, while the lines are those calculated from Equation 3 or 4 using the kinetic constants obtained previously (9) and the constants determined in this paper (Table I, Column 2) (see "Discussion").

1 The nomenclature and terminology used in this paper are those of Cleland (11).
RESULTS

Substrate Inhibition by α-Ketoglutarate and Lysine—A previous investigation has shown that saccharopine dehydrogenase is inhibited by high concentrations of a substrate, α-ketoglutarate (8). Substrate inhibition was also noted by lysine at very high concentrations, but no inhibition was observed with NADH up to 0.2 mM (about 8 times the Michaelis constant) at α-ketoglutarate and lysine concentrations of 0.5 mM and 2.0 mM, respectively. High concentrations of NAD⁺ (up to 5.0 mM) and saccharopine (up to 40 mM) showed no inhibition in the forward direction (Reaction 1) at pH 6.8. The possibility that inhibition by either α-ketoglutarate or lysine was due to inhibitory contaminant was ruled out because precisely the same degree of inhibition was obtained by use of the compounds which had been recrystallized several times.

Fig. 1 shows the plots of reciprocal of initial velocities against reciprocal of NADH concentrations at a constant concentration of lysine (1.8 times the Michaelis constant) and several fixed, high levels of α-ketoglutarate. As the figure shows, at high α-ketoglutarate concentrations the inhibition was uncompetitive, and the replot of vertical intercepts versus α-ketoglutarate was a linear function. When α-ketoglutarate inhibition was examined with lysine as the variable substrate at a constant level of NADH, the double reciprocal plots gave a family of curved lines concave upward, although at low lysine concentrations the lines were nearly parallel (Fig. 2). The curvature was more pronounced with higher α-ketoglutarate concentration. However, when plots were made of 1/v versus α-ketoglutarate concentrations at each concentration of lysine, they were linear at high concentrations of the inhibitor. Figs. 3 and 4 show the inhibition by high concentrations of lysine with NADH and α-ketoglutarate as the variable substrates, respectively. The inhibition pattern with NADH as the variable substrate was linear uncompetitive, and the double reciprocal plots with α-ketoglutarate as the variable again showed curved lines with linear 1/v versus lysine concentration plot. If it is assumed that substrate inhibition by α-ketoglutarate and lysine is due to the formation of abortive complexes with E:NAD⁺ and/or central complexes, they should give uncompetitive patterns with respect to both NADH and noninhibitory substrate. The curved double reciprocal plots in Figs. 2 and 4 show that the mechanism of inhibition is more complex. However, from the dependence of inhibitory effect of a variable substrate on the concentration of a substrate inhibitor, and the linearity of the plots of 1/v versus concentrations of a substrate inhibitor at each concentration of a variable substrate, the observed inhibition patterns may most simply be explained as arising from the combination of a variable substrate with the abortive complex(es) carrying a substrate inhibitor.

Substrate Inhibition in Presence of NAD⁺—Whereas the experiments of Figs. 1 to 4 are consistent with the assumption above, they do not give indication as to whether the inhibition is caused by the combination of a substrate inhibitor with the E-NAD⁺ or central complexes, or both. These alternatives may partly be distinguished by running the inhibition experiment in the presence of a constant level of an added product, NAD⁺. In its presence, the binding of a substrate inhibitor to the E-NAD⁺ complex will cause a slope effect with NADH as the variable substrate, while the binding solely to the central complexes will not. Fig. 5 shows the α-ketoglutarate inhibition in the presence of NAD⁺. The presence of NAD⁺ did give a
slope effect. Values of the apparent inhibition constants for slope ($K_{in}$) and intercept ($K_{0}$) were determined by fitting the initial velocity data obtained with α-ketoglutarate concentrations of more than 7.5 mM to Equation 2, and were found to be $15.5 \pm 1.5$ mM and $17.6 \pm 2.0$ mM, respectively.

**DISCUSSION**

Previous investigations have shown that the kinetic mechanism followed by saccharopine dehydrogenase is an ordered Ter Bi mechanism (the reverse direction, Reaction 1) and the sequence of addition of substrates is NADH, α-ketoglutarate, and lysine (8, 9, 12). In this mechanism substrate inhibition may arise in the following cases. If α-ketoglutarate binds twice to the E-NADH complex in a dead-end fashion, a substrate inhibition by α-ketoglutarate which is uncompetitive with respect to NADH and noncompetitive with respect to lysine will result. Likewise, the combination of 2 molecules of lysine with the E-NADH-α-ketoglutarate complex will give rise to uncompetitive substrate inhibition versus NADH and α-ketoglutarate. If lysine binds to the E-NADH complex as a dead-end inhibitor, the resulting inhibition is uncompetitive when NADH is the variable substrate and competitive when α-ketoglutarate is varied. Qualitatively the same inhibition patterns are observed when this complex is followed by the addition of α-ketoglutarate to form an unreactive quaternary E-NADH-lysine-α-ketoglutarate complex. These possibilities are incompatible with the results obtained.

The binding of α-ketoglutarate or lysine, at high concentrations, to the E-NAD$^+$* and/or central complexes gives an uncompetitive substrate inhibition versus NADH. The inhibition should also be uncompetitive when α-ketoglutarate is the substrate inhibitor and lysine is the variable substrate, and vice versa. Curved double reciprocal plots in Figs 2 and 4 show that this is not the case. Nonlinear double reciprocal plots with respect to variable α-ketoglutarate and lysine may be obtained when they both add simultaneously to the E-NAD$^+$* and/or central complexes. Simultaneous binding of the two substrates is unlikely because the concentrations of variable substrate are within the noninhibitory range. Apparently uncompetitive patterns at low concentrations of variable substrates and a strong dependence of inhibition by variable substrates on the level of inhibitory substrates rather suggest that the curved lines result from the interaction of variable substrate with the abortive complex(es) carrying the substrate inhibitor. The linearity of 1/v versus inhibitor concentration plots is consistent with this idea and excludes the possibility of any partial inhibition. The discussion above assumed that both α-ketoglutarate and lysine at high concentrations could form abortive complexes, and the resulting complexes in turn could absorb the other substrate. But the same inhibition patterns will be seen when only one substrate can bind to the E-NAD$^+$* and/or central complexes, and the other adds subsequently to the complex(es). The binding of lysine to the E-NAD$^+$* or central complexes is unlikely from the lack of interaction of lysine analogs with these complexes. It has been shown that the analogs of lysine such as ornithine, norleucine, and leucine are potent competitive inhibitors of lysine in the reverse direction and produced no inhibition in the forward direction even at concentrations more than 50 times their dissociation constants from the E-NADH-α-ketoglutarate-analog complex (8). If α-ketoglutarate binds to the E-NAD$^+$* complex, the product inhibition by this compound in the forward direction should theoretically give a noncompetitive pattern when saccharopine is the variable substrate. The combination with the central complexes would give rise to noncompetitive inhibitions for variable NAD$^+$* and saccharopine. Under experimental conditions, however, no appreciable slope effect was observed with respect to both NAD$^+$* and saccharopine (12). This could be due to a large dissociation constant of α-ketoglutarate from these complexes (see below).

If an alternate reaction path in which the order of addition of α-ketoglutarate and lysine is reversed (E $\rightarrow$ E-NADH $\rightarrow$ E-NADH-lysine $\rightarrow$ central complex) operates at high concentrations of lysine and if lysine forms dead-end complex(es) with either E-NAD$^+$* or central complexes, or both, substrate inhibition for which the double reciprocal plots are linear with respect to NADH and curved to α-ketoglutarate would be obtained. In this mechanism, however, the plots of 1/v versus NADH concentrations would not be linear, contrary to the experimental finding (Fig. 4). It is also difficult to visualize nonlinear reciprocal plots when α-ketoglutarate is the substrate inhibitor and lysine the variable substrate, since the concentrations of the latter are kept at low level. Furthermore, the combination of lysine with the E-NAD$^+$* or central complexes may be excluded from reasons mentioned above.

Thus the observed substrate inhibitions may best be interpreted as the result of combination of α-ketoglutarate with the E-NAD$^+$* and/or central complexes and subsequent binding of lysine to the abortive complex(es) inhibiting the dissociation of NAD$^+$* or catalysis. The rate equation for this mechanism may be written as

$$V = \frac{V_{MAX} [A][B][C]}{[A][B][C] + [A][B][C] + [A][B][C] + [A][B][C] + [A][B][C] + [A][B][C]},$$

where substrates A, B, and C correspond to NADH, α-ketoglutarate, and lysine, respectively; $K_{A}$, $K_{B}$, $K_{C}$ are the Michaelis constants; $K_{AB}$ and $K_{AC}$ are the dissociation constants of A from the EA complex, and of B from the EAB complex. $K_{AC}$ and $K_{AC}$ are the dissociation constants of B and C from the abortive complexes, $f$ represents a factor to be multiplied when the inhibitor combines exclusively with either E-NAD$^+$* or central complexes. Values of $K_{AB}/f$ and $K_{AC}$ were obtained by fitting the data of Figs. 2 and 4 to Equation 3 by the least squares method assuming equal variance for the velocities. Preliminary estimates of these values were made graphically, and products of various kinetic parameters and substrate concentrations were calculated from the values of parameters previously determined under the same conditions (9), and these were used as the initial values for iterative fits. The computer-calculated values of $K_{AB}/f$ and $K_{AC}$ are listed in Table I. As the table shows,

**Table I**

**Inhibition constants**

| Constant | Value |
|----------|-------|
| $K_{AB}/f$ | $26.6 \pm 0.9$ mM$^a$ |
| $K_{AC}$ | $1.4 \pm 0.1$ mM$^a$ |
| $f$ | $0.33$ |

$^a$From data of Fig. 2.

$^b$From data of Fig. 4.

$^c$From data of Fig. 5.
there was a good agreement between the values obtained with α-ketoglutarate and lysine as the substrate inhibitors. Although from data of Figs. 1 and 3, Equation 3 is not solvable for $K_{in}$/ and $K_{in}$, the initial velocity values calculated by substituting values for $K_{in}$/, $K_{in}$, and other kinetic parameters together with substrate concentrations of Figs. 1 and 3 into the equation were in excellent agreement with those obtained experimentally as indicated by calculated lines. Thus all the substrate inhibition data shown in Figs. 1 to 4 conform to Equation 3, verifying the mechanism considered above.

Fig. 5 shows a linear noncompetitive substrate inhibition by α-ketoglutarate with respect to NADH in the presence of an added reaction product, NAD+. This indicates that α-ketoglutarate in producing inhibition binds at least to the $E \cdot NAD^+$ complex. In the presence of $NAD^+$ ($Q$), Equation 3 may be expanded to

$$V_{OABC} = \frac{K_I}{[I]} \left( 1 + \frac{C}{K_C} \right) \left( 1 + \frac{Q}{K_Q} \right) \left( 1 + \frac{Q}{K_{EQ}} \right) \left( 1 + \frac{Q}{K_{EQ}} \right)$$

where $K_Q$ is the dissociation constant of $Q$ from the $EQ$ complex. At high concentrations of $B$ (α-ketoglutarate), the apparent inhibition constant for slope ($K_{in}$) is equal to

$$K_{in} \left( 1 + \frac{C}{K_C} \right) \left( 1 + \frac{Q}{K_Q} \right) \left( 1 + \frac{Q}{K_{EQ}} \right) \left( 1 + \frac{Q}{K_{EQ}} \right).$$

and that for intercept ($K_{in}$) to

$$K_{in} \left( 1 + \frac{C}{K_C} \right) \left( 1 + \frac{Q}{K_Q} \right) \left( 1 + \frac{Q}{K_{EQ}} \right).$$

From these equations, by substituting values for $C$, $Q$, $K_C$, $K_{in}$, $K_{in}$, $K_{in}$, and $K_Q$ (1.38 mM), the approximate value of $f$ was calculated as 0.63. The $f$ value of less than unity indicates that α-ketoglutarate binds only to the $E \cdot NAD^+$ complex, not to the central complexes, otherwise the value should be 1.

Although the inhibition patterns by α-ketoglutarate and lysine are rather complicated, in the absence of further data, it may thus be assumed that the inhibition results from dead-end combination of the former with the $E \cdot NAD^+$ complex and the secondary binding of the latter to this abortive ternary complex, and the prevention of dissociation of NAD+. This interpretation is consistent with a previous finding that α-ketoglutarate and lysine in the concentration range covered in the current investigation failed to show substrate inhibition when the coenzyme was NADPH instead of NADH. NADPH did support the reaction, although with much less efficiency than NADH, but no appreciable binding of NADP$^+$ to saccharopine dehydrogenase could be demonstrated (9). Therefore, in the reaction with NADPH, the steady state concentration of $E \cdot NADP^+$ complex would be too low to allow the formation of an abortive complex with α-ketoglutarate.

It is of interest to note that, while the dissociation constant of α-ketoglutarate from the $E \cdot NADP^+ \cdot \alpha$-ketoglutarate complex ($K_{in}$) calculated from the data of Table I is about 60 times as large as that from the $E \cdot NADH \cdot \alpha$-ketoletoglutarate complex ($K_{in}$), lysine can bind readily to the $E \cdot NADP^+ \cdot \alpha$-ketoglutarate complex with a dissociation constant comparable to its Michaelis constant. Apparently the conformation of enzyme protein induced by α-ketoglutarate binding is of primary importance in lysine binding.

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