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KINETIC FORMULATIONS FOR THE REDUCTION OF KETOMALONATE BY LACTATE DEHYDROGENASE

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Initial rate kinetic studies of lactate dehydrogenase with ketomalonate and NADH as substrates suggest that this enzymatic system is adapted to a rapid equilibrium ordered bi-bi ternary complex mechanism. The application of the reaction product inhibition method reveals the existence of the enzyme-NADH-hydroxymalonate and enzyme-NAD^+-ketomalonate abortive complexes. This kinetic behaviour is confirmed by the differential inhibition induced by several alternate products on the pyruvate-lactate dehydrogenase-NADH and ketomalonate-lactate dehydrogenase-NADH systems.

KEY WORDS: Ketomalonate, lactate dehydrogenase, kinetic mechanism, alternate product inhibition.

INTRODUCTION

Lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) catalyses the reduction of several 2-oxoacids and 2,4-dioxoacids to 2-hydroxyacids and 2-hydroxy-4-oxoacids respectively, and its kinetic mechanism with its physiological substrates is of the ordered bi-bi ternary complex type.[1] It has been suggested[2] that lactate dehydrogenase forms a homologous system with the cytosolic and mitochondrial isoenzymes of malate dehydrogenase and that these three enzymes are derived from a common ancestral gene. Recently, Roderick and Banaszak,[3] by X-ray diffraction methods, have confirmed the structural similarity of mitochondrial malate dehydrogenase to cytosolic malate dehydrogenase and lactate dehydrogenase.

Comparisons between kinetic studies with preferred and alternate substrates could give some information about reaction pathways, as well as indications on the molecular basis of specificity. The purpose of this paper is to formulate the kinetic mechanism of lactate dehydrogenase with ketomalonate, an unnatural substrate of mitochondrial malate dehydrogenase,[4,5] by the reaction product inhibition method. The study of the inhibitions induced by different hydroxyacids reported here has confirmed the postulated mechanism and permits an easy distinction of the kinetic behavior of lactate dehydrogenase with ketomalonate from that exhibited with its physiological substrate, pyruvate.

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MATERIAL AND METHODS

Materials

Skeletal muscle from freshly killed guinea-pigs (200–250 g) was used. Ketomalonic acid, L-lactic acid, L-malic acid and hydroxymalonic acid (Sigma); sodium pyruvate, NADH and NAD^+ (Boehringer). All other chemicals used were reagent grade. Guinea-pig skeletal muscle lactate dehydrogenase M_4 isoenzyme, free from malate dehydrogenase, was obtained by a modification of the method of Puig *et al.*,[6] that includes 5′AMP-Sepharose chromatography as the last step. The column (1.6 × 35 cm) was equilibrated with 50 mM sodium phosphate buffer, pH 7.4, 0.05% sodium azide. Lactate dehydrogenase elution was carried out with the same buffer containing 2 mM NADH. The specific activity of the purified enzyme was 1380 I.U./mg (determined with pyruvate and NADH) and 17 I.U./mg (determined with ketomalonate and NADH).

Kinetic studies

The initial velocities of enzyme reduction of ketomalonate or pyruvate by NADH were determined at 30 ± 0.1°C in 100 mM sodium phosphate buffer, pH 7.4, by measurement of the absorbance changes of the NADH at 340 nm in a Hewlett-Packard 8450 A spectrophotomer, in 3 ml and 1 cm light-path cells. One unit of lactate dehydrogenase activity is defined as the amount of enzyme producing the conversion of 1 μmol of substrate per minute, under the conditions of assay.

The kinetic mechanism of the system under study was established by the reaction product inhibition method[7] and was confirmed by the study of the inhibition induced by alternate products, which may be expected to give rise to "dead-end" complexes. All initial rate data were plotted in double-reciprocal form and the linear or non-linear nature of the inhibitions studied was established from the replots of the slopes and/or the intercepts versus the inhibition concentrations assayed. Kinetic parameters were determined by fitting data to the appropriate rate equation[8] by non-linear regression using a Basic program on a Olivetti M24 computer.[9] Parameter values are expressed as parameter ± S.D. (due to regression).

RESULTS AND DISCUSSION

Initial Velocity Studies in Absence of Products

Saturation curves of lactate dehydrogenase with ketomalonate and NADH as variable substrates were of the hyperbolic type. Saturating concentrations of ketomalonate and NADH were 13 mM and 0.14 mM, respectively.

Double-reciprocal plots with ketomalonate and NADH as substrates gave a family of straight lines with a common intercept to the left of the ordinate axis, above the X axis, if NADH is the variable substrate (Figure 1B). When ketomalonate was varied at different levels of NADH, however, the point of intersection was on the ordinates axis (Figure 1A). When the data were plotted with NADH as the variable substrate, a replot of the slopes versus the reciprocal of the ketomalonate concentration went through the origin. These patterns indicate that the reaction is equilibrium ordered with NADH being the first substrate added to the reaction sequence.[8] Kinetic par-

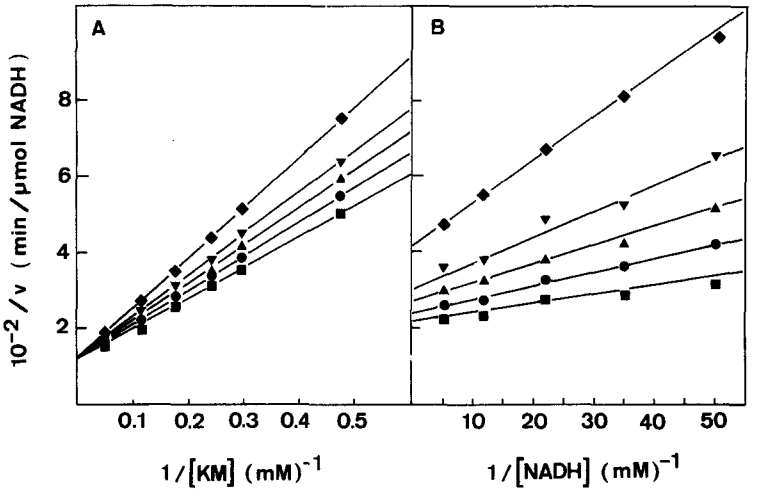

FIGURE 1 Double-reciprocal plots for guinea-pig lactate dehydrogenase. (A) Variation of the inital rate of reaction vs. ketomalonate concentration at different fixed concentrations of NADH. (◆) 0.036 mM; (▼) 0.06 mM; (▲) 0.072 mM; (●) 0.096 mM; (■) 0.14 mM. (B) Variation of the initial rate of reaction vs. NADH concentration at different fixed concentrations of ketamalonate. (◆) 2.1 mM; (▼) 3.6 mM; (▲) 4.4 mM; (●) 5.8 mM; (■) 7.2 mM. The assays were carried out in 100 mM sodium phosphate buffer, pH 7.4. [LDH] = 0.44 µg/ml.

ameters values, obtained by fitting the initial reaction rates to the rapid-equilibrium ordered bi-bi kinetic mechanism rate equation,[9] were: K_m (ketomalonate) = 5.7 ± 0.3 mM; K_m (NADH) = 0.078 ± 0.004 mM; V_{max} = 0.0080 ± 0.0003 µmol/min (about 1.2% of V_{max} obtained with physiological substrates).

Reaction Product Inhibition

The use of products of a reaction to investigate the kinetic mechanism of an enzyme through inhibition studies has become one of the most common tools of the kineticist. Reaction product inhibition of the lactate dehydrogenase by hydroxymalonate and NAD^+ with respect to each substrate was carried out in the presence of a constant concentration, saturating and non-saturating, of the other substrate. The results obtained (Table I) agree basically to that described for a rapid equilibrium ordered bi-bi ternary complex mechanism, with the existence of both enzyme-coenzyme binary complexes and enzyme-NAD^+-ketomalonate and enzyme-NADH-hydroxymalonate abortive complexes, the limiting step of the velocity being the release of hydroxymalonate.[10] The only discrepancies appear in the existence of hydroxymalonate inhibition versus NADH at saturating ketomalonate and NAD^+ inhibition with ketomalonate at saturating NADH, which according to the theoretical predictions[10] should not occur. Nevertheless, since the inhibition exercised by hydroxymalonate and ketomalonate or by NAD^+ and NADH between them was competitive (see Table I), the saturating concentrations of the substances determined in absense of products are no longer saturating under these conditions and it would be necessary to increase them considerably to cancel out the inhibition. This is in agreement with the increase in the inhibition constants on raising the concentration of the constant substrate (see Table I).[10]

TABLE I
Reaction product inhibition of ketomalonate-lactate dehydrogenase-NADH system

	Variable Substrate			
	Ketomalonate (1.5–15 mM)		NADH (0.02–0.2 mM)	
Inhibitor	NADH non-sat. 0.03 mM	NADH sat. 0.14 mM	KM non-sat. 2.5 mM	KM sat. 13 mM
Hydroxymalonate (10–100 mM)	C K_{is} = 60 mM	C K_{is} = 56 mM	UC K_{ii} = 14 mM*	UC K_{ii} = 70 mM*
NAD^+ (0.1–2 mM)	M K_{is} = 0.7 mM** K_{ii} = 0.2 mM**	M K_{is} = 4 mM** K_{ii} = 1.5 mM**	C K_{is} = 0.28 mM	C K_{is} = 0.31 mM

K_{is}: Inhibition constant determined from the slope vs. inhibitor concentration linear replot;
K_{ii}: inhibition constant determined from the intercept vs. inhibitor concentration linear replot.
C: Competitive inhibition; UC: uncompetitive inhibiton; M: mixed inhibition. (*) K_{ii} value increases with increasing concentration of fixed substrate (KM). (**) K_{is} ans K_{ii} values increases with increasing concentration of fixed substrate (NADH).
The values shown are the average of the results of 3 experiments. The variability observed is about 5%.

Inhibition by Alternate Products

The use of alternate products is a way of diagnosing or confirming a kinetic mechanism. For this reason we determined the influence of the nature of the substrates, pyruvate or ketomalonate, on the inhbition patterns caused by the hydroxyacids L-lactate, L-malate and hydroxymalonate on the lactate dehydrogenase activity.

According to the general rules for predicting the effect of normal or alternate products on the slope and intercept of double-reciprocal plots for a given varied substrate,[10] the different inhibitions obtained (Table II) show that all the hydroxyacids used can bind both to the enzyme-NAD^+ and enzyme-NADH complexes and form the ternary complexes enzyme-NAD^+-hydroxyacid and enzyme-NADH-hydroxyacid

TABLE II
Inhibition by several hydroxyacids of pyruvate-lactate dehydrogenase-NADH system

	Variable Substrate			
	Pyruvate (0.1–1 mM)		NADH (0.02–0.2 mM)	
Hydroxyacid	NADH non-sat. 0.03 mM	NADH sat. 0.14 mM	Pyr non-sat. 0.2 mM	Pyr sat. 1 mM
L-lactate (5–50 mM)	M[a] K_{ii} = 17 mM	M[a] K_{ii} = 19 mM	M[b] K_{is} = 14 mM	UC K_{ii} = 30 mM
L-Malate (20–200 mM)	M K_{is} = 90 mM K_{ii} = 52 mM	M K_{is} = 98 mM K_{ii} = 64 mM	UC[b]	UC K_{ii} = 79 mM
Hydroxymalonate (10 – 100 mM)	M K_{is} = 42 mM K_{ii} = 24 mM	M K_{is} = 48 mM K_{ii} = 20 mM	UC[b]	UC K_{ii} = 22 mM

[a]Replots of slopes vs. the inhibitor concentrations assayed were non-linear.
[b]Replots of intercepts vs. the inhibitor concentrations assayed were non-linear.
For abbreviations and comments, see legend of Table I.

TABLE III
Inhibition by several hydroxyacids of ketomalonate-lactate dehydrogenase-NADH system

	Variable Substrate			
	Ketomalonate (1.5–15 mM)		NADH (0.02–0.2 mM)	
Hydroxyacid	NADH non-sat. 0.03 mM	NADH sat. 0.14 mM	KM non-sat. 0.2 mM	KM sat. 1 mM
L-lactate (5–50 mM)	M K_{is} = 14 mM K_{ii} = 23 mM	M K_{is} = 16 mM K_{ii} = 29 mM	UC[a]	UC K_{ii} = 16 mM
L-Malate (20–200 mM)	C K_{is} = 109 mM	C K_{is} = 118 mM	UC K_{ii} = 73 mM	UC K_{ii} = 198 mM
Hydroxymalomate (10–100 mM)	C K_{is} = 60 mM	C K_{is} = 56 mM	UC K_{ii} = 14 mM	UC K_{ii} = 70 mM

[a]Replots of intercepts vs. the inhibitor concentrations assayed were non-linear. For abbreviations and comments, see legend of Table I.

when they act on the pyruvate-lactate dehydrogenase-NADH system. However, when ketomalonate is used as substrate instead of pyruvate, the inhibition patterns (Table III) show that hydroxymalonate and L-malate form exclusively the enzyme-NADH-hydroxyacid dead-end complex. This is in agreement with the rapid equilibrium established under these conditions and with the very low affinity that hydroxymalonate and L-malate possess for the enzyme-NAD^+ complex. On the contrary, L-lactate can bind both to the enzyme-NADH and to the enzyme-NAD^+complexes due to its higher affinity to the latter, so that the ternary complex enzyme-NAD^+ L-lactate can reverse the reaction as it is catalytically active, in contrast to enzyme-NAD^+-L-malate and enzyme-NAD^+-hydroxymalonate complexes which are inactive.

Our results show that alternate product inhibition experiments provide additional checks on the assumed mechanism for lactate dehydrogenase with ketomalonate as substrate and allow a kinetic differentiation of the pyruvate-lactate dehydrogenase-NADH and ketomalonate-lactate dehydrogenase-NADH enzymatic systems (see Tables II and III). Differences in inhibition patterns and linearity of secondary replots can be explained by a change from steady-state to rapid equilibrium ordered kinetics when lactate dehydrogenase uses a poor substrate like ketomalonate instead of its physiological substrate, pyruvate.

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