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KINETIC FORMULATIONS FOR THE OXIDATION AND THE REDUCTION OF GLYOXYLATE BY LACTATE DEHYDROGENASE

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Summary

Chicken liver lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) irreversibly catalyses the oxidation of glyoxylate (hydrated form) (I) to oxalate (pH = 9.6) and the reduction of (non-hydrated form) (II) to glycolate (pH = 7.4). (I) attaches to the enzyme in the pyruvate binding site and (II) attaches to the enzyme at the L-lactate binding site. The oxidation of (I) (pH = 9.6) is adapted to the following mechanism:

$$E \overset{NAD^+}{\rightleftharpoons} E\text{-}NAD^+ \overset{I}{\rightleftharpoons} [E\text{-}NAD^+\text{-}I] \rightarrow E\text{-}NADH\text{-}oxalate \overset{oxalate}{\rightleftharpoons} E\text{-}NADH \overset{NADH}{\rightleftharpoons} E$$
$$E\text{-}NADH \overset{I}{\rightleftharpoons} E\text{-}NADH\text{-}I$$

The reduction of (II) (pH = 7.4) is adapted to the following mechanism:

$$E \overset{NADH}{\rightleftharpoons} E\text{-}NADH \overset{II}{\rightleftharpoons} [E\text{-}NADH\text{-}II] \rightarrow E\text{-}NAD^+\text{-}glycol \overset{glycol}{\rightleftharpoons} E\text{-}NAD^+ \overset{NAD^+}{\rightleftharpoons} E$$
$$E\text{-}NAD^+ \overset{II}{\rightleftharpoons} E\text{-}NAD^+\text{-}II$$

The abortive complexes, E-NADH-I and E-NAD^+-II, are responsible for the inhibition by excess substrate in the reduction and oxidation systems, respectively. When lactate dehydrogenase and NAD^+ are preincubated, E-NAD^+-NAD^+ appears and causes inhibition by excess NAD^+ in the glyoxylate-lactate dehydrogenase-NAD^+ and L-lactate-lactate dehydrogenase-NAD^+ systems; the second NAD^+ molecule attaches to the enzyme at the L-lactate binding site.

Introduction

Oxalate is an end product in human metabolism; excess metabolite causes the appearance of calcium oxalate stones in the urine. The source of the pathogenesis of hereditary alterations associated with the continued, excess synthesis

of oxalate, that is hyperoxaluria type I (glycolic aciduria) and type II (L-glyceric aciduria), is unknown.

Endogenous oxalate proceeds, preferably, from the oxidation of glyoxylate; it is recognised that lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) is the enzyme playing the main role in the production of oxalate in the cell cytoplasm of the majority of mammal tissues [1,2], among them human liver [3], heart [3] and erythrocytes [4]. The oxidation of glyoxylate to oxalate, and its reduction to glycolate by the enzyme require NAD and NADH, respectively.

When comparing the activities of lactate dehydrogenase crystallised from ox heart, with respect to pyruvate and glyoxylate [5], it is observed that the activity exhibited with the latter is only 25% of that exhibited with pyruvate. It has been proposed [2] that the glyoxylate and the pyruvate are converted at the same site of the enzyme molecule and that the same functional groups take part in both conversions.

Lactate dehydrogenase from chicken liver [6] and from other sources [7,8] is adapted to an ordered bi-bi ternary complex mechanism in the conversion of pyruvate in L-lactate. From work with pig heart enzyme [2], it is shown that it behaves identically with pyruvate and glyoxylate, and a sequential mechanism for the reaction with both substrates is postulated. On the other hand, glyoxylate conversions catalysed by rabbit skeletal muscle lactate dehydrogenase are adapted to a Theorell-Chance mechanism [1].

In this paper the conditions are described under which lactate dehydrogenase from chicken liver catalyses the oxidation of glyoxylate to oxalate and its reduction to glycolate. The enzyme molecular site at which the glyoxylate is converted in both reactions are discussed and the kinetic mechanism is established. The formation of abortive complexes between the enzyme, glyoxylate and adenine nucleotides is shown. Likewise, an attempt has been made to determine the conditions under which inhibition for excess glyoxylate takes place.

Methods

Chicken liver lactate dehydrogenase crystallised according to the method of Lluis et al. [9] was used. The specific activity of the preparation is $102 \cdot 10^3$ units/mg protein (1 unit = 1 μmol NADH transformed/min); 1 g of purified preparation contains 200 mg protein. The substrates or inhibitors used were: glyoxylic acid and 50% sodium DL-lactate (Merck); sodium pyruvate, NAD^+ and NADH (Boehringer); sodium oxalate (UCB) and glycolic acid (Fluka). Freshly prepared solutions were used in the buffer required for each experiment and the pH was adjusted in each case (pH meter). 50 Mm glycine/NaOH buffer (pH = 9.6) and 50 mM sodium phosphate buffer (pH = 7.4) were used.

The rate of NADH oxidation at λ = 340 nm was followed to measure initial reaction velocities in a Beckman DBGT recording spectrophotometer, at 30 ± 0.1°C, in 3-ml cells and 1-cm light path. Addition of lactate dehydrogenase usually initiated the reaction.

The values of the kinetic parameters and of the enzyme-substrate complex dissociation constants were calculated according to the methods of Dalziel

[10] and Vestling & Florini [11]. The inhibitions were characterised graphically by plotting $1/v$ against $1/s$. The method of Dixon [12] was used for the determination of the binding site for two substrates present in one enzyme. The reaction mechanism was established by study of the substrate inhibition and product inhibition by the application of Cleland's rules [13,14].

Results

Oxidation and reduction of the glyoxylate at different pH

The optimum pH ranges for lactate dehydrogenase activity in the oxidation and reduction of glyoxylate lie between 9.4 and 9.6, and 7 and 7.4, respectively. Substrate inhibition appears in the glyoxylate-lactate dehydrogenase (6.6 μg/ml of purified preparation)/NADH system at pH 7.4 if the glyoxylate and NADH concentrations are above 24 mM and 0.1 mM respectively. The same system only acts at pH 9.6 if the enzyme concentration is at least 0.4 mg/ml of purified preparation.

In the glyoxylate-lactate dehydrogenase (41.6 μg/ml of purified preparation)/NAD^+ system at pH 9.6, only NAD^+ (4 mM or above) causes substrate inhibition. However in the same system at pH 7.4 with an enzyme concentration of 41.6 μg/ml of purified preparation, the glyoxylate (4 mM or above) causes substrate inhibition.

Oxidation and reduction of glyoxylate catalysed by lactate dehydrogenase and performed at pH 6; 7.4 and 9.6 do not appear reversible within the wide range of substrate concentrations used (from 1 to 100 mM).

Variation of the initial reaction rates with the concentration of substrate

The initial reaction rates for varying concentrations of substrate and coenzyme, below or equal to the kinetically saturating concentrations, in glyoxylate (4—24 mM)/lactate dehydrogenase (50 μg/ml of purified preparations)/NAD (0.5—3.7 mM) (pH = 9.6) and glyoxylate (4—15 mM)/lactate dehydrogenase (1.6 μg/ml of purified preparation)/NADH (0.005—0.1 mM) (pH = 7.4) systems, are non-parallel straight lines in both cases, suggesting that the kinetic mechanism is sequential.

Bonding sites of glyoxylate in lactate dehydrogenase

The site of the enzyme molecule to which the glyoxylate bonds when oxidised or reduced has been located by determining the behaviour of non-equimolar mixtures of glyoxylate and L-lactate or of glyoxylate and pyruvate in the glyoxylate or L-lactate/lactate dehydrogenase/NAD^+ system, at pH 9.6 and glyoxylate or pyruvate/lactate dehydrogenase/NADH system at pH 7.4, by application of Dixon's method [12]. The results are given in Table I.

It may be said that the glyoxylate and the L-lactate compete for the same site in the oxidation process whereas when glyoxylate is reduced, it competes for the pyruvate binding site of enzyme molecule.

In the process of reduction of glyoxylate (2.5 to 33 mM)/lactate dehydrogenase (6.6 μg/ml of purified preparation)/NADH (0.1 mM), pH 7.4, the glyoxylate does not compete with the L-lactate (8.3 and 40 mM); (K_{ip} = 14 mM,

TABLE I

NON-EQUIMOLECULAR MIXTURES OF L-LACTATE/GLYOXYLATE AND PYRUVATE/GLYOXYLATE

Theoretical rates for competing ($V_T^{(1)}$), or not competing ($V_T^{(2)}$) substrates for the same site; V_{exp} = experimental rates System L-lactate or glyoxylate/lactate dehydrogenase/NAD^+; pH = 9.6; [NAD^+] = 4 mM; [enzyme] = 41.6 μg/ml of purified preparation; K_m (L-lactate) = 3.5 mM; V (L-lactate) = $10.6 \cdot 10^{-2}$ ΔA/min; K_m (glyoxylate) = 10 mM; V (glyoxylate) = $20 \cdot 10^{-2}$ ΔA/min. System pyruvate or glyoxylate/lactate dehydrogenase/NADH; pH = 7.4; [NADH] = 0.1 mM; [enzyme] = 1.6 μg/ml of purified preparation; K_m (pyruvate) = 0.03 mM; V (pyruvate) = $4.8 \cdot 10^{-2}$ ΔA/min; K_m (glyoxylate) = 3.3 mM; V (glyoxylate) = $6 \cdot 10^{-2}$ ΔA/min.

L-lactate (mM)	Pyruvate (mM)	Glyoxylate (mM)	$V_T^{(1)}$ ($\times 10^2$)	$V_T^{(2)}$ ($\times 10^2$)	V_{exp} ($\times 10^2$)
5	—		7.4	12.2	6.7
6.6	—	1.6	7.6	12.9	7
8.3	—		7.9	13.5	7.7
16.6	—		8.4	14.4	8.3
—		1	3.3	4.4	3.5
—	0.05	3	3.7	5.8	4.4
—		5	4.1	6.6	4.7
—		10	4.6	7.5	5.7

K_{ii} = 34 mM) * or with the oxalate (0.1 and 0.25 mM) (K_{ip} = 0.05 mM, K_{ii} = 0.11 mM); these inhibitors interact with the enzyme in the L-lactate site itself. In the oxidation process of glyoxylate (2.5—33 mM)/lactate dehydrogenase

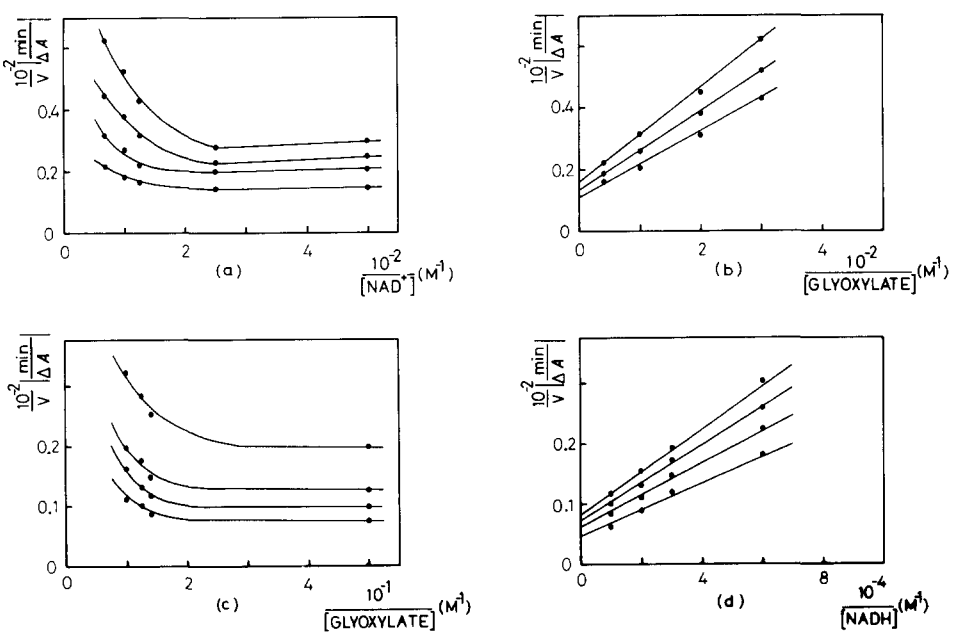

Fig. 1. Inhibition by substrates. Glyoxylate/lactate dehydrogenase/NAD^+ system (pH = 9.6); [enzyme] = 50 μg/ml of purified preparation; bottom to top: (a) [glyoxylate] (mM): 3; 5; 10; 24; (b) [NAD^+] (mM): 8; 10; 15. Glyoxylate/lactate dehydrogenase/NADH system (pH = 7.4); [enzyme] = 1.6 μg/ml of purified preparation; bottom to top: (c) [NADH] (mM): 0.1; 0.005; 0.03; 0.016; (d) [glyoxylate] (mM): 100; 80; 70; 20.

(41.6 μg/ml of purified preparation)/NAD (4 mM), pH = 9.6, the glyoxylate competes with the oxalate (1.5 and 7.5 mM); (K_{ip} = 1.2 mM) but not with the pyruvate (1 and 3.5 mM) (K_{ip} = 0.43 mM, K_{ii} = 1.3 mM). This shows that the glyoxylate is reduced in the lactate dehydrogenase site proper to the pyruvate whereas its oxidation takes place in this site characteristic to the L-lactate.

Inhibition for substrate

In the glyoxylate/lactate dehydrogenase/NAD^+ system (pH = 9.6) the cofactor (4 mM or above) inhibits by excess of substrate (Fig. 1a) wherein it differs from the glyoxylate. The inhibition induced by NAD^+ (Fig. 1b) is not competitive with respect to the glyoxylate.

The glyoxylate (glyoxylate/lactate dehydrogenase/NADH system, pH = 7.4) which inhibits by excess of substrate (24 mM or above) (Fig. 1c) is a non-competitive inhibitor with respect to NADH (Fig. 1d).

Inhibition by the reaction products

In the glyoxylate (2.5—33 mM)/lactate dehydrogenase (41.6 μg/ml of purified preparation)/NAD system, pH = 9.6, the oxalate (1.5 and 7.5 mM) is a competitive inhibitor with respect to the glyoxylate (K_{ip} = 1.2 mM) with saturating NAD (4 mM) or below this level (1.3 mM). The NADH (0.1 and 0.2 mM) inhibits the enzyme uncompetitively with respect to the glyoxylate (K_{ip} = 0.19 mM, K_{ii} = 0.12 mM) whether the NAD is saturating or non-saturating. In the other hand, oxalate (1.5 and 7.5 mM) inhibits uncompetitively (K_{ii} = 18 mM) and NADH (0.05 and 0.8 mM) inhibits competitively (K_{ip} = 0.021 mM) with respect to NAD (0.5 to 3 mM) against non-saturating glyoxylate (24 mM).

In the glyoxylate (2.5—20 mM)/lactate dehydrogenase (1.6 μg/ml of purified preparation)/NADH (0.025 to 1 mM) system (pH = 7.4), the NAD (1 and 2.5 mM) is a competitive inhibitor with respect to the NADH (K_{ip} = 0.12 mM) independently as to whether the glyoxylate is saturating (24 mM) or not (5 mM); whereas it is uncompetitive inhibitor with respect to glyoxylate (K_{ii} = 0.41 mM) for saturating NADH (0.1 mM) and non-saturating (0.05 mM). Glycolate (10 mM) does not cause inhibition in the system under study.

The secondary plots of the slopes and intercepts obtained from the corresponding inhibition graphs, are linear with respect to the inhibitor concentration in all cases, in both systems.

Kinetic parameters and dissociation constants of the enzyme-substrate complexes

The K_m, V, K(glyoxylate-cofactor) and the dissociation constants $\overline{K}$(cofactor) and $\overline{K}$(glyoxylate) values have been calculated from the secondary plots (slopes and intercepts) by application of Dalziel's [10] and Vestling-Florini's [11] methods (Table II). In all cases the dissociation constant of the lactate dehydrogenase-cofactor complex is less than that of the lactate dehydrogenase-glyoxylate complex, indicating that the cofactor is the first substrate to which the reaction sequence is added.

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* K_{ip}, inhibition constant referring to slope; K_{ii} that referring to intercept.

TABLE II

KINETIC PARAMETERS OF THE LACTATE DEHYDROGENASE

K_m (1) and K_m (2): true Michaelis constants for the glyoxylate and the cofactor respectively. K_{1-2}: $K_{glyoxylate-cofactor}$. $\bar{K}_1$ and $\bar{K}_2$: Dissociation constants of the enzyme-glyoxylate complex and enzyme-cofactor complexes respectively.

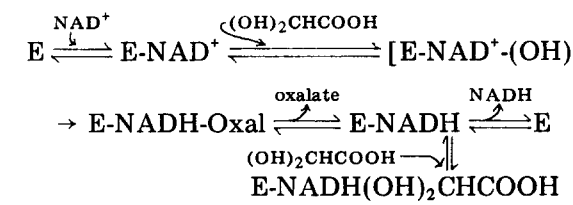
System	pH	K_M (1) (mM)	K_M (2) (mM)	V (ΔA/min · 10^2)	K_{1-2} (M · 10^7)	$\bar{K}_1$ (mM)	$\bar{K}_2$ (mM)
Glyoxilate lactate dehydrogenase NAD^+	9.6	5.1	0.2	12.5	42	15	0.83
Glyoxalate lactate dehydrogenase NADH	7.4	5	0.01	10	1.6	10	0.034

Analysis of inhibition by product. Reaction mechanism

The results obtained in the inhibition by product in both systems have been analysed by application of Cleland's rules [13,14]. The reaction mechanism is considered to be ordered sequential, since linearity is observed in all cases with respect to the substrate, and the double reciprocal plots of the rate and substrate concentration are straight lines having a common intersection point.

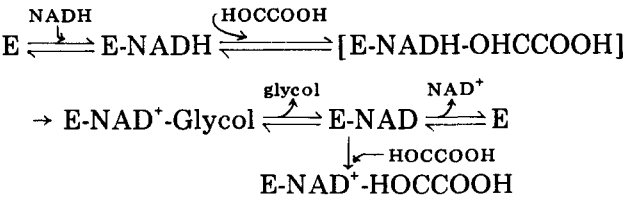
In the oxidation of glyoxylate, NADH is a competitive inhibitor with respect to NAD and non-competitive with respect to glyoxylate showing that NAD and NADH combine with the same enzyme form (free enzyme) whereas NADH and glyoxylate combine with different enzyme forms which are connected by irreversible steps.

The oxalate is an uncompetitive inhibitor with respect to NAD^+ and therefore combines with a form of enzyme different from the free enzyme; both forms must be connected by irreversible steps, which may be irreversible due to the irreversibility of the system itself and (or) to the release of NADH which does not act as an inhibiting product, all suggesting that the oxalate combines with the E · NADH enzyme form. In turn, the oxalate is a competitive inhibitor with respect to the glyoxylate. In a sequential mechanism, both would combine with different forms of the enzyme; nevertheless the inhibition induced in the system under study here is competitive, which may only occur if the ternary complexes formed are equivalent. Therefore, at least one of the two complexes cited has no kinetic significance. The above considerations are expressed in the following scheme:



If the glyoxylate · lactate · dehydrogenase · NAD^+ complex has no kinetic significance, it may be considered that the glyoxylate only combines with the E-NADH form in the presence of an excess of NADH, in other words, when this is acting as inhibitor by product.

A similar analysis of the glyoxylate/lactate dehydrogenase/NADH system allows the following sequence to be postulated:



In the sequence formulated, the E-NADH-HOCCOOH complex has no kinetic significance, whereby it is rapidly converted into E-NAD$^+$-glycolate. The formation of the terminal complex E-NAD$^+$-OHCCOOH is postulated.

Analysis of the inhibition by excess of substrate

In the glyoxylate/lactate dehydrogenase/NAD$^+$ (pH = 9.6) system, the excess NAD$^+$ is non-competitively inhibitory with respect to the glyoxylate which is not inhibited by substrate. The excess NAD$^+$ combines with the E-NAD$^+$ and E-NADH enzyme forms, which implies that the inhibition with respect to the glyoxylate is competitive if it combines with E-NAD$^+$ and uncompetitive if it combines with E-NADH. This would lead to a non-competitive linear inhibition, since the E-NAD$^+$ and E-NADH forms are connected by irreversible steps.

In the glyoxylate/lactate dehydrogenase/NADH (pH = 7.4) system, the excess glyoxylate is non-competitively inhibitory with respect to NADH, contrary to what Warren describes [2]. The hydrated and unhydrated forms of the substrate co-exists in equilibrium at pH = 7.4. In accordance with the suggested mechanisms, the unhydrated form of the glyoxylate combines with the binary complex E-NAD$^+$, while the hydrated form $(OH)_2CHCOOH$ combines with the E-NADH. Since the inhibition by excess substrate is non-competitive, the enzyme form combining with the excess glyoxylate is the binary complex E-NADH, the only one capable of inducing an inhibition of this type. It may be said that the hydrated form of the glyoxylate (which does not act as substrate and predominates at basic pH (2)) is responsible for the inhibition by excess substrate.

Inhibition of lactate dehydrogenase by preincubation with NAD$^+$

Concentrations below or equal to the kinetically saturating level of NAD$^+$ are preincubated with the enzyme at room temperature. An aliquot of the preincubating samples is poured into spectrophotometer cells containing L-lactate or glyoxylate (kinetically saturating concentrations). The initial rates found are compared with the calculated rates when the enzyme is added to the spectrophotometer cells containing NAD and L-lactate or glyoxylate.

The inhibition caused by the preincubation of NAD and the enzyme in the absence of the substrate (L-lactate or glyoxylate) (Table III) is observed in the systems studied. At pH 7.4 (L-lactate/lactate dehydrogenase/NAD$^+$ system) this inhibition is progressive with the time elapsed and stabilises as from 30 min.

The inhibiting effect of the NAD (0.6, 1, 2 and 3 mM) in samples where the enzyme (1.6 μg/ml of purified preparation) had been preincubated (20 min) with NAD$^+$, was determined for varying concentrations of L-lactate (5 to 20 mM). When the initial reaction rates are determined with a single concentration of NAD$^+$ (3 mM), with or without preincubation (30 min) with the en-

TABLE III

INHIBITION OF LACTATE DEHYDROGENASE BY PREINCUBATION WITH NAD^+

Enzyme-NAD^+ preincubation: 30 s; NAD^+ in preincubation three times greater than those in the table. System: L-lactate or glyoxylate/lactate dehydrogenase/NAD^+ (pH = 9.6); [enzyme] = 50 μg/ml of purified preparation; [L-lactate] = 16 mM; [glyoxylate] = 30 mM. System: L-lactate/lactate dehydrogenase/NAD^+ (pH = 7.4); [enzyme] 1.6 μg/ml of purified preparation; [L-lactate] = 16 mM.

System	pH	NAD^+ (mM)	Inhibition (%)
L-lactate lactate dehydrogenase NAD^+	7.4	3	48
		0.8	21
L-lactate lactate dehydrogenase NAD^+	9.6	3.3	49
		1	22
Glyoxylate lactate dehydrogenase NAD^+	9.6	4	44
		1	13

zyme for the same varying L-lactate concentrations, no variation of K_m is observed in either case. The above facts evidence the formation of a complex acting as inhibitor between the enzyme and the NAD and which may be formulated as: lactate dehydrogenase · NAD^+ · NAD^+.

To locate the enzyme site to which the second molecule of NAD^+ attaches, the co-enzyme was preincubated with the lactate dehydrogenase in the presence of oxamate and oxalate. The results obtained with the glyoxylate/lactate dehydrogenase/NAD (pH = 9.6) system (Table IV) show that the inhibition appearing in samples where the enzyme, the NAD and the oxalate are preincubated is similar to that induced by the oxalate without preincubation. On the other hand, in the samples where the enzyme, the NAD^+ and the oxamate are preincubated, the inhibition is similar to that in samples having an identical composition but where only the enzyme and the NAD^+ are preincubated. The results are the same with the L-lactate/lactate dehydrogenase/NAD^+ system.

TABLE IV

LACTATE DEHYDROGENASE PREINCUBATIONS WITH THE CO-ENZYME IN THE PRESENCE OF OXAMATE AND OXALATE

[Enzyme] = 1.6 μg/ml of purified preparation; pH = 9.6; [glyoxylate] = 24 mM; [NAD^+] = 4 mM; [oxalate] = 1 mM; [oxamate] = 0.5 mM; 10 min preincubation.

Preincubation of	System	Initial rate (ΔA/min · 10^2)	Inhibition (%)
—	glyoxalate lactate	7.5	
NAD^+ — +E	dehydrogenase NAD^+	4.1	45
—	glyoxalate lactate	6.1	19
NAD^+ + E	dehydrogenase NAD^+ oxalate	3.5	54
NAD^+ + E + oxalate		5.9	21
—	glyoxalate lactate	4.9	34
NAD^+ + E	dehydrogenase NAD^+ oxamate	2.3	69
NAD^+ + E + oxamate		3.1	59

Discussion

Lactate dehydrogenase catalyses the glyoxylate oxidoreduction [15,16]. The results described in this paper show some characteristics of these conversions and have allowed the enzyme binding site to which the glyoxylate attaches in the oxidation and reduction processes, as well as its kinetic mechanism, to be identified. Although it has been suggested that the glyoxylate attaches to the same lactate dehydrogenase binding site in the oxidation and reduction processes [2], the experiments described above allow is to be said that the metabolite attaches to two different sites on the enzyme molecule.

The foregoing results show that the conversion of the glyoxylate in oxalate and in glycolate conforms to a sequential mechanism, but it is a special type of ordered mechanism. Both reactions are irreversible, which limits the opportunity to determine the kinetic mechanism. The first substrate added to the reaction sequence is the cofactor, since in both systems this shows competitive inhibition with its own product and it gives rise to an enzyme-factor binary complex having a lower dissociation constant. The mechanisms postulated for both systems are partially Theorell-Chance, in which only one of the ternary complexes formed has no kinetic significance. The formation of abortive ternary complexes is shown. E-NADH-glyoxylate (non-hydrated form) and E-NAD^+-glyoxylate (hydrated form) which may be considered as analogous to the classical abortive pyruvate · E · NAD^+ and L-lactate · E · NADH complexes. These complexes are responsible for the substrate inhibition. In fact, at pH 7.4, the substrate of the glyoxylate/E/NADH system is the anhydrous form of the glyoxylate. Under these conditions, the hydrated and the anhydrous forms are in equilibrium; the former is present in the reaction medium and will form the E · NAD · $(OH)_2CHCOOH$ complex, producing inhibition when there is an excess of glyoxylate. On the other hand, at 9.6, the substrate of the glyoxylate/E/NAD^+ is the hydrated form of the glyoxylate. The equilibrium between the two forms of the substrate under these conditions lies well towards the hydrated form, whereby the E · NADH · OHCCOOH complex will hardly be formed. The assumption is in correlation with the fact that no substrate inhibition is observed when working with system at pH 9.6, but it does occur in the same system at pH 7.4.

The inhibition for excess of NAD^+ in the glyoxylate/lactate dehydrogenase/NAD^+ system (pH = 9.6) shows the formation of the ternary complexes E · NAD^+ · NAD^+ and E · NADH · NAD^+ originated in the joining of the new NAD^+ molecule to the E-NAD^+ and E-NADH enzyme forms which have already attained an appropiate configuration for the substrates to be able to combine. It suggests that NAD^+ has a certain affinity for a second site of the enzyme different from its own. The results obtained by preincubation of lactate dehydrogenase with NAD^+ could be the consequence of two different facts: (a) the attachment of the second NAD^+ molecule is independent of the L-lactate attachment site; or (b) the second NAD molecule attaches to the L-lactate site to give rise to an inactive complex; the consequence would be a reduction of the enzyme concentration capable of yielding products.

The blocking of L-lactate binding site by oxalate prevents the formation of the E · NAD^+ · NAD^+ complex, which implies that the second NAD^+ molecule

attaches to the L-lactate binding site in the lactate dehydrogenase molecule. The oxamate does not prevent the formation of this complex, which implies that the second NAD^+ molecule attaches to the lactate dehydrogenase independently of the pyruvate site.

In no case does the preincubation of the enzyme with NADH seem to cause an inhibiting effect. Considering the structural similarity between the NAD^+ and NADH, it may be suggested that the affinity of the former for the L-lactate binding site in the enzyme molecule is due only to the positive charge located in the nicotinamide ring. The formation of the $E \cdot NAD^+ \cdot NAD^+$ complex is independent of the substrate used by the lactate dehydrogenase. Nevertheless, its kinetic reaction mechanism, although it is always seen to be sequential, offers notable variations according to the substrates used.

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