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NAD-Linked L(+)-Lactate Dehydrogenase from the Strict Aerobe *Alcaligenes eutrophus*

2. Kinetic Properties and Inhibition by Oxaloacetate

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The L(+)-lactate dehydrogenase (EC 1.1.1.27) of *Alcaligenes eutrophus* catalyzes the NADH-dependent reduction of pyruvate and a few other 2-oxoacids. The K_m values for NADH, NAD, pyruvate and L(+)-lactate are 0.075 mM, 0.130 mM, 0.820 mM and 7.10 mM, respectively. The reaction follows a rapid equilibrium ordered bi-bi mechanism and involves the formation of a dead-end EBQ complex. The competitive inhibition of pyruvate reduction caused by NAD (with respect to NADH) is regarded to be of physiological importance.

The enzyme is strongly inhibited by oxaloacetate, oxalate and to a less extent by oxamate. Oxaloacetate was found to be the most powerful inhibitor of the enzyme and exerts an almost complete inhibition of the reduction of pyruvate and some 2-oxoacids at concentrations of 1 μM and less. At 0.1 μM oxaloacetate the inhibition of pyruvate reduction is about 90%.

The kinetics of pyruvate reduction in the presence of oxaloacetate is characterized by a burst phase followed by a decreased steady-state velocity. During the burst phase, which lasts from several seconds to some minutes, the enzyme undergoes transition to a less active enzyme form. The inhibition studies revealed the lactate dehydrogenase to be a hysteretic enzyme, due to its slow response to the ligand. The characteristics of the transient were examined.

The inhibition of lactate dehydrogenase from *A. eutrophus* by oxaloacetate is considered to be of great physiological importance, allowing its function only at a low oxaloacetate concentration and consequently at high NADH/NAD ratios.

An NAD-dependent L(+)-lactate dehydrogenase (EC 1.1.1.27) from the strictly respiratory bacterium *Alcaligenes eutrophus* has been purified 700-fold to homogeneity recently (preceding paper [1a]). In potassium phosphate buffer, pH 7.0, this lactate dehydrogenase is a dimer with a relative molecular mass of 74000. The enzyme is characterized by a broad substrate specificity. The enzyme does not belong to the type of lactate dehydrogenase which is activated by fructose 1,6-bisphosphate. It is responsible for the excretion of lactate by *A. eutrophus* when cultivated under conditions of restricted oxygen supply. The formation of this enzyme in *A. eutrophus* occurs over a wide range of oxygen deficiencies, i.e. when the cells are allowed to respire at 80% as well as at 3% of the maximum respiration rate. In contrast, L(+)-lactate is only excreted at severe oxygen deficiency, i.e. when the cells are allowed to respire only at 3–15% of their maximum respiration rate. The non-identity of the conditions of enzyme derepression and lactate excretion suggested that the enzyme must be subject to an efficient control on the level of enzyme activity.

Among a series of metabolites examined for affecting the activity of lactate dehydrogenase only NAD, phospho*enol*pyruvate, 3-fluoropyruvate, oxaloacetate, oxamate and oxalate exhibited a significant effect. Oxaloacetate was found to be a very powerful inhibitor of lactate dehydrogenase activity and exerted a strong inhibitory effect even at concentrations below 200 nM. The inhibitory action of oxaloacetate on lactate dehydrogenase activity may have a key function in controlling lactate formation in *A. eutrophus in vivo* and, therefore, required detailed investigation.

Examples for oxaloacetate affecting the activity of lactate dehydrogenases are rare. The L(+)-lactate dehydrogenase from *Bacillus subtilis* is inhibited by oxaloacetate in a competitive manner with respect to pyruvate [1], but the extent of inhibition is far from being as significant as in the case of *A. eutrophus* enzyme. The present study was aimed at describing the kinetics of the L(+)-lactate dehydrogenase from *A. eutrophus* with respect to reaction mechanism and regulation. Possible modes of the regulation of the enzyme by oxaloacetate involving a slow transition to an inactive state are discussed.

Abbreviations. v_i, initial steady-state velocity; v_{ss}, steady-state velocity; P_{IC}, intercept on [product] axis.

Enzymes. L(+)-Lactate dehydrogenase (EC 1.1.1.27); L(+)-malate dehydrogenase (EC 1.1.1.37); 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); malic enzyme (NAD-dependent) (EC 1.1.1.38); isocitrate dehydrogenase (NAD-dependent) (EC 1.1.1.41); isocitrate dehydrogenase (NADP-dependent) (EC 1.1.1.42); pyruvate dehydrogenase (EC 1.2.4.1); glutamate-oxaloacetate transaminase (EC 2.6.1.1); citrate lyase (EC 4.1.3.6).

MATERIALS AND METHODS

Purification of Lactate Dehydrogenase

The purification procedure for L(+)-lactate dehydrogenase from *Alcaligenes eutrophus* strain N9A-PHB$^-$02-HB$^-$1 has been described in the preceding paper [1a]. The homogeneous enzyme preparation resulting from affinity chromatography on Cibacron blue F3G-A Sepharose was sterilized by filtration and stored at 4 °C in 100 mM potassium phosphate buffer, pH 7.0, containing 200 mM potassium chloride. Aliquots of a diluted enzyme solution were used for kinetic studies. The specific activity of the preparation was about 800 U/mg protein.

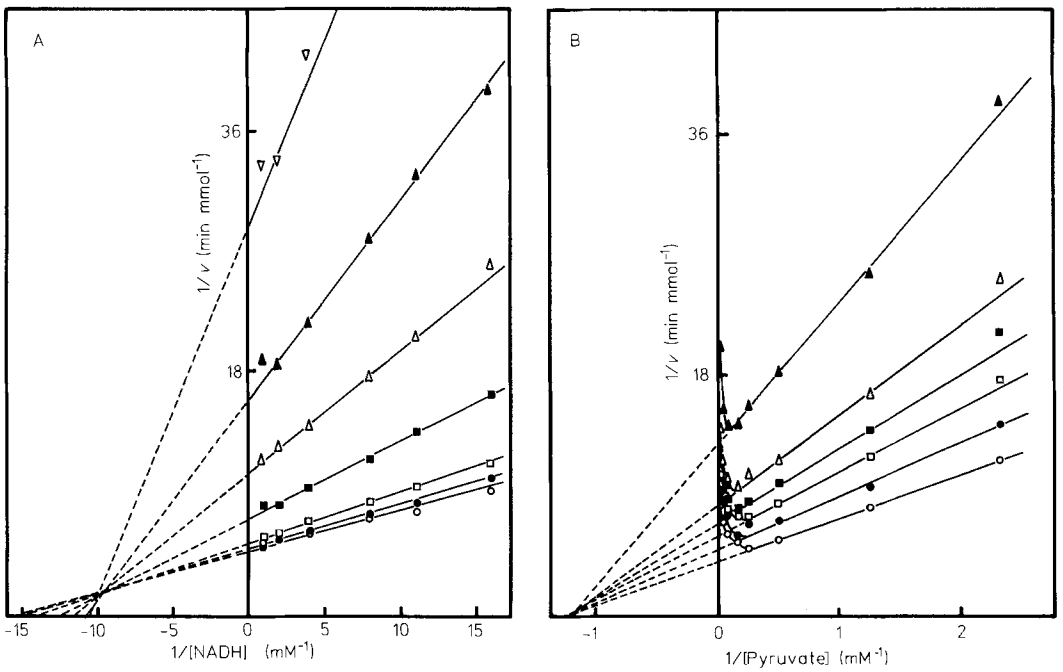


Fig. 1. *Double-reciprocal plots of lactate dehydrogenase activity (A) versus NADH at varied pyruvate concentration and (B) versus pyruvate at varied NADH concentration.* The reaction mixtures contained (total volume, 3.00 ml) 100 mM potassium phosphate buffer, pH 7.0, and 0.2 µg of purified enzyme. Pyruvate and NADH concentrations: (A) (○) 6 mM pyruvate; (●) 4 mM pyruvate; (□) 2 mM pyruvate; (■) 0.8 mM pyruvate; (△) 0.43 mM pyruvate; (▲) 0.21 mM pyruvate; (▽) 0.11 mM pyruvate. (B) (○) 1.000 mM NADH; (●) 0.250 mM NADH; (□) 0.125 mM NADH; (■) 0.090 mM NADH; (△) 0.062 mM NADH; (▲) 0.031 mM NADH

Kinetic Studies

Kinetic studies were performed at 30 °C in a total volume of 3.00 ml 100 mM potassium phosphate buffer, pH 7.0, by measuring rates of NADH oxidation at 366 nm, using an Eppendorff filter photometer connected to a recorder. Concentrations of the components of the reaction mixtures are given in the text. One unit of enzyme activity (U) is the amount catalyzing the conversion of 1 µmol of substrate/min. Specific activity is measured in U/mg protein.

Reagents

Pyruvate, oxaloacetate (for biochemical use), citrate, L-aspartate and 2-oxoglutarate were obtained from Merck (Darmstadt, FRG). Oxaloacetate solutions were prepared freshly and stored in ice, they were never used when older than 1 h. NAD, NADH, citrate lyase, glutamate oxaloacetate transaminase, and malate dehydrogenase were obtained from Boehringer (Mannheim, FRG).

RESULTS AND DISCUSSION

Determination of K_m *Values*

The determination of initial velocities at varied NADH or pyruvate concentrations and fixed concentrations of the second substrate resulted in non-parallel straight lines with common intersecting points on or above the 1/[NADH] or 1/[pyruvate] axis on Lineweaver-Burk double-reciprocal plots (Fig. 1 A and B). In potassium phosphate buffer, pH 7.0, there was no indication for cooperativity, and Hill plots revealed h values of 0.95 – 0.98. High pyruvate concentrations (> 5 mM) decreased initial velocities in an uncompetitive manner resulting in parallel straight lines in the Lineweaver-Burk plot with increasing values of $1/v$ intercepts for increasing pyruvate concentrations (not shown in Fig. 1 A). This kind of uncompetitive substrate inhibition is assumed to be due to the formation of an NAD · enzyme · pyruvate complex which is not reactive and is therefore designated as an abortive or dead-end complex [2]; it is characteristic for ordered systems. There are many reports on such dead-end complexes formed by lactate dehydrogenases [3] and by other dehydrogenases such as chicken liver mitochondrial malate dehydrogenase [4] or isocitrate dehydrogenase from *Rhodopseudomonas sphaeroides* [5]. From the corresponding replots of the Lineweaver-Burk plots the following K_m values were calculated: 0.075 mM (NADH), 0.820 mM (pyruvate), 0.130 mM (NAD) and 7.1 mM [L(+)-lactate]. The values for pyruvate and L(+)-lactate are in accordance with those for other lactate dehydrogenases and confirmed that pyruvate reduction is the physiological direction of enzyme catalysis. Nevertheless, lactate oxidation could be demonstrated, but pyruvate reduction occurred at a 15-fold higher rate. There were no significant differences between the K_m values for NADH and NAD compared to other lactate dehydrogenases [6]. Measurements of changes in intracellular concentrations or NAD/NADH ratios during a shift from aerobic conditions to conditions of restricted oxygen supply in *Alcaligenes eutrophus* should provide an answer, but relevant data are still lacking. It was only shown that under normal aerobic conditions the NAD/NADH ratio amounts to 1.5 – 3.0 depending on substrate and growth phase (M. Lohmeyer, unpublished results). In aerobic or facultatively aerobic bacteria the intracellular concentration of NAD is always higher than that of NADH. In *Klebsiella aerogenes*, NAD was reduced upon oxygen exhaustion and oxidized upon restoration of aerobiosis [7]. The transition of *Escherichia coli* and *K. aerogenes* from aerobic to anaerobic conditions resulted in a temporary increase of

Table 1. *Product inhibition pattern of L(+)-lactate dehydrogenase from* Alcaligenes eutrophus *in potassium phosphate buffer, pH 7.0*
Abbreviations: –, no inhibition; C, competitive inhibition; NC, noncompetitive inhibition

Inhibitor	Variable substrate			
	NADH		pyruvate	
	pyruvate (nonsaturating) 0.2 mM	pyruvate (saturating) 3.0 mM	NADH (nonsaturating) 0.05 mM	NADH (saturating) 0.5 mM
NAD (0–2 mM)	C K_{is} = 0.4 mM	C K_{is} = 0.4 mM	NC K_{ii} = 0.5 mM K_{is} = 1.2 mM (common intercepts below 1/[pyruvate] axis)	NC K_{ii} = 2.1 mM K_{is} = 1.6 mM (common intercepts on 1/[pyruvate] axis)

the intracellular NADH level, followed by a return to a constant value after a few minutes, whereas the intracellular NAD concentration decreased from a high aerobic to a low anaerobic level [8]. Most of the NAD disappeared due to turnover, and thus the NAD/NADH ratio dropped from 6 to 1 in *K. aerogenes* and 5 to 1.5 in *E. coli*. A decrease of the intracellular NAD/NADH ratio as a consequence of limited oxygen supply has also to be expected in *A. eutrophus*. Furthermore, the decrease of the NAD/NADH ratio should inhibit the pyruvate dehydrogenase complex, as all pyruvate dehydrogenases investigated so far are inhibited by NADH [9], and should therefore favour pyruvate reduction to lactate rather than pyruvate oxidation to acetyl-CoA.

Product Inhibition Pattern

After a sequential rather than a ping-pong mechanism for the function of L(+)-lactate dehydrogenase from *A. eutrophus* had been confirmed, the type of the sequential mechanism had to be studied using the product inhibition method [2]. NAD is competitive with respect to NADH at saturating as well as at nonsaturating pyruvate concentrations. Inhibition of NAD with respect to pyruvate at nonsaturating NADH concentrations was noncompetitive (Table 1). There was no or only negligible inhibition by L(+)-lactate even at high concentrations with respect to NADH or pyruvate. Therefore the enzyme follows the ordered 'standard' bi-bi mechanism as stated for all other lactate dehydrogenases examined for this purpose so far. The formation of an abortive EBQ (= NAD · enzyme · pyruvate) complex may be involved.

Inhibition by Phosphoenolpyruvate

Lactate dehydrogenase activity was inhibited in a noncompetitive manner by phospho*enol*pyruvate with respect to pyruvate. Inhibition was weak, and the K_i amounted to 1 mM. In view of the high inhibition constant, inhibition by phospho*enol*pyruvate probably does not play a major physiological role in *A. eutrophus*.

Inhibition by Oxalate and Oxamate

Activity of lactate dehydrogenase from *A. eutrophus* is strongly inhibited by oxalate and to a lesser extent by oxamate. Both substances are typical inhibitors of lactate dehydrogenases from animal as well as bacterial sources [6]. Inhibition by oxalate was uncompetitive with respect to both pyruvate and NADH. Inhibition constants of 5.3 µM and 8.5 µM, respectively, were calculated (Table 2) and indicated a remarkably strong inhibition compared to other lactate dehydrogenases. The type of inhibition indicates formation of an abortive NAD · enzyme · oxalate complex [2].

Table 2. *Inhibition of lactate dehydrogenase by oxamate and oxalate*
Abbreviations: NC, noncompetitive inhibition; UC, uncompetitive inhibition

Inhibitor	Variable substrate	
	NADH (5 mM pyruvate)	pyruvate (0.25 mM NADH)
Oxamate (0.6–4.0 mM)	UC K_{ii} = 2.2 mM	NC K_{ii} = 3.0 mM K_{is} = 8.0 mM
Oxalate (4.5–30 µM)	UC K_{ii} = 8.5 µM	UC K_{ii} = 5.3 µM

Inhibition by oxamate was uncompetitive with respect to NADH and noncompetitive with respect to pyruvate. Common intercepts lay below the 1/[pyruvate] axis. Inhibition by oxamate was weaker than that by oxalate and estimated inhibitor constants were in the millimolar range rather than in the micromolar range (Table 2). The inhibition pattern of oxamate indicates that abortive NADH · enzyme · oxamate as well as NAD · enzyme · oxamate complexes are formed.

INHIBITION BY OXALOACETATE

General Observations

The lactate dehydrogenase from *A. eutrophus* is inhibited by oxaloacetate in a unique fashion. Inhibition occurs even at a concentration of 50 nM. The progress curves of NAD reduction shown in Fig. 2 indicate that the initial velocity is not affected by oxaloacetate. The transient burst lasted from 10 s to several minutes. The velocity reached a low steady-state value which remained constant as long as no product inhibition occurred. Though only very low inhibitor concentrations were required for effective inhibition, oxaloacetate was not a tight-binding or slow tight-binding inhibitor because the molar concentrations of the enzyme used were about 100-times lower than those of oxaloacetate [10]. Because the length of the burst time was of the assumed magnitude of possible changes in intracellular oxaloacetate concentrations,

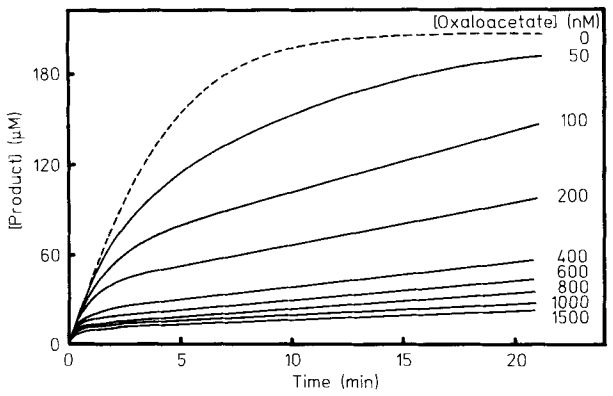


Fig. 2. *Progress curves of lactate-dehydrogenase-catalyzed pyruvate reduction in the presence of varied oxaloacetate concentrations and a fixed activity of lactate dehydrogenase.* Assay was carried out in 3.00 ml 100 mM potassium phosphate buffer, pH 7.0, containing 0.2 mM NADH, 3 mM sodium pyruvate and various concentrations of oxaloacetate as indicated. Reaction was started at zero time by the addition of 124 mU lactate dehydrogenase

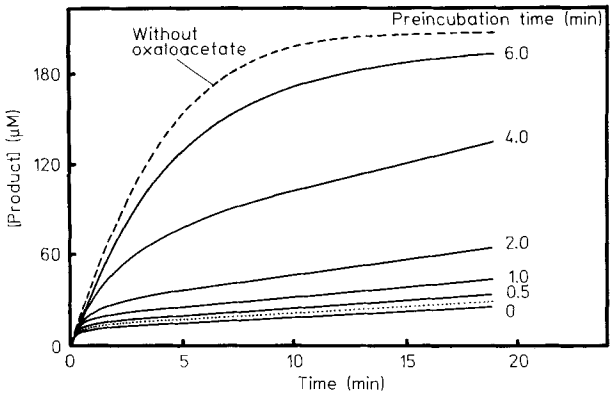


Fig. 3. *Effect of preincubation on inhibition of lactate dehydrogenase by oxaloacetate.* Progress curves demonstrate the effect of the kind of preincubation on oxaloacetate inhibition. Assays were carried out in 3.00 ml potassium phosphate buffer, pH 7.0, containing 0.2 mM NADH, 3 mM sodium pyruvate, 1 μM oxaloacetate and 100 mU lactate dehydrogenase. (– – –) Absence of oxaloacetate; (· · · · ·) lactate dehydrogenase was preincubated for 2, 4 or 6 min either in the presence of both pyruvate and oxaloacetate or of oxaloacetate alone before starting reaction either by the addition of NADH or by the addition of pyruvate and NADH at zero time; (——) lactate dehydrogenase was preincubated in the presence of oxaloacetate and NADH for the indicated times before adding pyruvate et zero time. Preincubations occurred in cuvettes at 30 °C

the lactate dehydrogenase from *A. eutrophus* is a hysteretic enzyme. The term 'hysteretic enzyme' was coined by Frieden [11] and refers to enzymes which respond slowly to a rapid change in substrate or modifier concentration. Kinetic aspects and physiological importance of hysteretic enzymes were recently reviewed [12, 13] and a large number of enzymes, especially those which are of importance in metabolic regulation, may fall into this category. Oxaloacetate affected the enzyme function in both directions, i.e. pyruvate reduction and lactate oxidation, to about the same extent. Furthermore, oxaloacetate inhibited the reduction of other substrates such as glyoxylate, 2-oxobutyrate, 2-oxovalerate, 3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate, 4-methyl-2-oxopentanoate and 2-oxoglutarate at a 1 μM concentration. Inhibition of pyruvate reduction occurred in potassium phosphate buffer, Tris/maleate buffer or imidazole/HCl buffer (each 100 mM, pH 7.0).

Specificity of Oxaloacetate

The inhibition was specific for oxaloacetate and was not due to possible impurities present in the oxaloacetate solution used. This was demonstrated by generation of oxaloacetate *in vitro* by the action of citrate lyase on citrate or by transamination of aspartate and 2-oxoglutarate by glutamate oxaloacetate transaminase. Relevant controls made sure that the inhibition was not due to other metabolites or the auxiliary enzymes. The high specificity of the inhibition by oxaloacetate was confirmed by applying other dicarboxylic acids. Compounds which were different from oxaloacetate with respect to one group, such as succinate (0.6 mM) or 2-oxoglutarate (3 mM), exerted no inhibition. The latter was even a good substrate for the enzyme. Malonate at 3 mM inhibited only slightly (18% inhibition). Only oxomalonate resulted in a 55% inhibition at 0.1 mM or 70% inhibition at 0.2 mM.

Inhibition by oxaloacetate (2 μM) could not be overcome or reversed by addition of Mn^{2+}, Mg^{2+}, fructose 1,6-bisphosphate, fructose 6-phosphate or a combination of divalent cations and a fructose phosphate (5 mM each). The absence of a regulatory mechanism involving fructose 1,6-bisphosphate has thus been confirmed again.

Effects of Various Kinds of Preincubation

The burst phase could not be overcome by preincubating the enzyme with both pyruvate and oxaloacetate or oxaloacetate alone for 2–6 min. If the enzyme, on the other hand, was preincubated with oxaloacetate and NADH, inhibition gradually disappeared depending on preincubation time. After preincubation with NADH and 1 μM oxaloacetate for 6 min, inhibition was negligibly low (Fig. 3). This relief of oxaloacetate inhibition was due to the low oxaloacetate reductase activity of the lactate dehydrogenase which at pH 7.0 was only about 0.05% of pyruvate reductase activity but was high enough to reduce oxaloacetate to malate which is not inhibitory.

Only rare examples for oxaloacetate reductase activities of lactate dehydrogenases have been reported. L(+)-Lactate dehydrogenase from *Bacillus subtilis* [1] is inhibited by oxaloacetate competitively with respect to pyruvate. The K_i value is high (1.4 mM). But oxaloacetate is also a poor substrate: the K_m is 1.5 mM, which is 175-times higher than the K_m value for pyruvate, and the rate of reduction is only 3.5% of that of pyruvate reduction. The oxaloacetate reductase activity of the DL-lactate dehydrogenase from *Leuconostoc mesenteroides* was smaller than 10% of pyruvate reductase activity [14]. Lower oxaloacetate reductase activities (about 0.1% of pyruvate reductase activity) were reported in lactate dehydrogenases from chicken liver, rabbit liver, pig skeletal muscle and dogfish skeletal muscle [15]. On the other hand, pig heart mitochondrial L(+)-malate dehydrogenase has little (1% of oxaloacetate reductase activity) pyruvate reductase activity [15]. In all cases K_m values for the alternative substrate were very high as compared with the ordinary substrate. There is another example of interference of oxaloacetate with lactate dehydrogenase: The lactate dehydrogenase-5 and lactate dehydrogenase-1 isoenzymes of dogfish are both protected to heat inactivation by oxaloacetate [16].

Characteristics of the Transient

A detailed analysis of the transient made sure that the transient is real and not artificial. Enzyme and inhibitor concentrations as well as initial substrate concentrations (five times K_m) were chosen to guarantee that steady-state velocities were reached in a region in which not more than 20% of the substrates were depleted during the burst phase. Under these conditions normally no decrease of the reaction rate due to substrate depletion or product accumulation occurs. This assumption was confirmed by adding the same amount of lactate dehydrogenase, which was initially used, to the reaction mixture again after the steady-state velocity had been reached. This resulted in a new burst phase which resembled the first one. Various experiments were aimed at establishing that the burst phase was not due to enzyme inactivation occurring in the course of the assay.

P_{IC}, i.e. the amplitude of the burst or the amount of excess product generated by the enzyme in going from the initial state to the steady state (P intercept of the elongated line for constant steady-state velocity with the P axis in Fig. 2), was proportional to enzyme concentration for different fixed concentrations of oxaloacetate and reciprocally proportional to the oxaloacetate concentration for various fixed enzyme concentrations. At pyruvate concentrations above the K_m value and with saturating NADH, the amplitude of the burst was independent of the pyruvate concentration but the amplitude decreased when the concentration of pyruvate decreased below 0.75 mM.

The detailed kinetic analysis of the transient phase resulted in many data. These data indicate that the transition process follows first-order reaction kinetics. However, these data could not be reliably interpreted in quantitative terms and at the present state of knowledge about the pyruvate reduction process did not lead to any mechanically interesting conclusions.

Steady-State Velocity

A linear relationship between steady-state velocities and enzyme concentrations examined for various fixed concentrations of inhibitor was obtained, and the ratio of v_{ss} to v_i (indicating the percentage of remaining activity after steady-state conditions are attained, if multiplied by 100) depended only on the oxaloacetate concentration (Fig. 4). For very high oxaloacetate concentrations the v_{ss}/v_i ratio becomes almost zero. The inhibitor concentration resulting in 50% inhibition, estimated by elongating the left-hand asymptote line to a ratio of 0.5, may occur in the region of 20 nM. Unfortunately, it was not possible to obtain meaningful values for the v_{ss}/v_i ratio when lower inhibitor concentrations were used because deviations were extensive. Standard procedures for analysing the effect of oxaloacetate on the steady-state velocity provided no better estimates of K_i for reasons which are inherent in the type of inhibition. Nevertheless, Fig. 4 clearly demonstrates the powerful inhibition of lactate dehydrogenase by oxaloacetate.

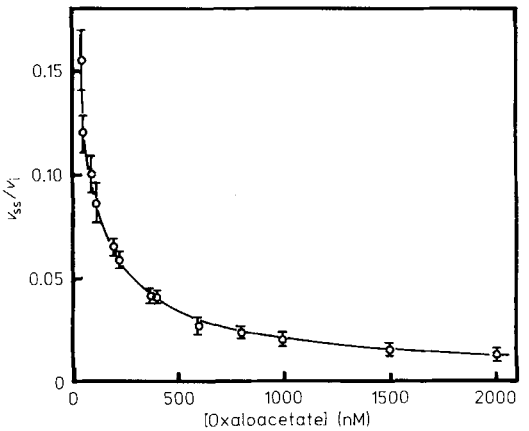
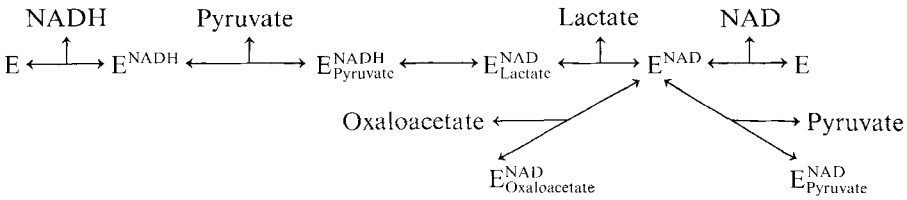


Fig. 4. *Dependence of the* v_{ss}/v_i *ratio on the oxaloacetate concentration.* Assay was carried out in 100 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM NADH, 3 mM sodium pyruvate and oxaloacetate concentrations as indicated. For each oxaloacetate concentration, varied activities of lactate dehydrogenase were used. The values (○) are means of about five measurements

Correlations of v_{ss} and the v_{ss}/v_i ratio to the pyruvate concentration showed that the v_{ss}/v_i ratio increased with decreasing pyruvate concentration. At pyruvate concentrations above the K_m value the ratio remained nearly constant. This means that the inhibition is less effective at nonsaturating substrate concentrations and reflects effects on the transient concentration of the reactive enzyme species.

The results obtained, especially those from preincubation studies, indicate that oxaloacetate has only a slight affinity to the free enzyme E or the enzyme · NADH complex. This can be concluded from the very low oxaloacetate reductase activity of the enzyme and the very slight effect of oxaloacetate on the initial reaction rate, on the one hand, and the relief of inhibition in the presence of NADH but maintenance of inhibition in the presence of pyruvate in either case, on the other hand. These arguments support the assumption that oxaloacetate combines either with the abortive NAD · enzyme · pyruvate complex, with the NADH · enzyme · pyruvate complex or with one of the subsequent enzyme states which usually appear in a sequential ordered bi-bi mechanism to cause its strong inhibition.



Inhibition may be caused by the formation of an exceptionally stable abortive NAD · enzyme · oxaloacetate complex. Equilibration proceeds slowly especially at low oxaloacetate concentrations, but steady-state conditions are ultimately attained. From this and other experiments (not shown here), it becomes clear that oxaloacetate does bind reversibly to the enzyme.

As lactate dehydrogenase from *A. eutrophus* is a dimeric enzyme with presumably more than one binding site for each ligand and it forms abortive complexes, the kinetic model for oxaloacetate inhibition is very complex. Continuation of relevant studies appears rewarding.

CONCLUDING REMARKS

A regulatory function of oxaloacetate has been repeatedly reported. Succinate dehydrogenase from various sources is inhibited by oxaloacetate in a competitive manner. The K_i value of oxaloacetate for succinate dehydrogenase from various rat tissues was estimated to be 1.5 μM [17]. NADP-dependent isocitrate dehydrogenases from *Escherichia coli*, *Bacillus subtilis*, *Brevibacterium flavum*, pig heart [18] and *Halobacterium cutirubrum* [19] are subject to a marked concerted feedback inhibition by glyoxylate and oxaloacetate. Both the NAD-dependent and NADP-dependent isocitrate dehydrogenases of *Alcaligenes eutrophus* H16 [20] are also subject to a concerted feedback inhibition by glyoxylate and oxaloacetate. In combination (0.5 mM each) these inhibitors exerted an almost complete inhibition. The 3-hydroxybutyrate dehydrogenase from the same strain had only 5% residual activity in the presence of 10 mM oxaloacetate [21]. The NAD-dependent malic enzyme from *E. coli* is inhibited in an allosteric manner; 10 μM oxaloacetate resulted in a 50% inhibition at 0.3 mM malate [22]. The malic enzyme from *Pseudomonas fluorescens* is also inhibited by oxaloacetate [23]. The present study on the lactate dehydrogenase of *A. eutrophus* added a further case to the examples of marked inhibitions by oxaloacetate.

The intracellular oxaloacetate concentrations are presumably controlled by alterations of the redox state of the cell [24]. If during the shift from aerobic conditions to conditions of oxygen limitation (see discussion above) the NAD/NADH ratio decreases, the intracellular oxaloacetate pool is expected to decrease due to the mediation of malate dehydrogenase. These shifts in turn should result in an increase of reactive lactate dehydrogenase. Both the raised intracellular pyruvate pool and the shift in NAD/NADH ratio would lead to an increase of lactate dehydrogenase activity in *A. eutrophus* and to lactate excretion. As shown previously [25,26], lactate is excreted only if oxygen deficiency is severe and the relative respiration rates are only 3–15% of the maximum rates. Unfortunately, there are only a few reports on intracellular oxaloacetate concentrations in bacteria or mammalian tissues, and examples for measurements of the oxaloacetate pool in response to changes of the oxygen supply rates are lacking. In the few cases where values for oxaloacetate among the intermediates of the citric acid cycle were provided, oxaloacetate was throughout present in the lowest concentrations. The oxaloacetate concentrations may, however, be high enough to affect the activity of the highly sensitive lactate dehydrogenase. Oxaloacetate pool size determinations have to be made to provide final proof for the involvement of oxaloacetate in the regulation of lactate dehydrogenase *in vivo*.

The data presented in this paper indicate that the strong inhibitory effect of oxaloacetate on pyruvate reduction at already low concentrations may provide a convenient method for the determination of small oxaloacetate concentrations in media and cell extracts. The intercept on the product axis, P_{IC}, which is inversely proportional to the oxaloacetate concentration, may be the parameter to be determined. Compared to photometric or radiometric methods a 10–20-fold increase and compared to fluorimetric methods a 2–4-fold increase of sensitivity may be expected.

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