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CONTROL OF GLUTAMATE DEHYDROGENASE FROM *LEMNA MINOR* BY DIVALENT METAL IONS

ADELHEID EHMKE and THOMAS HARTMANN

Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, D-3300 Braunschweig, BRD

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Key Word Index—*Lemna minor*; Lemnaceae; duck weed; glutamate dehydrogenase; metal ion activation; kinetics of calcium activation.

Abstract—The NADH and NAD^+ dependent reactions catalyzed by glutamate dehydrogenase (GDH) from sterile cultures of *Lemna minor* are completely inactivated by EDTA. The activities of both reactions can be fully restored by addition of Ca^{2+} and to a lesser extent Mn^{2+}, Zn^{2+}, Sr^{2+} or La^{3+}, whereas Mg^{2+} reactivates only the NAD^+ dependent reaction. Activation of the NADH reaction by Ca^{2+} has been studied by using partially purified, EDTA pretreated, and Mg^{2+} saturated GDH preparations. Saturation kinetic curves with Ca^{2+} were always sigmoidal, whereas saturation plots for the 3 substrates of the aminating reaction at various fixed Ca^{2+} concentrations showed normal Michaelis kinetics. However, a pronounced substrate inhibition at low Ca^{2+} levels was found, particularly with NH_4^+ and NADH. Product inhibition studies revealed unchanged enzyme substrate binding characteristics for NADH and 2-oxoglutarate in the Ca^{2+} free enzyme. A drastic alteration was established for the third substrate NH_4^+. The kinetic data suggest that Ca^{2+} governs an equilibrium between a catalytically inactive (Ca^{2+} free) and an active (Ca^{2+} saturated) enzyme form. Inactivation by removal of Ca^{2+} is related to an alteration in the binding characteristics or binding sequence of the substrate NH_4^+.

INTRODUCTION

In an earlier report [1] we demonstrated that the glutamate dehydrogenase (GDH) from *Lemna minor* (duck weed) displays some striking regulatory properties in respect to activation by divalent cations. For example, (a) GDH preparations treated by simple gel filtration on Sephadex G_{50} were markedly inactivated in the direction of NADH dependent reductive amination of 2-oxoglutarate, whereas the oxidative deamination of glutamate (NAD^+ dependent) remained unaffected. (b) GDH preparations pretreated with EDTA were completely inactive in both directions. (c) The oxidative reaction (NAD^+ dependent) was fully reactivated by addition of Ca^{2+} or Mg^{2+}; however the reductive reaction (NADH dependent) was reactivated only by Ca^{2+} but not at all by Mg^{2+}. (d) There was hardly any effect on the NADPH dependent GDH activity to be observed, neither by EDTA treatment nor by addition of divalent metal ions.

Although inhibition of GDH by EDTA [2] and other chelating agents and its reversal by various metal ions have been demonstrated for the GDH from various plants [3–8] the effects have never been studied in detail. The observed differential activation of the NAD^+/NADH dependent GDH reactions by Ca^{2+} and Mg^{2+} may represent a mechanism by which the direction of a reversible reaction could be controlled. The present studies were carried out to gain an insight into the mechanism of the metal ion activation.

RESULTS AND DISCUSSION

Stimulation by metal ions

The effect of various metal ions on an EDTA inactivated GDH preparation from *L. minor* is shown in Table 1. As already pointed out, Ca^{2+} in concentrations above 0.1 mM caused optimal reactivation of both the NADH and the NAD^+ dependent reactions, whereas

Table 1. Effect of various metal ions upon the GDH catalyzed reactions using an EDTA pretreated enzyme preparation

Metal ion	Final concn in assay (mM)	GDH activity (%)* NADH-reaction	NAD^+-reaction
no addition		<1	<1
Ca^{2+}	0.1	98	97
	1.0	100	100
	10.0	100	100
Mg^{2+}	0.1	<1	65
	1.0	<1	100
	10.0	5	97
Zn^{2+}	0.1	2	68
	1.0	82	—
	2.0	62	99
Mn^{2+}	1.0	22	56
	5.0	82	72
Sr^{2+}	0.1	2	80
	1.0	32	82
	10.0	97	90
Cs^{2+}	0.1	<1	<1
	1.0	<1	<1
	10.0	<1	<1
La^{3+}	0.1	50	90
	1.0	55	—

* Activity in presence of 1 mM Ca^{2+} was set as 100%. Mg^{2+}, Zn^{2+}, Mn^{2+} were supplied as sulphates, all other cations as chlorides.

Mg^{2+} was effective only in the NAD^+ reaction. Stimulation of activity by Zn^{2+}, Mn^{2+}, Sr^{2+} and La^{3+} was similar to that of Ca^{2+} but less pronounced, especially in the direction of reductive amination with NADH. Cs^{2+} up to 10 mM was without activating effect in both directions.

In contrast to the GDH of *Blastocladiella*, which is more sensitive to inhibition by EDTA at alkaline pH values than at pH 7 [9], with *Lemna* GDH the inhibition patterns of the GDH catalysed reactions by EDTA as well as their reactivation by Ca^{2+} are pH independent at pH values between 7.2 and 8.8.

To obtain more evidence on the specificity of the metal ion effects kinetic studies were performed. These studies were focused on the activation of the aminating reaction by Ca^{2+} ions using EDTA pretreated, Mg^{2+} saturated enzyme preparations which are essentially inactive with NADH but fully active with NAD^+.

Kinetics of Ca^{2+} activation of the NADH dependent reaction

Ca^{2+} saturation. Saturation kinetics with Ca^{2+} always produces sigmoidal curves, which vary considerably in shape depending on the substrate concentrations used in the assay system. At low substrate concentrations Ca^{2+} activation occurs in a concentration range which is much smaller than that observed at high substrate concentrations (Fig. 1). As shown in Fig. 2 this effect is mainly due to variations in the NADH and NH_4^+ concentrations, whereas the influence of 2-oxoglutarate is less pronounced. This indicates that the observed substrate dependent fluctuations in the Ca^{2+} saturation kinetics are not caused by the chelating properties of the keto acid.

Substrate kinetics. From the foregoing results it seems reasonable that Ca^{2+} acts as a positive allosteric effector of the aminating reaction. If this is true either the substrate affinity of one or more of the substrates or the maximal velocity (V_{max}) should be altered with the Ca^{2+} free enzyme. Fig. 3 presents the saturation curves of the 3 substrates in the presence of different Ca^{2+} concentrations. The figures show normal Michaelis–Menten kinetics for all substrates, but a pronounced substrate inhibition at low Ca^{2+} concentrations, particularly with NH_4^+ and NADH. The Lineweaver–Burk plots of the data given in Fig. 3 revealed that Ca^{2+} mainly affects V_{max}. However, the exact interpretation is difficult because of the substrate inhibition. To obtain clear evidence whether the substrate binding is altered, product inhibition studies according to the method of Cleland [10] with both the Ca^{2+} saturated and the Ca^{2+} free enzyme were carried out.

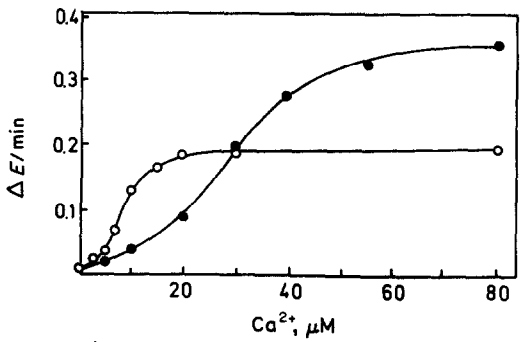

Fig. 1. Activation of the NADH dependent reactions of EDTA pretreated *Lemna* GDH by various Ca^{2+} concns. ●—● all substrates added at saturating concn (standard assay conditions). ○—○ all substrates added at concns near to their K_m values (NADH = 0.1 mM; NH_4Cl = 50 mM; 2-oxoglutarate = 4 mM).

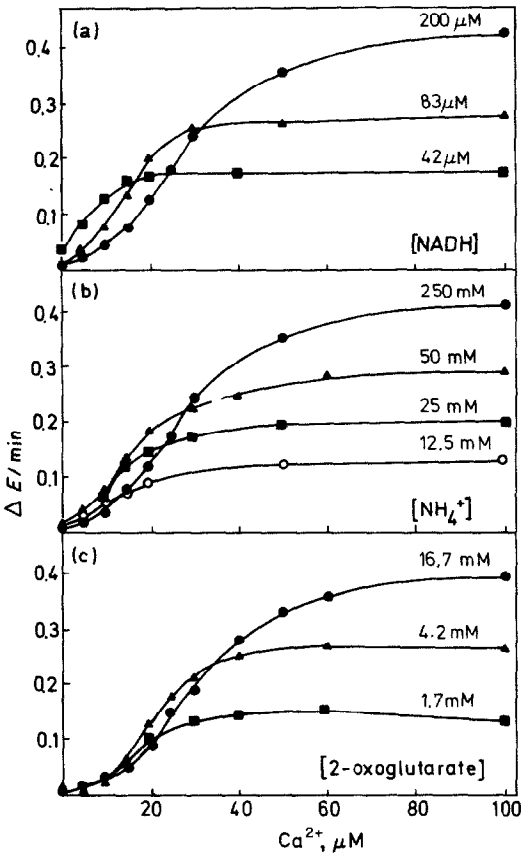

Fig. 2. Activation of the NADH dependent reaction of EDTA pretreated *Lemna* GDH by Ca^{2+} at various fixed concns of (a) = NADH; (b) = NH_4^+; (c) = 2-oxoglutarate. In each system the other substrates were added in the standard assay concns.

Product inhibition studies

In the product inhibition studies NADH, ammonium and 2-oxoglutarate were taken as product inhibitors of the oxidative reaction when NAD^+ and glutamate were used as substrates. The respective second substrate was always held fixed and at standard assay concentration. The inhibition patterns elicited were as follows. (a) With both enzyme preparations, NADH served as a competitive inhibitor of NAD^+ binding and as noncompetitive inhibitor of glutamate. (b) 2-oxoglutarate is a competitive inhibitor of glutamate and a noncompetitive inhibitor of NAD^+. The same patterns were found with the Ca^{2+} free and Ca^{2+} saturated enzyme preparation. (c) When ammonium was used as the product inhibitor the two GDH preparations were significantly different. Ammonium inhibition of glutamate binding is noncompetitive (almost competitive) with the Ca^{2+} saturated enzyme but uncompetitive with the Ca^{2+} free GDH (Fig. 4). The inhibition of NAD^+ binding is noncompetitive with

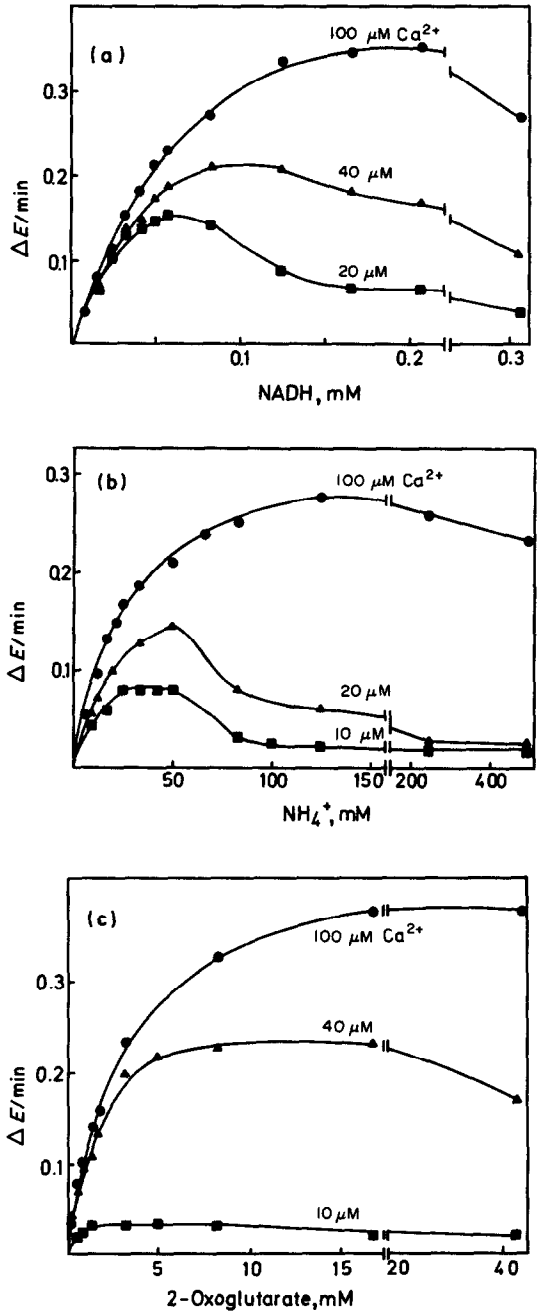

Fig. 3. Substrate saturation curves of EDTA pretreated *Lemna* GDH at various fixed concns of Ca^{2+}. (a) = NADH; (b) = NH_4^+; (c) = 2-oxoglutarate. In each system the other substrates were added in standard assay concns.

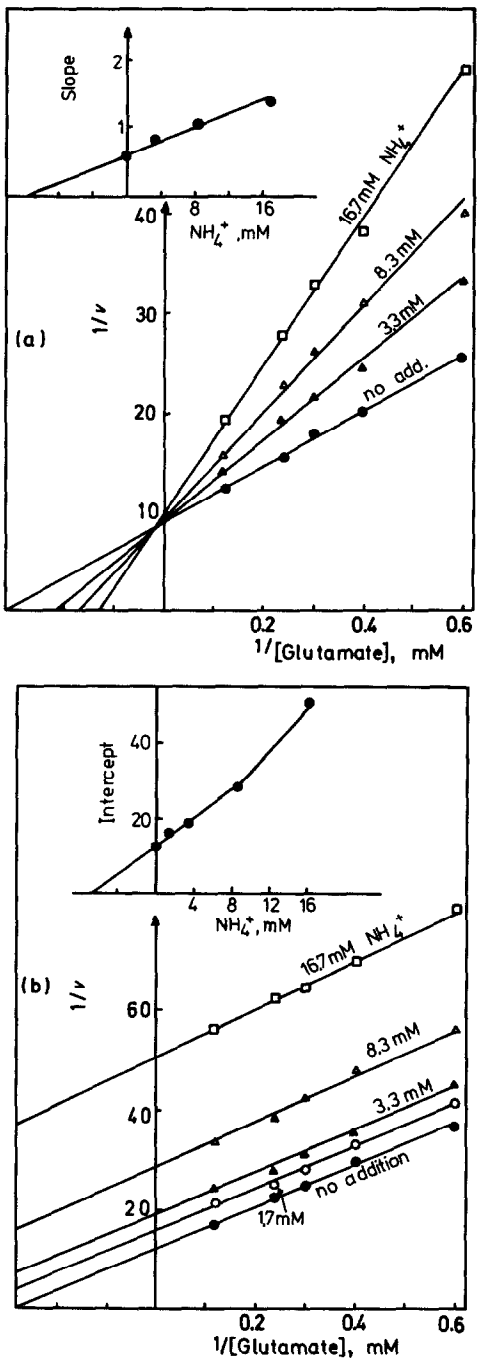

Fig. 4. Double reciprocal plots of NH_4^+ inhibition of the oxidative reaction catalysed by *Lemna* GDH. NAD^+ was saturating (2.1 mM) and glutamate was varied against several fixed concns of NH_4^+. Inset, replot of slope or intercept against inhibitor concn. (a) Ca^{2+} satd GDH prepn. (b) Ca^{2+} free GDH prepn.

both enzyme preparations as shown by the primary plots in Fig. 5. However, the secondary plots (intercept and slope against inhibitor concentration) were linear only with the Ca^{2+} saturated enzyme (Fig. 5a). In the case of the Ca^{2+} free GDH the secondary plots were parabolic (Fig. 5b).

The kinetic data obtained from the product inhibition studies indicate that the removal of Ca^{2+} does not affect the substrate binding affinity of the enzyme for NADH and 2-oxoglutarate. The inhibitor constants (K_i) for the two substrates can be calculated from the Dixon-plots giving values of 13 μM (Ca^{2+} saturated enzyme) and 14 μM (Ca^{2+} free enzyme) for NADH, and 3.3 mM (Ca^{2+} saturated enzyme) and 3.5 mM (Ca^{2+} free enzyme) for 2-oxoglutarate.

However, removal of Ca^{2+} clearly alters the binding characteristics of the third substrate, ammonium. The

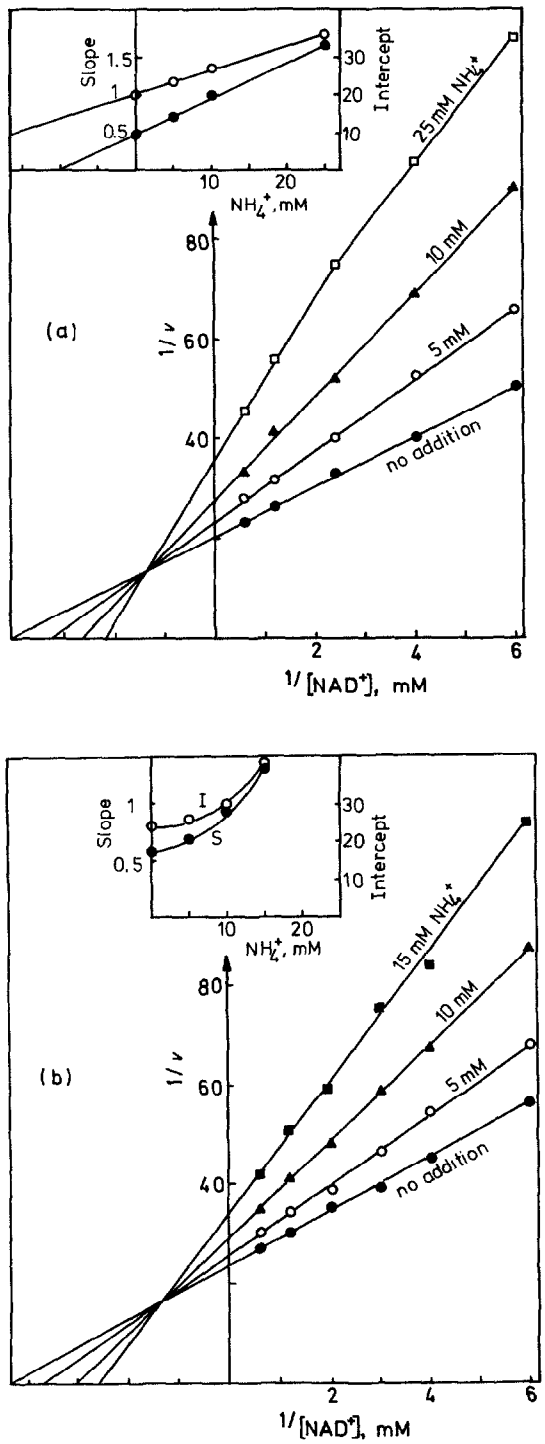

Fig. 5. Double reciprocal plots of NH_4^+ inhibition of the oxidative reaction catalysed by *Lemna* GDH. Glutamate was saturating (16.7 mM) and NAD^+ varied against several fixed concns of NH_4^+. Inset, replot of slope and intercept against inhibitor concn. (a) Ca^{2+} satd GDH prepn. (b) Ca^{2+} free GDH prepn.

alteration from a noncompetitive to an uncompetitive inhibition pattern with ammonium as product inhibitor against glutamate as the variable substrate (Fig. 4) indicates that in contrast to the Ca^{2+} saturated enzyme the Ca^{2+} free GDH binds ammonium to an enzyme form substrate and that there is no reversible connection between the two forms.

The observed alteration from a linear noncompetitive to a parabolic noncompetitive inhibition pattern with ammonium as inhibitor and NAD^+ as the variable substrate (Fig. 5) indicates that in the Ca^{2+} free enzyme ammonium not only combines with the enzyme form with which it is associated during the normal reaction sequence, but in addition functions as a powerful dead end inhibitor. Such a dead end inhibition by ammonium may also explain the pronounced substrate inhibition observed in the presence of subsaturating Ca^{2+} concentrations (see Fig. 3).

Based on the observed kinetic behaviour of the GDH from *Lemna* we propose that Ca^{2+} governs a reversible equilibrium between two enzyme forms only one of which —the Ca^{2+} saturated form—is effective to catalyse the reductive reaction. Obviously the inactivation of the reaction by removal of Ca^{2+} is related to an alteration in the binding characteristics or binding sequence of the substrate ammonium.

Since activation by divalent metal ions has been demonstrated for GDHs from other plant sources one can argue that the observed kinetic mechanism may represent a more general feature of the GDH from higher plants. However with other enzymes the effects were found to be rather unstable. Thus, mitochondrial pea GDH loses much of its susceptibility to regulation by chelators and metal ions during storage and purification [6]. Indeed it is an advantage of the *Lemna* enzyme that its metal ion dependency remains stable during various treatments. Unaltered Ca^{2+} activation can still be demonstrated in highly pure GDH preparations recently obtained in our laboratory by a purification procedure including gel filtration on Sepharose, DEAE cellulose ion exchange chromatography and affinity chromatography.

EXPERIMENTAL

L. minor L. was grown as described earlier [1] in a nutrient soln containing ammonium succinate (10 mM) as sole nitrogen source.

Enzyme prepn. For most of the kinetic expts a partially purified enzyme prepn was used. The purification procedure was mainly as in [1] involving Me_2CO powder prepn, extraction with 0.1 M Tris–HCl buffer pH 8.1 and centrifugation at 30000 *g* for 10 min. The supernatant was fractionated by $(NH_4)_2SO_4$ pptn (45–65%) and desalted on a column of Sephadex G_{50}.

Prepn of Ca^{2+}-free enzyme. EDTA was added to the Sephadex G_{50} eluate to give a final concn of 1 mM, then the extract was dialysed for 16 hr against 0.01 M Tris–HCl buffer pH 8 containing 0.5 mM $MgSO_4$. The resulting Ca^{2+} free, Mg^{2+} saturated prepn was fully active with NAD^+ but had no measurable activity with NADH. Ca^{2+} saturated enzyme: *Lemna* GDH was saturated with Ca^{2+} by addition of $CaCl_2$ at a final concn of 1 mM to the enzyme assay mixture.

Enzyme assays. Details of enzyme assays have been reported earlier [1]. The standard reaction mixture contained the following components: NADH reaction: 0.08 M Tris (pH 8.1), 0.21 mM NADH, 0.3 M NH_4Cl, 16.7 mM 2-oxoglutarate. NAD^+ reaction: 0.08 M Tris (pH 8.8), 2.1 mM NAD, 16.7 mM glutamate. The reaction was started by addition of 2-oxoglutarate or glutamate, respectively.

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