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MALATE DEHYDROGENASE OF SPINACH: SUBSTRATE KINETICS OF DIFFERENT FORMS

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; malate dehydrogenase; molecular weight; conformation; kinetics; reaction rate; action of ions and cosubstrate.

Abstract—*Sephadex G-200 gel filtration of an ammonium sulfate fraction, containing the bulk of NAD-dependent* malate dehydrogenase, yields forms of differing MW. Both Mg^{2+} and NADH stabilize the 127000 daltons MW form. K^+, or incubation with dithioerythritol, cause splitting and partial reaggregation, resulting in MWs ranging between 35000 and 180000 daltons. Chromatography in the presence of dithioerythritol and NADH results in an enzyme with a non-linear reaction rate at low substrate concentrations. Plots of initial velocity vs substrate and cofactor concentration respectively are characterized by two slopes of positive cooperativity separated by an intermediary plateau of negative cooperativity. Gel chromatography in the presence of Mg^{2+} or K^+ or drastic dilution of the enzyme results in an enzyme with linear reaction rates also at low substrate concentration. Its kinetics are consistent with the view that the enzyme undergoes conformational changes when the substrate concentration is varied.

INTRODUCTION

IN RECENT years numerous publications have dealt with the separation of NAD-dependent malate dehydrogenase (E.C. 1.1.1.37) of plant origin by chromatographic and electrophoretic methods.[1] In most cases these enzymes were designated as isoenzymes. Evidence for their characterization as products of different genes and hence of their possessing different aminoacid compositions is incomplete and mostly of an indicative nature.[2] In part these isoenzymes were revealed as species of differing MW, which varied from 17000[3] or approximately 700000[1] daltons. In part they were found to have the same MW (e.g. 60000;[1] 58000[4] and 66000[5] daltons) and to be charge isomers which differ in their conformation.

A modification of MW by mono-and divalent cations has recently been reported. Mg^{2+} and Ca^{2+} reverse the aggregation of *Neurospora crassa* malate dehydrogenase which takes place in Tris buffer (pH 9) of low ionic strength and reduce the various isoenzymes to one species of 65000 daltons.[6] In contrast the MWs of malate dehydrogenase of *Lemna minor*,

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[6] BENVENISTE, B. P. and MUNKRES, K. D. (1973) *Biochem. Biophys. Res. Commun.* **50,** 711.

grown on high Ca^{2+} levels, are greater (140000 and 70000 daltons) than when growth takes place on low Ca^{2+} levels (35000 and 17000 daltons respectively).[3] These differences are not due to the synthesis of isoenzymes sensu stricto but rather to the variable aggregation of subunits. Isoenzymes of peroxisomes and mitochondria,[5] which differ in their electrophoretic behaviour, exhibit different K_m values and are inhibited by different oxaloacetate concentrations.[5,7] The following paper is concerned with the modifications of MW species by cations and by NADH, resulting in changes in reaction rate and substrate kinetics.

RESULTS

Variation of MW forms by Mg^{2+}, K^+, NADH and dithioerythritol

(a) *The action of Mg^{2+}.* If 10 mM Tris buffer pH 8 + $MgCl_2$ (2 mM) is used for Sephadex chromatography, with respect to initial velocity, a small peak corresponding to MW of 105000 (±3000) daltons separates from the main peak [MW 127000 (±3000) daltons]. Although the relative amounts of the two peaks may vary considerably in different runs, both MW species are always present.

(b) *The action of K^+.* If the ammonium sulfate fraction is chromatographed in 50 mM Tris buffer pH 7·7 + 0·1 N KCl, four shoulders are present in all runs; they correspond to MWs of 186000 (±8000), 127000 (±3000), 91000 (±3000) and 64000 (±3000) daltons respectively. Though their relative amounts may vary, the latter peaks always predominate.

In agreement with the findings of Johnson and Hatch,[8] the NADP-dependent malate-dehydrogenase was found to be activated by incubation of the enzyme with dithioerythritol (DTE) (0·8 mg/ml) at 30° for 1 hr to about the 3 times its normal activity. In the NAD-dependent malate dehydrogenase this treatment causes no or only a very slight increase in activity (<5%). However it results in a partial splitting of the enzyme into lower MW species. Besides the peaks of the untreated enzyme two further marked peaks results which correspond to MWs of 39000 (±2000) and 32000 (±2000) daltons. The relative heights of these two peaks may vary considerably and in some preparations, especially after incubation with DTE at 37°, they may comprise about half the total activity.

(c) *The action of NADH and DTE.* Dissolution and chromatography of the enzyme fraction in 50 mM Tris buffer pH 8 + DTE (0·8 mg/ml) + NADH (0·5 mM) results almost exclusively in a peak which corresponds to MW of 127000 (±3000) daltons. This indicates that NADH stabilizes the MW species of 127000 daltons.

The time-course of the reaction

At substrate saturating conditions all enzyme forms show normal linear rate constants, whether they were obtained after chromatography in the presence of either K^+, Mg^{2+} or NADH. Moreover, the same reaction rate/min, obtained by the same amount of protein confirms that identical amounts of enzyme were used in the assay (Fig. 1). In contrast, at non saturating substrate conditions, the MW species of 127000 daltons, stabilized by NADH, is characterized by a relatively rapid initial rate which decreases with time to a slower one. The curvilinearity of the reaction rate depends on the substrate concentration and is shown, as an example in presence of 20 μM oxaloacetate (Fig. 1).

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Kinetics with respect to oxaloacetate as the substrate varied

At 0·1 mM NADH and with oxaloacetate as the substrate varied, the form of 127000 daltons, obtained after chromatography with NADH + DTE, shows two sigmoidal slopes with an intermediate plateau (Fig. 2). The standard error (s.e.) demonstrates that the determination of the reaction rate is more critical at the two inflexion points (5 and 30 μM oxaloacetate respectively) than in the section between them. The Hill plot indicates a positive

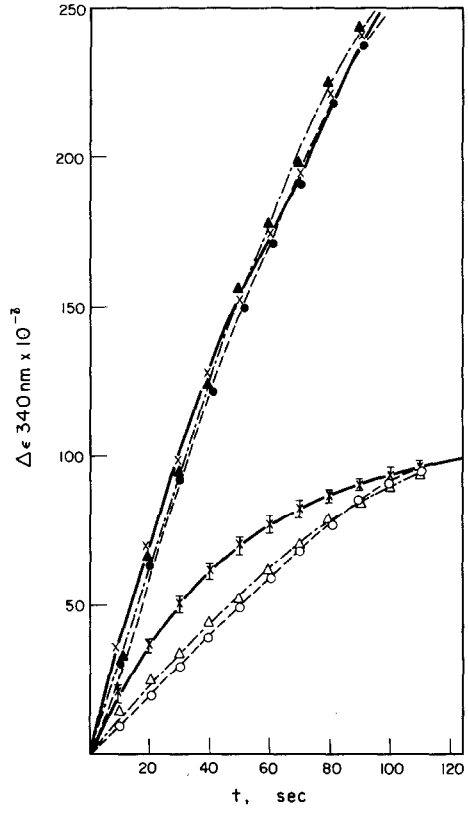

FIG. 1. REACTION RATE OF MALATE DEHYDROGENASE; at 0·1 mM NADH + 0·1 mM oxaloacetate—×——× 127000 daltons MW form after gel chromatography with Tris buffer/dithioerythritol/NADH, ▲-·-·-▲ 91000 daltons MW form after gel chromatography with Tris buffer/dithioerythritol/KCl, ●----● 127000 daltons MW form after gel chromatography with Tris buffer/dithioerythritol/$MgCl_2$; at 0·1 mM NADH + 0·02 mM oxaloacetate—⁎——⁎ 127000 daltons MW form after gel chromatography with Tris buffer/dithioerythritol/NADH, △-·-·-△ 91000 daltons MW form after gel chromatography with Tris buffer/dithioerythritol/KCl, ○---○ 127000 daltons MW form after gel chromatography with Tris buffer/dithioerythritol/$MgCl_2$, ⁎ = s.e. The reaction was started by addition of the enzyme (50 μg protein).

cooperative interaction with the substrate (n = 3·6), with an intermediary plateau of 'negative cooperativity'[9] (n = <1). Substrate kinetics corresponding to this enzyme form are also obtained when the ammonium sulfate fraction is incubated with 50 mM Tris buffer pH 8 + DTE (0·8 mg/ml) for 1 hr at 30°. However, the protein concentration is critical and

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incubation of dilute solutions (<4 mg protein/ml) results in substrate kinetics which are obtained after prior dilution of the enzyme (see below). Moreover, the concentration of NADH, present in the assay medium, seems to be essential for the production of this

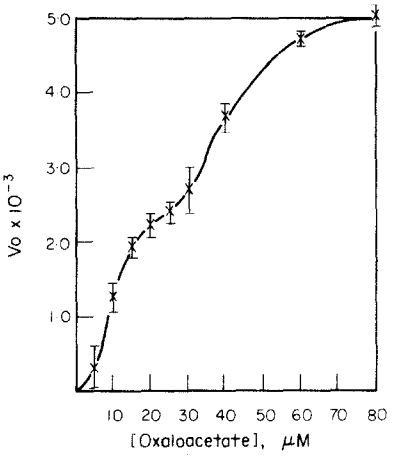

FIG. 2. SUBSTRATE KINETICS OF THE 127000 DALTONS MW FORM AFTER GEL CHROMATOGRAPHY WITH TRIS BUFFER/DITHIOERYTHRITOL/NADH IN THE PRESENCE OF 0·1 mM NADH.
Reaction was started by addition of the enzyme: ⊦ = s.e.

enzyme form; Fig. 3 demonstrates the modification of substrate kinetics by NADH, since at 25 μM NADH the first slope does not show positive cooperativity any longer.

The MW species corresponding to 127000, 105000 and 64000 daltons, obtained by chromatography with either K^+ or Mg^{2+}, do not differ in their substrate kinetics from

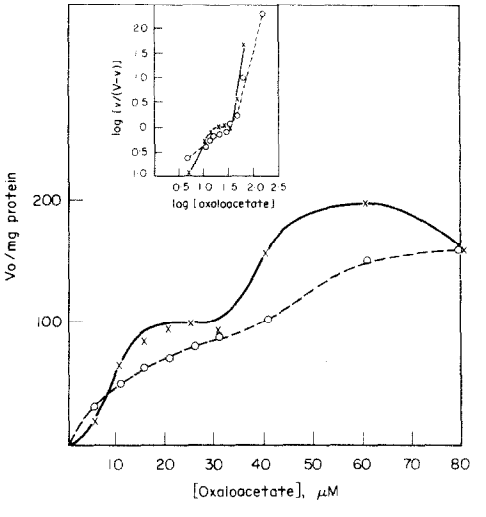

FIG. 3. SUBSTRATE KINETICS OF MALATE DEHYDROGENASE (60–70% AMMONIUM SULFATE FRACTION AFTER INCUBATION WITH DTE) IN THE PRESENCE OF ×——× 0·1 mM NADH; O- - -O 0·025 mM NADH.
Reaction was started by addition of the enzyme. Insert: Hill plot of Fig. 3.

each other if statistically evaluated. Their kinetics are characterized by four maxima and minima, as Fig. 4 demonstrates, for the enzyme form obtained by chromatography with Mg^{2+}.

Kinetics with respect to NADH as the cosubstrate varied

Two slopes with positive cooperativity are separated by an intermediate plateau of negative cooperativity (Fig. 5). Analogously a decrease in oxaloacetate concentration to 0·025 mM removes the positive cooperativity of the first slope.

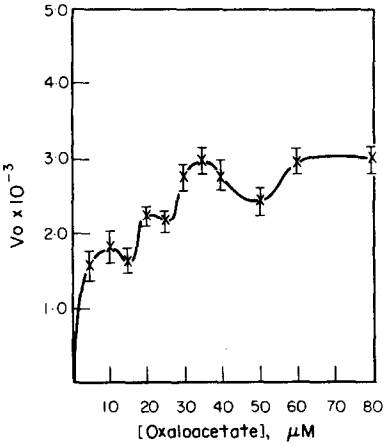

FIG. 4. SUBSTRATE KINETICS OF THE 127000 DALTONS MW FORM AFTER GEL CHROMATOGRAPHY WITH TRIS BUFFER/DTE/$MgCl_2$ IN THE PRESENCE OF 0·1 mM NADH.
Reaction was started by addition of the enzyme: ⊀ = s.e.

The effect of enzyme dilution

Using the ammonium sulfate fraction (60–75% saturation) the amount of enzyme was adjusted to a turnover rate at substrate saturating conditions as given in Fig. 1. If this enzyme is drastically diluted by adding it to the test buffer (2·8 ml) and then the reaction

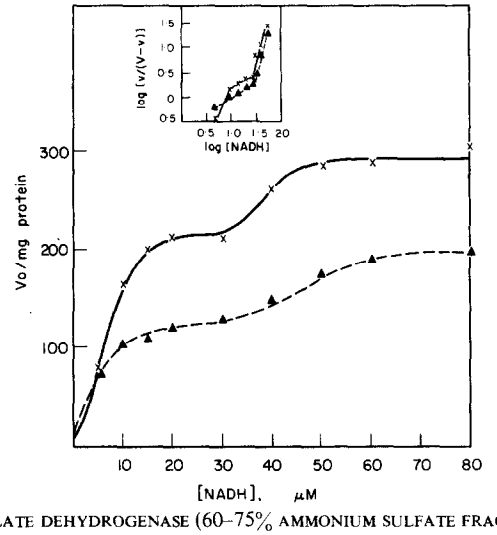

FIG. 5. KINETICS OF MALATE DEHYDROGENASE (60–75% AMMONIUM SULFATE FRACTION AFTER INCUBATION WITH DITHIOERYTHRITOL) VS NADH IN THE PRESENCE OF ×——×0·1 mM OXALOACETATE; ▲----▲ 0·025 mM OXALOACETATE.
Reaction was started by addition of the enzyme. Insert: Hill plot of Fig. 5.

started by simultaneous addition of NADH + oxaloacetate, the time course of the reaction is linear, not only at substrate saturating conditions but also at non-saturating ones.

This corresponds to the enzyme forms obtained after chromatography with either K^+ or Mg^{2+} (see Fig. 1). The substrate kinetics (Fig. 6) also indicate that dilution gives rise to enzyme forms, similar to those obtained by chromatography with Mg^{2+} or K^+ (see Fig. 4). The increased s.e. indicates enhanced variability in enzyme forms present, due to random splitting by dilution.

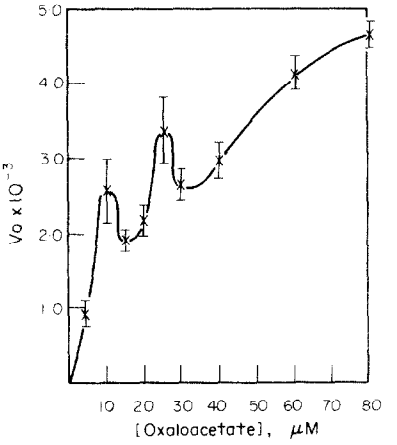

FIG. 6. SUBSTRATE KINETICS OF MALATE DEHYDROGENASE (60–75% AMMONIUM SULFATE FRACTION) AFTER DILUTION OF THE ENZYME WITH BUFFER. THE REACTION WAS STARTED BY ADDITION OF NADH (0·1 mM FINAL CONCENTRATION) + OXALOACETATE; ⫲ = S.E.

DISCUSSION

The gel chromatographic studies demonstrate that the NAD dependent malate dehydrogenase of plant origin can readily be modulated in its MW by ions, reducing agent or cosubstrate. Of the series of multiple MW forms, that of 64000 daltons seems to represent the most stable unit. It alone was found when crude animal or plant extracts were repeatedly frozen and thawed before gel filtration.[10] Moreover this form exhibits a number of charge isomers.[1,4] The MW form of 127000 daltons, obviously the double aggregate of this stable unit, is stabilized by Mg^{2+} as well as by NADH, whereas K^+ favours the appearance of the whole range of MW species, and excessive treatment with DTE causes the formation of the two lowest MW species. These two peaks, corresponding to MWs of 39000 and 32000 daltons, consistently appeared in all of four separations; however, it is not certain whether the double peak is indeed due to different MW species or whether different shape of the molecules may influence their emergence from the column. As in *Lemna*,[3] divalent, in contrast to monovalent, cations cause aggregation of green leaf malate dehydrogenase, whereas the enzyme of *Neurospora crassa* reacts in a contrary manner.[6]

Though the tertiary structure of an enzyme is known to be determined by the aminoacid sequence, it can also be influenced by the cosubstrate available during the process of reassociation and refolding.[11] NADH, which effectively fixes the 127000 daltons MW species, seems to stabilize an enzyme form with special reaction rate and substrate kinetics. This suggests that an alteration in conformation under the influence of NADH takes place.

The curvilinear progress rate may be explained in two different ways: It may be caused

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by a change in the enzyme from an unstable configuration with a relatively fast rate at low substrate concentrations, to a form with reduced activity. Such transient changes in reaction progress curves of yeast hexokinase[12] and glycogen synthase I from bovine heart[13] or skeletal muscle[14] have been interpreted in this way. On the other hand the product inhibition by malate may be involved; in agreement with the findings of Coombs *et al.*,[15] preliminary studies (unpubl.) have shown that malate acts as a competitive inhibitor, but additionally, marked changes in the type of reaction rate were found. This indicates that malate reacts not only at the substrate binding site but also at a regulatory site. Interaction of malate with an allosteric site is also indicated by the studies of Coombs.[15] Finally, both these mechanisms may eventually cooperate, since malate accumulation induces transient kinetics of the enzyme. As a consequence of the nonlinear reaction rate, initial velocities cannot be obtained by graphical but only by analytical methods.[16]

The MW species of 127000 daltons obtained after chromatography with NADH shows unusual substrate kinetics which recently were published for a number of enzymes (e.g. bacteria and sheep kidney phosphoenol-pyruvate carboxylase,[17,18] honey bee glyceraldehyde 3-phosphate dehydrogenase,[19] pea seed fructose-6-phosphate kinase in the presence of its inhibitor phosphoenolpyruvate[20]).

In the case of cytidine triphosphate synthetase the intermediate plateau is interpreted[9] as negative cooperativity, whereas Gelb *et al.*[19] explain the sigmoid section by an activation of a metastable state of the enzyme. The conformational changes which, in the case of the MW forms not stabilized by NADH, take place at various substrate concentrations are difficult to explain at present. Such conformational changes are also known from other enzymes, e.g. *Neurospora* phosphofructokinase.[21]

By the action of cations, S–S reducing agents or NADH, the enzyme readily undergoes changes between different MW forms and conformations. Thus the exceedingly high turnover rates, found in extracts at saturating substrate conditions,[22,23] are possibly of no physiological significance and the initial velocities are effectively reduced according to the levels of oxaloacetate and NADH respectively.

EXPERIMENTAL

Ammonium sulfate fractionation. Fully grown spinach leaves (*ca* 100 *g*) were harvested from the greenhouse and the NAD-dependent enzyme was extracted at 4° in the presence of N_2 with the medium described for the NADP-dependent enzyme.[9] After filtration through muslin and centrifugation at 25000 *g* for 20 min, fractionation with solid $(NH_4)_2SO_4$ showed the bulk of activity at 60–75% saturation. The protein was dissolved in 50 mM Tris buffer pH 8, containing DTE (8 mg/10 ml). The soln, containing 15–20 mg protein/ml, was saturated to 80% with $(NH_4)_2SO_4$ and stored at 4°. No change in activity could be detected within 1 month. Gel chromatography. After centrifugation, the protein of the $(NH_4)_2SO_4$ fraction (5–7 mg) was redissolved in the solvent systems as indicated in Results. For gel-chromatography,[24] Sephadex G-200 superfine was soaked separately with each of the

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various solvent systems used. The columns were 1·6 × 35 cm. Each run was repeated at least 3×. Dextran blue and a set of proteins with MWs from 13500–5400000 daltons were used for calibration of the column. Each of the columns, differing in their solvent systems was calibrated separately even identical migration values (V_e/V_0) for all solvent systems resulted. Flow rates of 2–3·6 ml/hr were employed in the different runs. Determination of enzyme activity. The change in E at 340 nm was measured with a Leitz Digital double beam spectrophotometer with automatic printing of the values every 10 sec. The assay system (total vol. 3 ml) contained: 25 mM Tris buffer pH 8, 1 mM EDTA, oxaloacetate and NADH as indicated. For substrate saturating conditions 0·1 mM oxalacetate and NADH respectively were used. The initial velocity was calculated according to ref. 16, using a computer program developed for this purpose. For determination of s.e. 8–10 measurements were used. Protein determinations were done according to ref. 25.

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