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PURIFICATION AND PROPERTIES OF MALATE DEHYDROGENASES FROM CAULIFLOWER

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Abstract—Two isozymes of L(−)-malate dehydrogenase (E.C. 1.1.1.37) were separated by ion-exchange and extensively purified from cauliflower (*Brassica oleracea* L. var. *botrytis*). Each of the purified isozymes was characterized by the following criteria: molecular size, Michaelis constants for all substrates, reactivity with NAD analogs, pH optima, heat stability and electrophoretic mobility. The isozymes differ significantly only in their K_m values for malate and NAD, and in their affinity for DEAE-cellulose. Their properties are similar to those of other plant and animal malate dehydrogenases, suggesting that this enzyme is evolutionarily conservative.

INTRODUCTION

KAPLAN[1,2] and Wilson[3,4] and their co-workers have done extensive comparative enzymological studies on the NAD- and NADP-requiring dehydrogenases. Most of these studies, however, have dealt with animals and microorganisms, while higher plants have been relatively neglected. Since molecular evolution is undoubtedly as important in plants as in other organisms, we undertook extensive studies on the properties of isozymes of the enzyme L-malate dehydrogenase (MDH) isolated from a variety of higher plants. This paper reports the properties of two MDH's[5] from cauliflower.

Multiple forms of MDH have been found in a number of higher plants.[6] In barley seedlings,[7] two purified MDH isozymes were shown to be associated with mitochondria and cytoplasm, respectively, while in spinach[8] and *Opuntia*,[9] three isozymes were shown to be associated respectively with microbodies, mitochondria and cytoplasm. Davies[10] has reported the presence of two partially separable forms of MDH activity in cauliflower extracts, and examined some properties of one of the isozymes. We have further purified and

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[1] N. O. KAPLAN, in *Evolving Genes and Proteins* (edited by V. BRYSON and H. J. VOGEL), p. 243, Academic Press, New York (1965).
[2] N. O. KAPLAN and M. M. CIOTTI, *Ann. N.Y. Acad. Sci.* **94**, 701 (1961).
[3] A. C. WILSON, N. O. KAPLAN, L. LEVINE, A. PESCE, M. REICHLIN and W. S. ALLISON, *Fedn. Proc.* **23**, 1258 (1964).
[4] A. C. WILSON and N. O. KAPLAN, in *Taxonomic Biochemistry and Serology* (edited by C. A. LEONE), p. 321, Ronald Press, New York (1964).
[5] Abbreviations used: MDH, L(−)-malate dehydrogenase; OAA, oxaloacetate; AP-NAD, 3-acetylpyridine-adenine dinucleotide; TN-NAD, thionicotinamide-adenine dinucleotide; DTT, dithiothreitol.
[6] L. M. SHANNON, *Ann. Rev. Plant Physiol.* **19**, 187 (1968).
[7] S. B. YUE, *Phytochem.* **5**, 1147 (1966).
[8] V. ROCHA and I. P. TING, *Arch. Biochem. Biophys.* **140**, 398 (1970).
[9] S. K. MUKERJI and I. P. TING, *Arch. Biochem. Biophys.* **131**, 336 (1969).
[10] D. D. DAVIES, *Biochem. J.* **80**, 93 (1961).

characterized the two forms of cauliflower MDH. The properties reported in this paper serve as 'reference standards' for comparative studies with MDH's from other higher plants; we shall report those results in another communication.

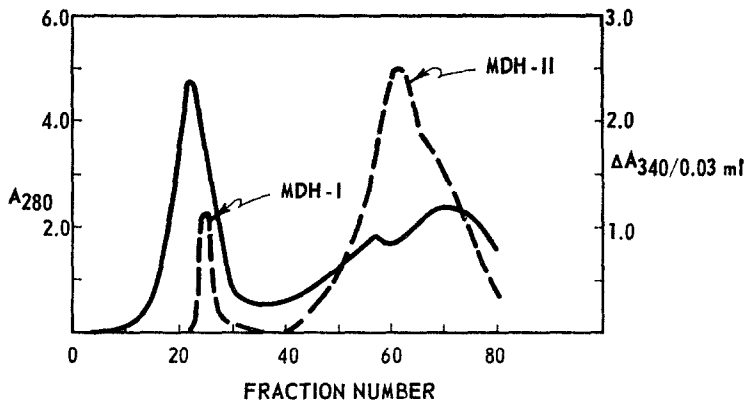

FIG. 1. SEPARATION OF CAULIFLOWER MDH ISOZYMES ON DEAE-CELLULOSE.
A linear gradient of potassium phosphate buffer, pH 7·5, from 0·01 M to 0·2 M was used for elution. Solid line = A_{280}; dashed line = MDH activity (ΔA_{340}/0·03 ml of enzyme fraction). See Experimental.

RESULTS

Separation of Isozymes

Figure 1 shows clearly that two isozymes of cauliflower MDH can be separated on microgranular DEAE-cellulose; hereafter these two isozymes will be called 'MDH-I' and 'MDH-II'. The separation is interesting in itself since it is not merely a charge phenomenon; the two peaks cannot be separated or distinguished by starch-gel electrophoresis at pH 6·5, 7·5 or 8·5. We do not yet know the intracellular location of the two isozymes. For a similar elution pattern found with barley MDH Yue[7] has shown that peak I is the mitochondrial and peak II the cytoplasmic enzyme. The same kind of elution pattern is shown by the MDH's of *Opuntia*.[9]

Molecular Size

Each of the purified peaks was subjected to sucrose density-gradient centrifugation, using catalase ($s_{20,w}$ = 11·35S) as a marker. MDH-I was found to have an approximate $s_{20,w}$ of 5·4 S, while the value for MDH-II was found to be 5·7 S. These sedimentation coefficients are not significantly different for the two peaks, and are somewhat larger than those found for other MDH's.[11–14] Attempts to separate the two enzymes into subunits have so far been unsuccessful.

The two enzymes also showed identical peaks of activity when subjected to gel filtration chromatography (Fig. 2), again indicating that they are of the same molecular size.

[11] L. SIEGEL and S. ENGLARD, *Biochim. Biophys. Acta* **54**, 67 (1961); S. ENGLARD and H. H. BREIGER, *Biochim. Biophys. Acta* **56**, 571 (1962).
[12] G. B. KITTO and N. O. KAPLAN, *Biochem.* **5**, 3966 (1966).
[13] G. B. KITTO and R. G. LEWIS, *Biochim. Biophys. Acta* **139**, 1 (1967).
[14] W. H. MURPHY, C. BARNABY, F. J. LIN and N. O. KAPLAN, *J. Biol. Chem.* **242**, 1548 (1967).

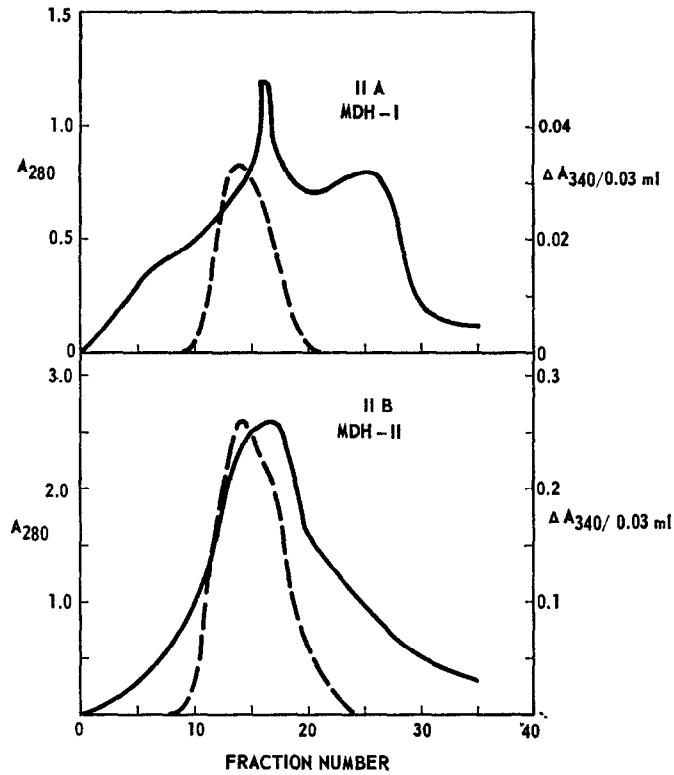

FIG. 2. GEL FILTRATION OF CAULIFLOWER MDH ISOZYMES ON BIOGEL A-0·5 M.
(A): Gel filtration of MDH-I. (B): Gel filtration of MDH-II. The same column was used for both isozymes. Fraction volume was 3 ml. The eluting buffer was 0·01 M potassium phosphate, pH 7·5, containing 1 mM EDTA and 1 mM DTT. Solid lines = A_{280}; dashed lines = MDH activity (ΔA_{340}/0·03 ml enzyme fraction).

Kinetic Constants

The Michaelis constants for malate, oxaloacetate (OAA), NAD and NADH were determined for each of the isozymes. The results are shown in Table 1. Both K_{malate} and

TABLE 1. MICHAELIS CONSTANTS OF CAULIFLOWER MDH ISOZYMES

Isozyme	K_{malate} (mM)	K_{OAA} (μM)	K_{NADH} (μM)	K_{NAD} (μM)
MDH-I	7·0	25	64	25
MDH-II	20	19	53	280

K_{NAD} are significantly higher for MDH-II than for MDH-I. K_{OAA} and K_{NADH} are essentially the same for the two isozymes. Our K_{OAA} for MDH-I is lower than that reported by Davies[10] (25 vs. 90 μM) but different conditions of pH and temperature were used for the determinations. Considerably higher concentrations of malate than of the other three substrates were required to saturate the enzyme.

Both isozymes showed significant substrate inhibition at high concentrations of malate and OAA. These inhibitions, especially those by OAA, are more marked with MDH-II. Further investigation on the kinetic properties of the enzyme is in progress.

Analog Ratios

Besides the natural coenzyme, the cauliflower MDH's will also utilize analogs of NAD as electron acceptors. The ratios of the activities with two of these cofactors, 3-acetylpyridine-adenine dinucleotide (AP-NAD) and thionicotinamide-adenine dinucleotide (TN-NAD), relative to the activity with NAD, are shown in Table 2. Since the relative activities vary

TABLE 2. THE ACTIVITY OF CAULIFLOWER MDH ISOZYMES WITH NAD ANALOGS, RELATIVE TO THE ACTIVITY WITH NAD

Isozyme	[Malate] (mM)	R^*_{AP-NAD}	R^*_{TN-NAD}
MDH-I	3·33	6·9	0·20
	66·7	4·8	0·38
MDH-II	3·33	2·8	0·16
	66·7	0·98	0·18

* R = rate with analog at indicated malate concentration/rate with NAD at 3·33 mM malate.

with substrate concentration, the rates with the two analogs relative to the rate with NAD were determined at two different malate levels, one non-saturating and one saturating. The relative rates do not appear greatly different for the two isozymes, but the rate for MDH-I is significantly higher than for MDH-II with AP-NAD at both low and high malate. As we will report in detail elsewhere, much greater differences are seen among various members of the genus *Brassica*.

Optimum pH

The pH optima of the cauliflower MDH's are difficult to determine, since their activities vary according to the relative cationic and anionic compositions of the buffer used. A similar phenomenon has been reported by Weimberg[15] for an MDH from pea seeds. As measured in either 0·05 M bicine or HEPES buffer, both peaks show a pH optimum between 7·5 and 8·0 in the OAA-reduction assay. The pea seed MDH already mentioned gave essentially the same result at low cation concentration.

Heat Stability

The ability of the two isozymes to withstand heating at various temperatures for 20 min was determined. The temperature at which 50% inactivation occurred for the MDH-I enzyme was 47·5° and for the MDH-II it was 53·0°. The MDH-II enzyme is slightly more heat resistant, but the difference is probably not significant. There is apparently no protective effect of added protein, since the presence of bovine serum albumin at concentrations of 0·1, 1·0 or 10·0 mg/ml during the heating period had no effect on the inactivation of the two enzymes.

[15] R. WEIMBERG, *J. Biol. Chem.* **242**, 3000 (1967).

Electrophoretic Mobility

Both isozymes migrate toward the anode in both starch and acrylamide gels at pH values between 6·5 and 8·5. The mobilities of the two are identical under all conditions tried. The purest preparations show only one band of protein for each isozyme, corresponding exactly to the zone of enzyme activity on the gel.

DISCUSSION

The properties of the two forms of MDH from cauliflower presented in this study are remarkably similar to each other. MDH-I and MDH-II show practically identical electrophoretic mobilities, molecular sizes (both by density-gradient centrifugation and by gel filtration), pH optima and heat stabilities. The Michaelis constants for OAA and NADH are very similar, as are the reaction rates with NAD analogs relative to the rates with the natural coenzyme. The only significant differences found were in the K_m values for malate and NAD, and in the strength of binding to DEAE-cellulose. Also, no properties examined were found to be strikingly different from those of mitochondrial and cytoplasmic MDH's from other higher plants and from animals. All these findings lead us to suggest that MDH is evolutionarily a rather conservative molecule. Thus biochemical properties of MDH's that may prove to be useful taxonomically or phylogenetically will no doubt be characters distinguishable quantitatively, rather than qualitatively. A later paper will discuss some of the properties that are useful characters.

Two properties of the cauliflower MDH's appear significantly different from those reported for enzymes from other sources. First, the molecular size as determined by sucrose density gradient centrifugation appears to be somewhat greater than that of animal MDH's,[11–13] but this remains to be confirmed by more sophisticated methods. Second, the two isozymes are inseparable by gel electrophoresis, while many animal MDH isozymes are easily separable. Our finding is puzzling in the light of the ease of separation of the two isozymes by ion-exchange.

The temperatures required for 50% inactivation of MDH-I and -II may appear rather low, but they are not too different from the heat stabilities reported by Mukerji and Ting[9] for *Opuntia* MDH's and by McNaughton[16] for MDH of cat-tail (*Typha latifolia*) ecotypes.

EXPERIMENTAL

Chemicals. NAD, NADH and NAD analogs were purchased from P-L Biochemicals. OAA, L(−)-malic acid, and Cleland's reagent (dithiothreitol; DTT) were purchased from Calbiochem. Whatman microgranular DEAE-cellulose was purchased from Reeve-Angel and Bio-Gel A-0·5 M from Bio-Rad. All other chemicals were reagent grade.

Plant materials. Cauliflower (*Brassica oleracea* L. var. *botrytis*) heads were obtained at local markets, kept refrigerated and used within 24 hr of purchase. No significant differences were seen among results obtained with different lots of cauliflower.

Enzyme purification. All steps were carried out at 4°. Heads of cauliflower were stripped of green leaves and stem tissue, broken into small pieces and rinsed in ice-cold distilled H_2O. The pieces were then placed in a large Waring blender with 1 ml/g of 0·2 M potassium phosphate buffer, pH 7·5, containing 1 mM EDTA and 10 mM mercaptoethanol, and ground at top speed for 2 min. The extract was squeezed through either one layer of Miracloth (Calbiochem) or 4 layers of cheesecloth. In large-scale purifications (5 or more heads) the strained extract was centrifuged in a Servall RC-2B centrifuge at 10 000 *g* for 20 min to remove gelatinous material. $(NH_4)_2SO_4$ was added to the clarified extract to 30% saturation;[17] the pH was maintained at 7·5 during this operation by addition of conc. NH_4OH. The resulting precipitate was sedimented by centrifugation at 10 000 *g* for 30 min and discarded. The supernatant fluid was brought to 70% saturation

[16] S. J. McNaughton, *Plant Physiol.* **41**, 1736 (1966).

[17] All $(NH_4)_2SO_4$ concentrations are given relative to 100% saturation at 0° (730 g/l.).

with $(NH_4)_2SO_4$; the pH was again maintained at 7·5. The resulting precipitate was centrifuged at 10 000 *g* for 30 min and the supernatant discarded. The precipitate was dissolved in a minimal volume of 0·01 M potassium phosphate, pH 7·5, containing 1 mM EDTA and 1 mM DTT, and either dialyzed overnight against 20–50 vol. of the same buffer, or reduced to about one-fifth of its original volume by ultrafiltration in a Diaflo apparatus using a UM-10 membrane. In either case, the desalted protein solution was placed on a column of microgranular DEAE-cellulose equilibrated with 0·01 M potassium phosphate buffer, pH 7·5, containing 1 mM EDTA and 1 mM DTT, and eluted with a linear concentration gradient of potassium phosphate buffer, pH 7·5, from 0·01 M to 0·2 M; both buffer reservoirs contained 1 mM EDTA and 1 mM DTT. Two distinct peaks of activity were obtained (Fig. 1). With fibrous DEAE cellulose the peaks overlap considerably. Fractions showing activity in each peak were pooled, taking care not to use fractions in the possible overlap zones between MDH peaks I and II. The pooled fractions in each peak were then concentrated to 10–20 ml by ultrafiltration as described above, and each peak was passed through a column of Bio-Gel A-0·5 M equilibrated with 0·01 M potassium phosphate buffer, pH 7·5, containing 1 mM EDTA and 1 mM DTT; elution was carried out with the same buffer. Fractions showing significant activity were pooled and again concentrated by ultrafiltration to *ca.* 5 ml. The pooled, concentrated fractions were brought to 70% saturation with $(NH_4)_2SO_4$ and stored at 0°. The enzyme is stable for several months under these conditions. These $(NH_4)_2SO_4$ suspensions provided the source of enzyme activities for the experiments described below.

Protein assays on crude fractions were performed by the method of Lowry *et al.*[18] using bovine serum albumin as a standard. Purer fractions were assayed for protein using the method of Warburg and Christian.[19] The degree of purification varied from preparation to preparation, but was usually about 50-fold. The best preparation showed roughly a 120-fold purification over the crude extract for both peaks, and both peaks were homogeneous by starch- and acrylamide-gel electrophoresis and sucrose density-gradient centrifugation. These highly purified peaks were used for the determination of kinetic constants.

Enzyme assays. All enzyme assays were monitored at 340 nm with a Gilford model 2400 recording spectrophotometer. The reaction mixture for the standard assay during purification contained: 0·1 M potassium phosphate buffer, pH 7·5, containing 0·5 mM EDTA; 0·13 mM NADH; and sufficient enzyme to give a ΔA_{340} of 0·02–0·2/min. The blank contained only buffer and water. The reaction was started by adding an aliquot of an OAA solution (prepared and neutralized daily) to give a final concentration of 0·15 mM and a total vol. of 3·0 ml. The reaction is usually linear for at least 1 min. Assays for the determination of kinetic constants were performed under the following conditions: (1) Variable OAA; same conditions as the standard assay, except the OAA concentration was varied. (2) Variable NADH; same conditions as the standard assay, except the NADH concentration was varied. (3) Variable malate; 0·05 M bicine buffer, pH 8·5, 0·2 mM NAD and enzyme. Varying amounts of a neutralized solution of L(−)-malic acid were added to start the reaction. Total reaction volume was 3·0 ml. (4) Variable NAD; Same conditions as for variable malate, except malate concentration was held constant at 30 mM and variable amounts of NAD solution were added to start the reaction.

Kinetic constants were calculated from plots of v/s against v,[20] rejecting points showing obvious substrate inhibition, which was seen with all substrates except NAD. The plots were calculated by the least-squares method and drawn using a Wang 700B programmable calculator with a plotter output.

In the determination of the rates of reaction with NAD analogs as compared to the natural coenzyme, the following conditions were used: (1) Low malate; 0·05 M bicine buffer, pH 8·5, 3·33 mM L(−)-malate, and enzyme. The reaction was started by adding an aliquot of a solution of NAD, AP-NAD or TN-NAD to give a final concentration of 0·1 mM. Reaction vol. was 3·0 ml. The reaction with NAD was monitored at 340 nm, that with AP-NAD at 363 nm and that with TN-NAD at 398 nm. Reaction rates were calculated using molar extinction coefficients of $6{\cdot}22 \times 10^3$, $8{\cdot}8 \times 10^3$ and $11{\cdot}4 \times 10^3$, all at their reduced absorption maxima, for NAD, AP-NAD and TN-NAD respectively.[21] (2) High malate: Same conditions as for low malate, except the malate concentration was 66·7 mM.

Heat stabilities. Stock solutions of MDH were diluted in 0·1 M HEPES, pH 7·5, containing 1 mM EDTA and 1 mM DTT. The diluted samples were incubated in a shaking water bath at various temperatures from 25 to 65°. After 20 min aliquots were removed and assayed for remaining enzyme activity. Temperatures at which 50% inactivation occurred in 20 min (T_{50}) were determined from plots of log percent activity lost *vs.* temperature.

Electrophoresis. Starch gel electrophoresis and enzyme staining were performed according to Fine and Costello[22] using a citrate-phosphate buffer at pH 6·5, 7·5 or 8·5. Power was supplied by a Beckman Duostat

[18] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[19] O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1942).
[20] B. N. J. HOFSTEE, *Nature, Lond.* **184**, 1296 (1959).
[21] T. KAWASAKI, personal communication.
[22] I. H. FINE and L. A. COSTELLO, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. VI, p. 958, Academic Press, New York (1963).

set at a constant current of either 15 or 30 m-amp. All runs were for 18 hr. Acrylamide gel electrophoresis was performed using an Ortec pulsed-power apparatus and a Tris-citrate buffer.

Density gradient centrifugation. Density gradient centrifugation was performed using a 0–30% linear sucrose gradient in a Spinco SW-39 rotor.[23] Catalase was used as a marker.

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[23] R. G. MARTIN and B. N. AMES, *J. Biol. Chem.* **236**, 1372 (1961).

Key Word Index—Brassica oleracea; Cruciferae; cauliflower; malate dehydrogenase; isozymes.