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VARIANT MALATE DEHYDROGENASE ISOENZYMES IN MITOCHONDRIAL POPULATIONS*

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SUMMARY

Variations in isoenzymes of malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) of mitochondrial populations were detected after polyacrylamide gel electrophoresis. Two distinct mitochondrial populations of a single barley genotype were resolved using sucrose density gradient equilibrium centrifugation of purified mitochondrial preparations. A third population, contiguous with one of the physically separable populations, was distinguishable on the basis of differences in malate dehydrogenase isoenzyme content of the mitochondria. These studies provide a means of detecting hitherto undetectable variant isoenzymes, as well as demonstrating biochemical methods for distinguishing polymorphic mitochondrial populations.

INTRODUCTION

Spectrophotometric assays of enzymes which are associated with specific subcellular organelles serve as an accepted means of identifying these organelles during physical separation and purification techniques. The spectrophotometric assays of enzymes which we had been using as mitochondrial markers had both the necessary sensitivity and specificity, but were tedious. We believed they were also lacking in that they offered no way to investigate the possibility that multiple molecular forms of the enzymes were present. Such enzymes could possess different kinetics, substrate affinities or co-factor requirements, which could alter the results of the assay.

In order to test such possibilities, we initiated studies on malate dehydrogenase. Isoenzymes of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) have been described in both plants[1] and animals[2]. Malate dehydrogenase isoenzymes have been reported to occur in the cytoplasm[1], and in subcellular organelles, including mitochondria[3], glyoxysomes[4], peroxisomes[5], and chloroplasts[6]. Presence of malate dehydrogenase activity in some organelles may be due to contamination[5]. Variant isoenzymes of malate dehydrogenase have also been found in mitochondria[7-12], glyoxysomes[11] and cytoplasm[1]. Multiple molecular forms of malate dehydrogenase

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may be separated using electrophoresis and characterized by localized formazan formation in gels incubated with substrate and specific cofactors. In this paper we have demonstrated the existence of variant malate dehydrogenase isoenzymes in mitochondrial populations of barley. In addition, we have presented evidence for the occurrence of physically contiguous but biochemically distinguishable mitochondrial populations within a single genotype.

MATERIALS AND METHODS

Seeds of a barley (*Hordeum vulgare* L.) F_1 hybrid (experimental line 63j-18-17 × cv. 'Benton', C.I. 1227) were soaked for 2 h in aerated water. Mitochondria were isolated from about 175 scutella (diploid non-emergent cotyledon) as described previously[20]. The washed mitochondrial pellet was resuspended in 0.5 ml of 0.95 M sucrose and 0.005 M EDTA and layered on a linear sucrose density gradient (0.95 1.9 M sucrose and 0.005 M EDTA). Centrifugation was carried out in a Spinco SW-50L rotor for 60 min at 35 000 rev./min. The rotor was decelerated with no braking. After centrifugation, 48 three-drop fractions were collected by removing the gradient from the top of the tube using an Isco density gradient fractionator.

Individual fractions were layered above polyacrylamide gels, a 5-mm gel plug was polymerized in place above the sample to prevent possible loss of the unpolymerized sample into the reservoir, and disc electrophoresis was carried out according to standard methods[13]. A pH 8.8 Tris–glycine buffer was used. Constant current was 5 mA per gel. All operations were carried out in the cold (0–6°). Malate dehydrogenase activity was assayed by a modification of published methods[14], by rinsing the gels in ice water and incubating them in a reaction mixture containing 25 mM malate; 0.98 mM nitro blue-tetrazolium; 13 μM nicotinamideadenine dinucleotide; 14 μM phenazine methosulfate and 0.82 mM Tris (pH 7.5).

Activity of malate dehydrogenase isoenzymes was detected by formazan formation following incubation of the gels at 37° for 15 min. Optical density of the individual formazan bands was measured at 650 nm using a Gilford spectrophotometer with a linear transport gel scanning attachment. Cytochrome *c* oxidase activity was measured spectrophotometrically[15].

The possibility of glyoxysomal contamination of mitochondrial populations was considered, and assays of a glyoxysomal marker enzyme, isocitrate lyase (EC 4.1.3.1), were carried out to evaluate the extent of contamination[16].

RESULTS

Equilibrium ultracentrifugation of purified barley mitochondria on sucrose density gradients demonstrated the occurrence of distinct mitochondrial populations in an F_1 hybrid. Fig. 1 illustrates the polymorphism of mitochondria separated on linear sucrose gradients. When mitochondria were isolated from scutella of barley seedlings at very early stages of germination (12 18 h) using the same techniques as for density gradient preparations, and assayed polarographically; the ADP:O ratios and respiratory control ratios of these mitochondria, utilizing α-ketoglutarate averaged 3.0 and 12.0, respectively[18]. Thus, we were assured that the gradient preparations represented the active mitochondrial populations of the cells.

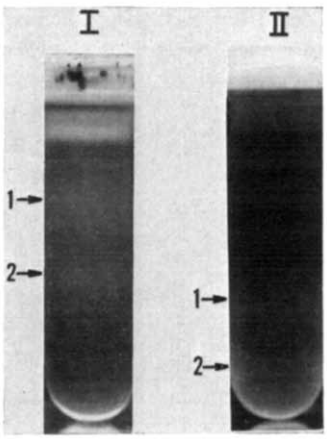

Fig. 1. Separation of barley mitochondria on sucrose density gradients. I. "Expanded" gradient, 1.4 to 1.9 M sucrose. II. Conventional gradient, 0.95 to 1.9 M sucrose. Numbers 1 and 2 indicate the two physically separable mitochondrial populations.

Spectrophotometric assay of cytochrome *c* oxidase served as a mitochondrial marker enzyme in initial experiments. In the work reported here, mitochondrial malate dehydrogenase was used as a mitochondrial marker enzyme. The pattern of malate dehydrogenase activity found in individual aliquots of the fractionated density gradients is shown in Fig. 2. Selected fractions representative of the different malate dehydrogenase isoenzymes found, (1) in the less dense portions of the gradient, (2) in the portion of the gradient where the "lighter" mitochondrial population was located, and (3) in the region of the gradient where the more dense mitochondrial population was found, are shown. It is significant that the malate dehydrogenase isoenzymes of the fractions show distinctive differences. As shown previously, the fastest migrating malate dehydrogenase isoenzyme is the cytoplasmic[19,20] form. The other bands repre-

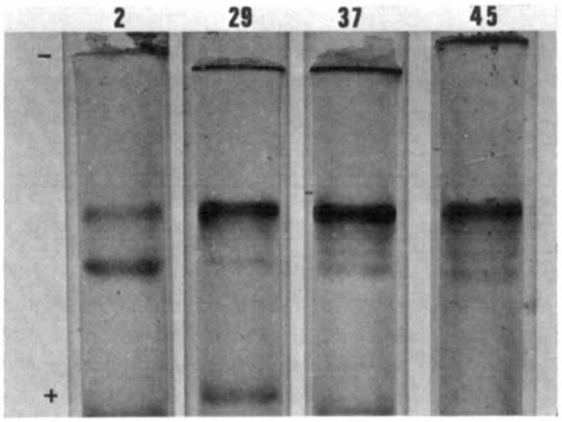

Fig. 2. Visualization of malate dehydrogenase isoenzymes on polyacrylamide gels. Tube numbers denote the gradient fraction layered on each gel. Specific isoenzyme nomenclature is detailed in the text.

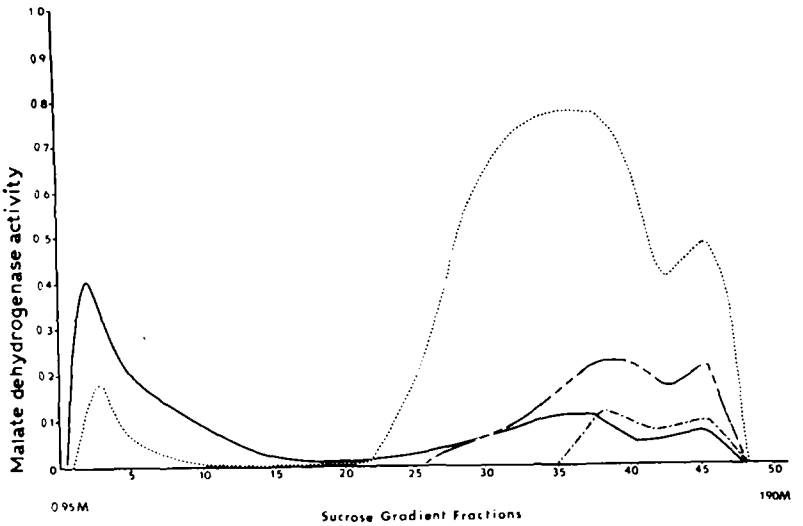

Fig. 3. Distribution of malate dehydrogenase activity after sucrose density gradient centrifugation of barley mitochondria. Curves are drawn for best fit to the data (48 specific observations determined each curve). — · —, cytoplasmic malate dehydrogenase; · · · · · ·, mitochondrial malate dehydrogenase-1; — — — —, mitochondrial malate dehydrogenase-2; — · —, mitochondrial malate dehydrogenase-3.

sent isoenzymes of mitochondrial malate dehydrogenase. The band at the interface of the gel may be either a high molecular weight malate dehydrogenase which failed to migrate under our conditions of electrophoresis[21] or enzyme activity associated with mitochondrial fragments which were also unable to penetrate the gel.

Fig. 3 presents the activity of malate dehydrogenase isoenzymes as they occur through the range of the density gradient. The pattern of mitochondrial malate dehydrogenase-1 serves to identify the location of the mitochondrial populations on the gradient. Note that two additional malate dehydrogenase isoenzymes are observed in the portion of the gradient containing mitochondria. The use of density gradients in this manner allows the visualization of additional malate dehydrogenase isoenzymes not normally seen in differential centrifugation preparations. The contrast

TABLE I

DISTRIBUTION OF MALATE DEHYDROGENASE ISOENZYMES IN MITOCHONDRIAL POPULATIONS SEPARATED ON SUCROSE DENSITY GRADIENTS

Activity of malate dehydrogenase isoenzymes is expressed as percentage of total enzyme activity recovered from individual fractions.

Mitochondrial population	*Fraction No. (peak)*	*Cytoplasmic malate dehydrogenase*	*Mitochondrial malate dehydrogenase-1*	*Mitochondrial malate dehydrogenase-2*	*Mitochondrial malate dehydrogenase-3*
	3	66	34	0	0
I	33	9	82	9	0
II	38	7	66	19	8
III	45	7	57	26	10

in isoenzyme content of the mitochondrial populations is shown by the quantitative differences in mitochondrial malate dehydrogenase-2 and mitochondrial malate dehydrogenase-3. These differences can be seen in Table I, where the percentage distribution of malate dehydrogenase isoenzymes of selected portions of the gradient is shown.

Qualitative differences in the malate dehydrogenase isoenzymes are evident. Based on visualization of the mitochondrial populations, on the quantitative measurement of mitochondrial protein, and on the assay of cytochrome *c* oxidase activity, the mitochondria may be divided into two populations. Because of the differences in specific mitochondrial malate dehydrogenase isoenzymes found in these populations we have further divided the less dense mitochondrial population into two populations (I and II). We have tentatively categorized the particles associated with a peak of mitochondrial malate dehydrogenase activity in the upper portion of the gradient as "pro-mitochondria"[18], on the basis of enzymatic and other biophysical properties.

DISCUSSION

Polymorphism of plant mitochondria has previously been demonstrated in corn[17], as well as in barley[22]. Polymorphism of mitochondria has been associated with heterosis. A heterotic hybrid of maize was found to have types of mitochondria in common with its parents, in addition to a new type of mitochondria presumably formed *de novo* as a result of some sort of gene action in the hybrid. These results suggested that mitochondrial polymorphism could act in producing fitness and contribute to homeostasis[17]. The barley hybrid studied here also exhibits mitochondrial polymorphism. Its mitochondria are physically separable into two types, and enzymatically represent three classes of particles.

Multiple malate dehydrogenase enzymes have previously been reported in barley[19,20,23]. This report demonstrates the occurrence of additional malate dehydrogenase isoenzymes of barley and their intracellular localization. Earlier work had shown an enzymatic interaction between cytochrome *c* oxidase preparations of aliquots of different maize mitochondrial populations separated on density gradients[24]. The present study reaffirms the enzymatic heterogeneity of such populations and provides evidence for a functional role of variant mitochondrial malate dehydrogenase isoenzymes in polymorphic mitochondrial populations. Genetic studies of the pattern of inheritance of these barley isoenzymes are in progress. Work in preparation using a primary trisomic tester set in barley has demonstrated the chromosomal location of genes specifying two malate dehydrogenase isoenzymes[25,26]. This work adds to the evidence for nuclear genetic control of malate dehydrogenase[11].

Glyoxysomes have not previously been found in barley, although the lipid metabolism of the scutellum of barley is sufficiently similar to that of maize, in which the glyoxylate cycle has been reported[27], that one would suspect their possible occurrence. In order to check on the possibility of contamination of our mitochondrial preparation by glyoxysomes, we assayed fractions from mitochondrial gradients using a glyoxylate cycle marker enzyme, isocitrate lyase. No isocitrate lyase activity was found in the gradients. When older barley scutella (24–48 h germination) were used as source of mitochondria, some isocitrate lyase activity was found associated with the pellet at the bottom of the density gradient after centrifugation. This demon-

strates the presence of the glyoxylate cycle in barley and supports the findings of other workers on the progressive development of glyoxylate cycle activity in germinating seedlings[28].

As mentioned, two of the malate dehydrogenase isoenzymes, mitochondrial malate dehydrogenase-2 and mitochondrial malate dehydrogenase-3 have not previously been visualized in barley[20,23]. The separation and resolution of isoenzymes from preparations on sucrose density gradients may be important in genetic studies of isoenzymes, where recognition of all molecular forms of a given enzyme is extremely critical in establishing inheritance, as well as in providing evidence for the polymeric nature of the enzymes studied.

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