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CYTOPLASMIC AND MITOCHONDRIAL MALATE DEHYDROGENASES OF NEUROSPORA

REGULATORY AND ENZYMIC PROPERTIES

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SUMMARY

The metabolic regulation of *Neurospora crass* cytoplasmic malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) is described. Synthesis of the cytoplasmic isozyme is subject to glucose repression whereas the mitochondrial forms are constitutive. Mycelia upon transfer from glucose to acetate as sole carbon source differentially synthesize cytoplasmic malate dehydrogenase, other enzymes of the glyoxylate cycle, and cytoplasmic aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1). The kinetics of cytoplasmic malate dehydrogenase synthesis indicate that the apparent increase in activity is 7-fold above the basal level of mitochondrial malate dehydrogenase activity and the absolute increase is several hundred-fold; maximal derepression occurs at the onset of growth on acetate; glucose repression after maximal derepression is not a rapid process; inhibition of derepression by antibiotics indicates that the isozymes are synthesized by cytoplasmic and not mitochondrial ribosomes; mineral starvation does not release glucose repression of either cytoplasmic malate dehydrogenase or aspartate aminotransferase but leads to gross cytological abnormalities.

A purification procedure for the cytoplasmic and mitochondrial isozymes is described. The enzymes are demonstrated to differ in stability, ethanol solubility, isoelectric point, kinetics with substrate inhibition, coenzyme and substrate analogs, and inhibition by either low-molecular-weight compounds, antiserum, or mitochondrial structural protein. Differential association of the two isozymes of Neurospora and porcine heart with homologous structural protein is described.

INTRODUCTION

At least two positions of a number of given enzymes in regard to subcellular localization in eucaryotes are encountered, mitochondrial and extramitochondrial[1].

Abbreviation: DNS, 1-dimethylaminonaphthalene 5-sulfonyl.

Such "heterotopic" enzymes meet the criteria of isozymes first suggested by MARKERT AND MØLLER[2] in that even though they ostensibly catalyze the same reaction, they generally differ in other properties. Although such heterotopes have been the subject of numerous biochemical investigations, the molecular and genetic basis of the most obvious property, differential subcellular localization, has received scant attention either within the limited context of isozymes or within the broader context of membrane specificity.

One of the more intensively investigated pair of heterotopes is the mitochondrial and so-called "soluble" forms of malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37). Subsequent to the studies of mitochondrial malate dehydrogenase and aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) by MUNKRES *et al.*[3–5], KITTO *et al.*[6] reported that two major electrophoretically separable forms of malate dehydrogenase could be obtained from Neurospora, one from the cytosol and the other from mitochondria. In addition, two forms of aspartate aminotransferase, cytoplasmic and mitochondrial, accompanied the corresponding forms of malate dehydrogenase during most purification procedures.

Recently the "soluble" form of malate dehydrogenase is found in association with another cellular organelle called "glyoxysome" or "microbody"[7]. Thus the physiological significance of cytoplasmic malate dehydrogenase lies in the association with glyoxylate cycle enzymes[7,8] and perhaps not in a malate–aspartate shuttle to maintain H^+ balance[9]. Moreover, in green plants, members of an isozyme trio of malate dehydrogenase are uniquely localized with chloroplasts, mitochondria and glyoxysomes[10].

In Neurospora, glyoxylate cycle enzymes are repressed during culture with glucose as the carbon source and derepressed by culture with acetate carbon source[11–13]. Concomitantly, malate dehydrogenase activity in the cytosol increases[13,14].

This report describes preliminary observations on the metabolic regulation, simultaneous purification and some enzymic properties of cytoplasmic and mitochondrial malate dehydrogenase from mycelia of Neurospora. Additional experiments bearing upon the molecular genetics and mechanics of localization of these enzymes are reported elsewhere[15].

MATERIALS AND METHODS

Stock cultures

Wild-type Neurospora (74A, FGSC No. 936) was maintained in storage at 4° on silica gel[16]. Periodically, new vegetative cultures were established in Fries minimal agar[17]. Conidia from 7-day-old slants were harvested in water and the number estimated by absorbance measurements calibrated by a hemocytometer. Under these conditions, between 90 and 100% of the conidia formed colonies when plated on Fries minimal.

Chemicals

D- and L-malic acid, oxaloacetic acid, *meso*-tartrate, phenazine methosulfate, pyridoxal phosphate, succinic acid, D,L-*threo*-isocitrate, L-aspartic acid, α-ketoglutaric acid, ATP, *p*-chloromercuribenzoate, dimethylaminonapthalenesulfonate, 2-hydroxy-5-nitrobenzyl bromide, and Aquacide I were products of CalBiochem. NAD^+, NADH,

NADP+, NADPH, chloramphenicol, cycloheximide, dithiothreitol, phenazine methosulfate, nitroblue tetrazolium and CoASAc were products of Sigma Chemical Co. Coenzyme analogs were obtained from Pabst Laboratories. $(NH_4)_2SO_4$ (enzyme grade), sucrose (enzyme grade), and crystalline porcine cytoplasmic and mitochondrial malate dehydrogenase were obtained from Mann Research Lab. 4-(2-bromo-acetamido)-salicylate was a gift from D. Ott, Los Alamos Scientific Laboratory. Glucose and sodium acetate were A.R.

Standard enzyme induction and growth measurements

Wild-type conidia ($1 \cdot 10^8$) were transferred to 100 ml of Fries minimal medium containing 10% glucose in 500 ml Florence flasks and incubated at 30° on a rotary shaker in semi-darkness at 225 cycles/min for 16 h. Mycelial pads were harvested aseptically on a Buchner funnel, washed with Fries minimal medium containing no carbon source, and transferred to 100 ml Fries minimal medium containing 0.5% sodium acetate in 500 ml Florence flasks. After dispersing mycelia, the cultures were returned to the same growth conditions.

Cells were removed periodically with a wide bore 10-ml pipette, collected on a Buchner funnel, washed with cold buffer (0.50 M sucrose, 1 mM EDTA, and 50 mM Tris–HCl (pH 7.4 at 4°) and blotted dry. The pads were either dried overnight at 40° for weight analysis or ground in a mortar and pestle on ice for 30 sec in the weight proportions: 1 mycelia: 2 sand: 10 buffer. Extracts were centrifuged at 2000 × *g* for 10 min and debris was discarded. The supernatant was centrifuged at 16 000 × *g* for 30 min. The supernatant (cytosol) was set aside and the mitochondrial pellet was resuspended in the same buffer, recentrifuged at 16 000 × *g* for 30 min and the mitochondrial pellet was suspended in phosphate buffer (50 mM, pH 7.0). Latent mitochondrial enzyme was released for assay with 1% digitonin.

Large-scale culture and induction of cytoplasmic malate dehydrogenase for purification purposes were in scale with the above procedure. Mycelia from 15 l of Fries minimal medium containing 2% sucrose after 24 h growth at 30° were transferred to 150 ml of Fries medium supplemented with 0.5% sodium acetate and incubated at 30° for 8–10 h.

Parallel purification of the isozymes

All of the following steps were at 4°.

Step 1. Extraction. Fresh mycelia were suspended in 7 vol. of Tris–sucrose–EDTA buffer and homogenized at full speed for 1 min in a quart Waring Blendor. An equal weight of glass beads (500–800-mμ diameter) were added to the slurry and mixed for 3 h in a ball mill. Glass beads were removed by decantation and washed with Tris–sucrose–EDTA buffer. The wash and supernatant were combined, filtered through cheesecloth, and centrifuged for 10 min at 12 000 × *g*. Cell debris was discarded and the supernatant(S1) was centrifuged at 12 000 × *g* for 30 min. The mitochondrial pellet(M1) was suspended in 50 ml Tris buffer and stored at −20°.

Step 2. $(NH_4)_2SO_4$ precipitation (0–90%). Fraction S1 was adjusted to 90% saturation in $(NH_4)_2SO_4$, centrifuged at 12 000 × *g* for 30 min, and the precipitate(SP1) was collected in sodium phosphate buffer (50 mM (pH 7.0), 1 mM EDTA and 1 mM dithiothreitol). A second precipitate was obtained in a similar manner after storing the supernatant overnight once again and combined with SP1.

Step 3. $(NH_4)_2SO_4$ precipitation (30–75%). Fraction SP1 was dialyzed against the same buffer and adjusted to 30% saturation with $(NH_4)_2SO_4$. The precipitate was removed and discarded after centrifugation and the supernatant (SS1) adjusted to 75% $(NH_4)_2SO_4$. Precipitate (SP2) was collected, suspended in the above buffer and dialized against 20% (w/v) $(NH_4)_2SO_4$ (containing 1 mM EDTA and 1 mM dithiothreitol). The slight precipitate was discarded after centrifugation and the supernatant (SS2) was set aside for the next step.

Step 4 and 4A. Ethanol fractionation. Fraction SS2 was adjusted to 10 mM in L-malate and chilled to 0° on an ice bath. Absolute ethanol (−20°) was slowly added to the enzyme solution in a thin stream which was chilled in a dry-ice–acetone bath until 52% ethanol was added at −15°. The solution was stored at 0° for 30 min and centrifuged. The precipitate (SP3, cytoplasmic malate dehydrogenase) was collected in phosphate buffer (10 mM (pH 7.6), containing 1 mM dithiothreitol) and set aside. The supernatant was adjusted to 67% ethanol by the above procedure, stored at 0° for 30 min and centrifuged. The precipitate (MP4, mitochondrial malate dehydrogenase) was collected in phosphate buffer (10 mM (pH 7.6), containing 1 mM dithiothreitol) and set aside. Immediately after preparation, Fractions SP3 and MP4 were dialyzed against the same buffer with two changes. Precipitate was removed by centrifugation and discarded.

Step 5. Anion-exchange chromatography. DEAE (Cellex D, BioRad, 0.9 mequiv/g) was prepared by the procedure of PETERSON AND SOBER[18] washed with distilled water, and equilibrated with sodium phosphate buffer 10 mM (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol). Columns (1 cm × 22 cm) were packed and washed with 10 vol. of buffer. Enzyme (either Fraction SP3 or MP4) was applied to the column, developed at 15 ml/h and 5-ml fractions were collected. Fractions 2–16 from SP3 or Fractions 16–32 from MP4 were pooled and concentrated in dialysis tubing with Aquacide I. Finally, the samples were dialyzed against buffer with two changes. Mitochondrial malate dehydrogenase could be stored at −20° for several months, however cytoplasmic malate dehydrogenase became inactive after a month's storage at −20°. Most of the measurements to be described were carried out within 2 weeks after preparation.

Other preparations

Partially purified antibody to mitochondrial malate dehydrogenase from rabbit antisera was prepared as described previously[19].

1-Dimethylaminonaphthalene 5-sulfonyl (DNS)-labeled malate dehydrogenase was prepared as follows. Enzyme (0.3 mg in 0.5 ml 75% ethanol and 15 mM phosphate buffer (pH 7.0)) was incubated with 70 μg of DNS chloride at 4° for 24 h. The precipitate was collected by centrifugation, resuspended in 1 ml cold 50 mM phosphate buffer at pH 7.0 and passed through a 1 cm × 10 cm column of BioGel P-2 (BioRad) equilibrated with buffer at 4°.

Structural protein from Neurospora and porcine mitochondria were prepared as previously described[20] with the following additional steps. The insoluble protein was dissolved in a freshly prepared solution of 8 M urea (enzyme grade) and 0.1 M NaOH at room temperature (1 mg/10 ml), centrifuged at 20 000 × *g* for 30 min to remove a trace of insoluble material, neutralized in an ice bath to pH 6.0 with HCl, collected by centrifugation, redissolved in urea–NaOH solution and dialyzed against 5 l of 50 mM Tris–HCl buffer (pH 8.5) containing 10 mM 2-mercaptoethanol in acetyl-

ated dialysis tubing for 24 h. The tubing was then transferred to dialyze against 5 l of 50 mM phosphate buffer (pH 7.4) with 10 mM mercaptoethanol for 12–24 h. The opaque colloidal suspension of protein was either stored in solution at 4° under toluene (if to be used in a few days) or precipitated with 10 vol. of cold acetone, collected by centrifugation, air-dried and stored at 4°, in a dessicator over $CaCl_2$. For titration experiments, the dry protein (10 mg/ml) was suspended in 50 mM phosphate buffer (pH 7.4) at room temperature in a 3-ml test-tube, stirred with a Vortex mixer, and stored overnight at 4°. The colloidal supernatant (containing about 1 mg/ml) was withdrawn with a syringe and used for titration experiment.

Enzyme and protein assay

Malate dehydrogenase was generally assayed in the forward reaction as previously described[4]. One unit of activity is equivalent to the formation of 1 μmole NADH/ml per min. Specific activity is defined as units/mg protein. Aspartate aminotransferase was assayed as previously described[21]. Isocitrate lyase (*threo*-DS-isocitrate glyoxylate-lyase, EC 4.1.3.1) activity was determined by the method of KOBR *et al.*[11]. Succinate dehydrogenase (EC 1.3.99.1) was estimated spectrophotometrically with phenazine methosulfate by the method of SINGER AND KEARNEY[22] with extrapolation to v_{max} in respect to electron carrier. Protein was determined by the method of LOWRY *et al.*[23] for the induction kinetics experiments. In the experiments of Tables I, II, III, protein was determined by a macro- or a microbiuret method[24]. Where sucrose was present trichloroacetic acid precipitation was used.

Immuno-neutralization

Purified cytoplasmic and mitochondrial malate dehydrogenases were incubated with purified rabbit anti-mitochondrial malate dehydrogenase in 50 mM phosphate buffer (pH 7.4) at 0° for various time intervals in parallel with controls of enzyme and antibody alone. Data were corrected for the slight quantity of endogenous rabbit malate dehydrogenase and for slow inactivation of cytoplasmic malate dehydrogenase alone during the experiment.

Isoelectric focusing

Polyacrylamide gel isoelectric focusing experiments were carried out by the method described by LEABACK AND RUTTER[25] at 12° with 3–10 or 5–8 range "Ampholine" carrier electrolyte (LKB-Producter, Sweden). Gels were stained for malate dehydrogenase activity after focusing or electrophoresis in a solution containing: potassium L-malate (1.0 M, pH 7.6), 4 ml; potassium phosphate buffer (1.0 M, pH 7.6), 4 ml; NAD^+, 12 mg; nitroblue tetrazolium, 20 mg; phenazine methosulfate (0.6 mg/ml), 0.1 ml; and distilled water, 22 ml. After being stained for 10–60 min in the dark, gels were fixed in 1% acetic acid. Densitometer tracings of stained gels were made with a Gilford recording spectrophotometer. A "blank" gel without sample or stain was cut into 0.5-cm segments, eluted with distilled water and the relation of pH to gel length was determined.

Electrophoresis

Polyacrylamide gel electrophoresis was performed in a Büchler Polyanalyst

apparatus with twelve columns 5 mm internal diameter × 65 mm long at constant temperature of 12° and constant voltage (approx. 4 mA per column). The directions of the manufacturer's handbook were followed for cationic and anionic gels. For mobility measurements at a given pH, at least three time intervals were employed. Mobility was expressed as mm/h in 7.5% gel at 12° and estimated from distance *vs.* time plots. Mobilities at pH 9.9, 7.0 and 4.2 (constant ionic strength) were normalized to a common power factor, 400 mW/h per twelve gel tubes.

Fluorescence polarization titrations

Fluorescence measurements were made with an Aminco–Bowman spectrophotometer equipped with glan-polarizer prisms and constant temperature regulator as outlined by CHEN AND BOWMAN[26].

RESULTS AND DISCUSSION

Kinetics of derepression and repression of cytoplasmic malate dehydrogenase

Fig. 1 illustrates the increase of cytoplasmic malate dehydrogenase activity as a function of time after transfer from glucose to acetate medium. An isometric plot[27] of the same data shows that 10 units of cytoplasmic malate dehydrogenase are synthesized per mg of protein during induction. Parallel cultures with glucose *plus* acetate (Fig. 1) reveal no differential increase in activity in the cytosol, indicating glucose (or metabolites thereof) is the repressor rather than acetate being an inducer. The basal level of activity at zero time can be demonstrated to be largely mitochondrial

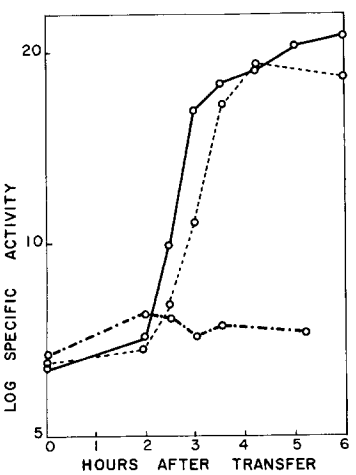

Fig. 1. Differential inhibition of the derepressed synthesis of cytoplasmic malate dehydrogenase by chloramphenicol, cycloheximide or glucose as a function of time. Wild-type Neurospora (74A) was grown for 16 h on 100 ml Fries minimal medium containing 10% glucose and transferred to Fries containing 0.5% sodium acetate alone (○—○) or supplemented with cycloheximide (8 μg/ml) (○—·—○), chloramphenicol (3 mg/ml) (○— — —○), or Fries containing 0.5% sodium acetate and 10% glucose (○— · —○). Samples of 10 ml were collected at timed intervals after transfer, filtered, and ground in a mortar and pestle containing 0.2 g sand and 1 ml of buffer. The extracts were centrifuged at 16 000 × *g* for 25 min. The supernatants were assayed for enzyme and protein as described in MATERIALS AND METHODS.

in origin by isoelectric focusing and is probably released from mitochondria during extraction of cells.

The constitutive level of cytoplasmic malate dehydrogenase is a function of glucose concentration and physiological age of the culture. With 5% glucose in an 18–20-h culture started from adequate and viable inocula, electrophoretic analysis of cytosol indicates the presence of cytoplasmic and mitochondrial malate dehydrogenase. The former represents approx. 1% or less of the total activity. Conversely, in older cultures or in cultures with lower initial glucose concentration, significant derepression may occur due to diminished glucose concentration and possibly the utilization of accumulated products such as acetate or ethanol. Thus, although the apparent increase in cytoplasmic malate dehydrogenase activity above the basal contaminating level of mitochondrial enzyme in the cytosol is between 3- and 7-fold, the actual increase is on the order of several hundred-fold. Conversely, activity in the mitochondrial fraction remains relatively constant during induction on acetate. (Table II).

During the first 4–6 h after transfer to acetate, the rate of cytoplasmic malate dehydrogenase synthesis is more rapid than the overall rate of protein synthesis and is reflected by the increase in specific activity. However, after maximal derepression is reached, although the total cytoplasmic malate dehydrogenase activity remains constant, the specific activity of the cytosol declines about 10%. This is attributed to a failure of cytoplasmic malate dehydrogenase production to parallel protein synthesis. It is difficult to determine whether the maintained high level of the total cytoplasmic activity in the cytosol during this period results from continued but relatively slower production of the enzyme or a stability of the existing enzyme to catabolism.

Another explanation for these results arises from consideration of the work of KOBR *et al.*[28]. They found malate dehydrogenase activity present in glyoxysomes only when mycelia are cultured on acetate. Thus the malate dehydrogenase activity in the glyoxysomes may be the cytoplasmic form. More direct evidence for this "packaging" hypothesis was demonstrated by GERHART AND BEEVERS[29]. In the experiments described here, the glyoxysomes (if not broken by the extraction) would be centrifuged down with the mitochondria and a fraction of the cytoplasmic enzyme would no longer be found in the cytosol. Therefore, even if the enzyme continued to be synthesized at the rapid rate observed during the first 4–6 h, the cytosol pool of free enzyme would level off or decrease as it was incorporated into glyoxysomes. This hypothesis might be tested by examining the cell extracts for glyoxysomes and monitoring their malate dehydrogenase activity during induction. Such investigations are presently in progress.

Addition of glucose to a culture at the time of maximal induction leads to a decline in specific activity at the onset of the massive increase in total protein synthesis (Fig. 2). However, the specific activity then remains constant for at least 2 h indicating that cytoplasmic malate dehydrogenase is still being synthesized at a rapid rate.

The apparent synthesis of both isozymes is associated with cytoplasmic and not mitochondrial ribosomes as indicated by the effects of chloramphenicol and cycloheximide. As illustrated in Fig. 1, chloramphenicol (a specific inhibitor of mitochondrial ribosomes of Neurospora *in vivo* and *in vitro*[30,31]) alters neither the time-course nor differential synthesis of cytoplasmic enzyme. Similar results were obtained with

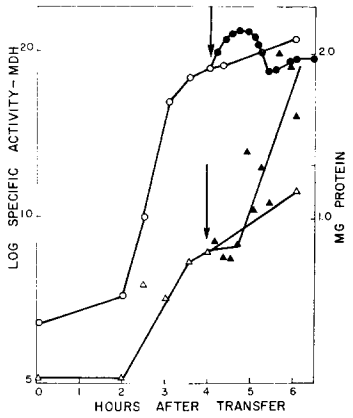

Fig. 2. Absence of short-term glucose repression of cytoplasmic malate dehydrogenase in derepressed cells. The experimental procedure described in Fig. 1 was repeated. After 4 h of culture on Fries containing 0.5% sodium acetate, the mycelium was transferred to 100 ml Fries containing 10% glucose. This transfer is indicated by the heavy arrow. Sampling was continued for an additional 2.5 h. One unit of enzyme activity (× 0.161) equals one I.U. ○—○, specific activity of malate dehydrogenase from cells induced on 0.5% sodium acetate; △—△, total mg protein in cytosol per 10 ml sample of cells induced on 0.5% sodium acetate; ●—●, specific activity of malate dehydrogenase of induced cells transferred to 10% glucose; ▲—▲, total mg protein in cytosol per 10 ml sample of induced cells transferred to 10% glucose.

the mitochondrial enzyme in glucose culture with conditions allowing anaerobic fermentation[32]. Conversely, cycloheximide (specific for cytoplasmic ribosomes of Neurospora[30]) essentially completely abolishes synthesis of both enzymes. A similar situation is found for these isozymes in Saccharomyces[33,34].

The capacity for derepressed synthesis of cytoplasmic malate dehydrogenase has been apparently conserved in short-term evolution since only one of twelve strains of *Neurospora crassa* from a world collection was found to be incapable of derepressed synthesis of the enzyme.

Nutritional and cytological factors in metabolic regulation of cytoplasmic malate dehydrogenase

In the preliminary experiments reported by KITTO *et al.*[6], the authors were apparently unaware of the possibility of enzyme repression and presumably inadvertently cultured mycelium on substandard medium deficient in a number of essential mineral nutrients. However, their description of cytoplasmic and mitochondrial malate dehydrogenase from mycelium cultured under these conditions suggested to us that some nutritional conditions might overcome the repression of the cytoplasmic isozyme by glucose. This view is not supported by the results of the experiments to date. On mineral starvation medium of KITTO *et al.*[6], a differential increase of malate dehydrogenase, aspartate aminotransferase, and isocitrate lyase activities occurs in the cytosol relative to the nutritionally adequate control, even in the presence of sucrose (Table I) (the repression on Fries medium containing 2% sucrose is essentially equivalent to the repression obtained with 5% glucose in the previous experiments). These results superficially resemble the results after derepression on Fries medium containing acetate (compare Tables I and II) with four major exceptions.

TABLE I

DIFFERENTIAL REGULATION OF THE SOLUBLE AND MITOCHONDRIAL ISOZYMES OF NEUROSPORA MALATE DEHYDROGENASE BY STARVATION CULTURE

*Culture conditions**	*Growth (g fresh wt. per flask)*	*Specific activity (μmoles/min per mg protein at 37°)*						
		Soluble fraction				*Mitochondrial fraction*		
		Isocitrate lyase	*Malate dehydrogenase*	*Aspartate aminotransferase*	*Succinate dehydrogenase*	*Isocitrate lyase*	*Malate dehydrogenase*	*Aspartate aminotransferase*
Standard	1.4	0.047	2.74	0.065	0.0187	0.160	10.1	0.920
Starvation	0.1	1.13	4.37	0.230	0.0254	0.715	1.25	0.0025
		Total activity (μmole units per g fresh mycelial wt.)						
			× 10^{-2}	× 10^{-1}			× 10^{-2}	× 10^{-1}
Standard	1.4	6.75	0.392	0.925	2.68	3.35	0.211	1.92
Starvation	0.1	158	0.615	3.24	3.56	18.7	0.033	0.006

* The standard culture conditions were with Fries minimal medium supplemented with with incubation from conidial inoculum for 26 h at 27°. The starvation culture was with a medi in Mg^{2+}, SO_4^{2-}, PO_4^{3-} and NH_4^+ (see below) with 2% sucrose as described by KITTO *et al.*[6]. Incu conidial inoculum for 48 h at 27°. Strain: 74A (wild-type).

Adjunct	*Concentration (mg/ml)*		*mg N/l*	
	Standard	*Starvation*	*Standard*	*Starvation*
NH_4^+	2230	0.5	1740	0.4
NO_3^-	780	730	177	165
PO_4^{3-}	700	0		
Mg^{2+}	48	0		
SO_4^{2+}	167	2.5		

First, under these starvation conditions, the growth rate is less than 10% the normal growth rate on Fries medium containing 0.5% sodium acetate (Tabl and after 48 h in a shake flask at 27° the mycelium appears grossly slimy, choc brown and is obviously disintegrating and undergoing autolysis. Furthermore, c logically these cells have gross subcellular abnormalities indicative of statio phase cells grown on nutritionally adequate Fries medium. In contrast, ace cultured cells have normal ultrastructure (K. D. MUNKRES, K. BENVENISTE AN ZUICHES, unpublished observations).

Bearing in mind the possibility of cellular pathology, a third apparent m difference in the distribution of malate dehydrogenase and aspartate aminotransfe between the two cell fractions from acetate and starvation cells is that the "n chondrial" fraction of the latter is virtually devoid of the dehydrogenase and an transferase activity, whereas little difference in the mitochondrial activities observed between glucose and acetate cells (Tables I, II). Conceivably, the sta cells could have fewer or more labile mitochondria; however, the comparati normal activities and distribution of succinate dehydrogenase suggest that th

TABLE II

REGULATION OF THE ACTIVITIES OF NEUROSPORA ISOCITRATE LYASE, MALATE DEHYDROGENASE AND AMINOTRANSFERASE IN THE SOLUBLE CELL FRACTION BY ACETATE AND GLUCOSE CARBON SOURCES

*Carbon source**	*Specific activity (μmoles/min per mg protein at 37°)***						
	Soluble fraction				*Mitochondrial fraction*		
	Isocitrate lyase	*Malate dehydrogenase*	*Aspartate aminotransferase*	*Succinate dehydrogenase*	*Isocitrate lyase*	*Malate dehydrogenase*	*Aspartate aminotransferase*
Glucose	0.047	2.74	0.065	0.0187	0.160	10.1	0.920
Acetate	1.77	8.20	0.650	0.0405	1.00	6.4	0.460
	Total activity (μmole units/g fresh mycelial wt.)						
		$\times 10^{-2}$	$\times 10^{-1}$			$\times 10^{-2}$	$\times 10^{-1}$
Glucose	6.75	0.392	0.925	2.68	3.35	0.211	1.92
Acetate	262	1.21	9.60	6.00	20.9	0.133	0.96

* Mycelia of wild-type 74A were precultured from conidial inoculum with Fries minim containing 2% glucose for 26 h at 27°, then transferred to minimal medium supplemented with eit (2%) or acetate (0.5%) and incubated for 8 h at 27°.

** Malate dehydrogenase in reverse reaction.

not true. A fourth major apparent quantitative difference between starvation acetate cells is that the total activity of malate dehydrogenase and aspartate am transferase in the former does not differ from glucose controls and the activities apparently simply redistributed between the two cell fractions.

Purification of mitochondrial and cytoplasmic malate dehydrogenase

A simultaneous purification of the isozymes from extracts of derepre mycelia is outlined in Table III. In this procedure, electrophoretic analyses indic that the mitochondrial isozyme activity comprises about half of the total activit the initial cytosol due to breakage of mitochondria during the initial extraction. Tl the overall purification factors for cytoplasmic and mitochondrial forms are ab

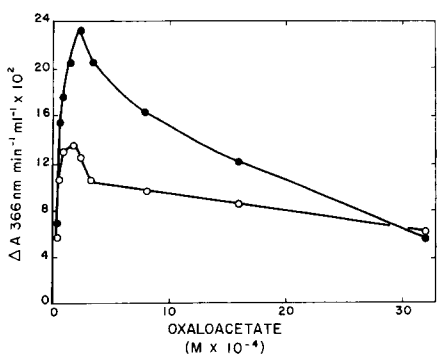

Fig. 3. Relative initial velocities as a function of oxaloacetate concentration. ●—●, mitochond ○—○, cytoplasmic. Conditions: triethanolamine buffer (pH 7.6, 66 mM), NADH (0.18 mM) purified enzyme (Step 5, Table III) in a cuvette of 1 cm light path at 37°.

TABLE III

PURIFICATION OF SOLUBLE AND MITOCHONDRIAL MALATE DEHYDROGENASES OF NEUROSPORA FROM EXTRACTS OF MYCELIA INDUCED WITH ACETATE

Mycelia were precultured with Fries minimal medium supplemented with 2% sucrose, harvested and incubated for 8 h with Fries minimal supplemented with 0.5% acetate. The purification outlined is from 250 g fresh weight of mycelia.

Step	*Protein (g)*	*Specific activity**	*Total units ($\times 10^{-6}$)*	*Purification factor*	*Substrate inhibition H/L ratio***
1. Extract (soluble)	8.4	480	4	1	
2. $(NH_4)_2SO_4$ (0–90%)	4.0	950	4	2	1.5
3. $(NH_4)_2SO_4$ (30–75%)	0.60	3300	2	7	
4. Ethanol (0–52%)	0.16	13000	2	27	1.5
5. DEAE-cellulose (from Step 4)	0.017	118000	2	250	1.7
4A. Ethanol (52–67%)	0.06	7000	0.4	15	2.7
5. DEAE-cellulose (from Step 4A)	0.011	55000	0.6	115	3.4

* μmoles NADH oxidized per min per mg protein at 37°.

** The ratio of initial velocities at oxaloacetate concentrations of $3.2 \cdot 10^{-3}$ M(H) and $3.2 \cdot 10^{-4}$ M(L) (see Fig. 3).

500- and 230-fold, respectively. The latter is comparable to the degree of purification obtained in a previously described procedure for mitochondrial malate dehydrogenase from repressed cells in which purity was judged to be at least 90% (ref. 24). Electrophoretic analysis of the purified isozymes at pH 9.9 by a specific histochemical stain for activity indicated that the preparations were relatively ($\geqslant 80\%$) free of the heterologous isozyme.

The distribution of the isozymes during purification can be monitored by assays employing differential substrate inhibition by oxaloacetate (Table III, Fig. 3).

Attempts were made to extract the enzymes from acetone powder of derepressed

TABLE IV

COMPARATIVE KINETICS OF MALATE DEHYDROGENASE ISOZYMES WITH SUBSTRATES AND COENZYME ANALOGS

*Substrates**	*Relative initial velocity (%)***	
	Cytoplasmic malate dehydrogenase	*Mitochondrial malate dehydrogenase*
L-Malate (0.1 M); NAD^+	100	100
thio NAD^+	3	25
$NADP^+$	0.6	1.5
L-Aspartate (0.1 M); NAD^+	1.5	4.4
Meso-tartrate (0.1 M); NAD^+	18	69
Meso-tartrate (0.1 M); thio NAD^+	3	25
D-Malate (0.1 M); NAD^+	0	0

* Coenzymes were 0.44 M.

** In 0.075 M glycine–NaOH buffer (pH 9.9) at 37° at appropriate wavelength. Purified enzyme (Step 5, Table III).

mycelia as previously described for the preparation of mitochondrial malate dehydrogenase from glucose-repressed mycelia[4]. However the cytoplasmic isozyme, unlike the mitochondrial, was inactivated in cells or in the purified state by exposure to cold acetone. Even in the procedure finally employed, greater instability of the cytoplasmic isozyme was encountered.

Comparative kinetic properties

Mitochondrial malate dehydrogenase is relatively more sensitive than the cytoplasmic isozyme to inhibition by high concentrations of oxaloacetate (Fig. 3). Similar results are obtained with these enzymes from animals[35] and yeast[36]. In addition significant differences in relative initial rates were observed with various coenzymes and substrate analogs (Table IV). These isozymes of several animal sources[35] and yeast[36] may also be distinguished in this manner.

Comparative inhibition properties

Differential inhibition of the isozymes is obtained with a variety of compounds (Table V). The non-competitive, instantaneous, reversible inhibition of Neurospora mitochondrial malate dehydrogenase by pyridoxal phosphate was previously described[37]. Differential instantaneous inhibition of initial rates were obtained with *p*-chloromercuribenzoate, ATP, and CoASAc. Differential inhibition by the mercurial may indicate differences in accessibility, reactivity or number of sulfhydryl groups. Differential inhibition by ATP and CoASAc are of interest in terms of allosteric control of gluconeogenesis and respiration. Others have observed that ATP and ADP are allosteric effectors of porcine[38,39] and plant [40] mitochondrial malate dehydrogenase.

A time dependence of irreversible inhibition of the isozymes by the bifunctional reagent, 4-(2-bromoacetamido)-salicylate was observed. Thus, 2 min of preincubation of enzyme with 25 μM inhibitor and 0.1 mM NADH caused 55% inactivation of the

TABLE V

DIFFERENTIAL INHIBITION OF MALATE DEHYDROGENASE ISOZYMES

Inhibitor	*Inhibitor Concn. (mM)*	*Inhibition (%)**	
		Cytoplasmic malate dehydrogenase	*Mitochondrial malate dehydrogenase*
Pyridoxal phosphate	1.0	48	86
p-Chloromercuribenzoate	0.1	37	9
4-(2-Bromoacetamido)salicylate	0.025	55	5
ATP	5	5	50
CoASAc	1	34	5
2-Hydroxy-5-nitrobenzyl bromide	0.25	98	45
Rabbit antisera to Neurspora mitochondrial malate dehydrogenase		0	75
Mitochondrial structural protein	0.0025	0	70–98

* Enzyme from Step 5 (Table III). Inhibition is relative to initial rates of control in assay with mixture containing 50 mM phosphate buffer (pH 7.4), 0.6 mM oxaloacetate and 0.1 mM NADH. Where inhibitor concentration or time of inhibition effects were observed, the tabulated values represent maximal differences between cyloplasmic and mitochondrial malate dehydrogenase.

cytoplasmic form and 5% or less of the mitochondrial enzyme. However, no difference in instantaneous inhibition of the isozymes was observed. The inhibition constant was 0.2 mM and was competitive with NADH. Salicylate also inhibited both enzymes equally and instantaneously but no time-dependent inhibition was observed. The inhibition constant was 14 mM and was competitive with NADH. These results imply that this inhibitor initially reacts rapidly and reversibly with the active center of both enzymes and secondarily reacts at a differentially slower rate to form a covalent bond.

BARMAN AND KOSHLAND[41] first introduced the reagent, 2-hydroxy-5-nitrobenzyl bromide, as a means of modification and identification of tryptophanyl residues in proteins. In alkaline media (pH 7.4) such as used here, other amino acids such as cysteine, methionine, or tyrosine may also react with 2-hydroxy-5-nitrobenzyl

TABLE VI

DIFFERENTIAL IMMUNO-NEUTRALIZATION OF SOLUBLE AND MITOCHONDRIAL MALATE DEHYDROGENASE OF NEUROSPORA

Rabbit anti-mitochondrial malate dehydrogenase (μg/ml)	*Inhibition (%)**			
	Cytoplasmic malate dehydrogenase		*Mitochondrial malate dehydrogenase*	
	Preincubation time (min):			
	15	*45*	*15*	*45*
2.8			20	
28			28	
280	0	0	44	75
2800	0	0	45	60
28000	0	0	43	67

* Enzyme (from Step 5, Table III) See MATERIALS AND METHODS for other conditions.
** Concentrations of immune protein in the assay were 1/10 the given concentration in preincubation.

bromide. Hence, the observation of differential concentration dependence of 2-hydroxy-5-nitrobenzyl bromide in the inhibition of the isozymes could reflect a composite of differences between these two enzymes in accessibility, reactivity, or number of such residues, including tryptophan. Recent studies by SCHELLENBERG AND MCLEAN[42] indicated that native porcine mitochondrial malate dehydrogenase is inert toward inhibition at pH 6–8 by HNBB, but titration of reversibly denatured enzyme indicated that one reactive tryptophan, essential for activity, normally exists in a sterically inaccessible environment.

The most specific and reactive differential inhibitors of the Neurospora isozymes encountered are either immune rabbit antisera to the mitochondrial form or Neurospora mitochondrial structural protein (Table VI, Fig. 4). Of the two proteins, mitochondrial structural protein preparations were at least equal to and in some cases more effective inhibitors than immune antisera. Thus a weight ratio of 50:1 structural protein:enzyme yielded 50% inhibition of the mitochondrial enzyme. If the functional molecular weight of structural protein aggregates is taken to be 23 000 (refs. 20, 43) the inhibition constant in a number of experiments was about 1 μM. Conversely, no inhibition of the cytoplasmic enzyme was obtained with a 6-fold greater excess of

structural protein even after prolonged incubation. Nearly identical results were obtained with the porcine isozymes in association with porcine structural protein (Fig. 4). Preliminary experiments with the bovine isozymes and homologous structural protein yielded similar results. Previous analyses of the association of wild-type or mutant mitochondrial malate dehydrogenase with wild-type or mutant structural protein by kinetic and fluorescence polarization criteria indicated that the association was reciprocally stereospecific[44,45].

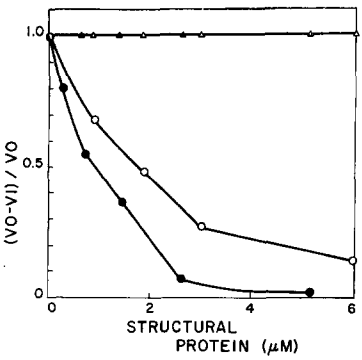

Fig. 4. Differential inhibition of malate dehydrogenase isozymes of porcine heart and Neurospora by homologous mitochondrial structural protein. ▲—▲, cytoplasmic malate dehydrogenase (Neurospora); ●—●, mitochondrial malate dehydrogenase (Neurospora); △—△, cytoplasmic malate dehydrogenase (porcine); ○—○, mitochondrial malate dehydrogenase (porcine). Purified enzymes were preincubated with homologous structural protein in phosphate buffer (50 mM, pH 7.0) at 25° for 10 min and assayed at 37° in 50 mM phosphate buffer, pH 7.4, containing 0.6 mM oxaloacetate and 0.1 mM NADH. Given concentration of structural protein is that in assay. At half-maximal inhibition, the weight ratio of structural protein to enzyme was about 50:1. Functional molecular weight of structural protein is taken to be 23 000 (refs. 20, 40).

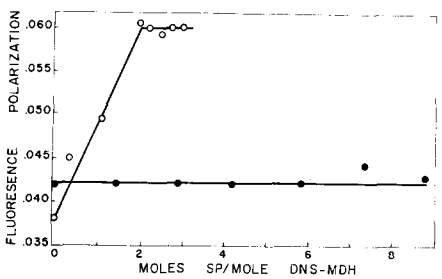

Fig. 5. Fluorescence polarization titration of DNS-labeled isozymes with mitochondrial structural protein of Neurospora. Activation at 370 nm, emission at 495 nm, 23°. DNS-enzyme (200 μg) in 2 ml 50 mM phosphate buffer (pH 7.4) containing 0.44 M sucrose was titrated with 50-μl aliquots of a solution of structural protein (SP) containing 70 μg protein per ml, 50 mM phosphate buffer (pH 7.4) and 0.44 M sucrose. ○, mitochondrial isozymes; ●, cytoplasmic isozyme. DNS-MDH, DNS-malate dehydrogenase.

At low concentrations (equimolar) of structural protein no inhibition or alteration of the Michaelis constant for malate of wild-type mitochondrial malate dehydrogenase was observed[44,45]; however, protein fluorescence polarization titration indicates an association of the proteins at a stoichiometry of near 1:1 and by the use of mutants of either the enzyme or structural protein, alterations in the Michaelis constant for malate were observed. Protein fluorescence polarization titration in the present experiments confirmed the association of the mitochondrial isozyme and structural protein, but failed to reveal association of the cytoplasmic form. To examine in greater detail the association and to obtain greater sensitivity in the measurements, fluorescence polarization titrations of DNS-labeled isozymes with structural protein were performed. As illustrated in Fig. 5, essentially the same relation as in protein fluorescence titration was obtained; namely, structural protein associates with the mitochondrial but not with the cytoplasmic isozyme. However, unlike the protein titration experiments of unmodified enzyme, here two moles of structural protein

associated with one mole of mitochondrial enzyme. The reason for this difference is unknown.

Electrophoretic mobilities and isoelectric points

Extracts of mycelial homogenates contain one active cytoplasmic form and three sub-forms of mitochondrial malate dehydrogenase after electrophoresis or isoelectric focusing in polyacrylamide gel. The isoelectric points from focusing and

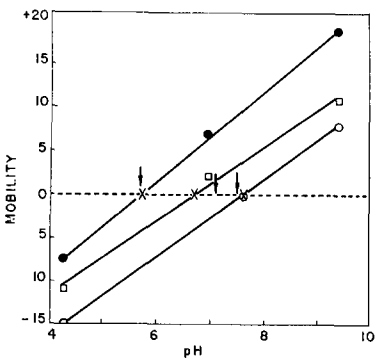

Fig. 6. Determination of isoelectric points of malate dehydrogenases by mobility measurements. ×, apparent isoelectric point from mobility measurement. Arrows indicate isoelectric point determined by isoelectric focusing. ●, cytoplasmic; □, mitochondrial-2; ○, mitochondrial-1.

mobility measurements were in good agreement (Fig. 6). The subcellular origin of the isozymes was identified by differential centrifugation of mycelial homogenates.

Previous analyses of the mitochondrial enzyme in starch zone electrophoresis indicated that the three sub-forms were simultaneously altered in charge in a malate assimilation mutant and that the three sub-forms were interconvertible[5]. Other studies (K. BENVENISTE AND K. D. MUNKRES, unpublished observations) of the mitochondrial sub-forms indicate they are members of an interconvertible oligomeric series differing in both charge and molecular weight and probably not "conformers" as previously suggested[5]. The cytoplasmic isozyme, in contrast to the mitochondrial isozymes, migrates as a single component in electrophoresis and isoelectric focusing and is a more acidic protein with isoelectric point near 5.6 (Fig. 6). The maximum difference between the isoelectric points of the cytoplasmic enzyme and the most acidic mitochondrial form ($pI = 7.0$) is thus 1.4 units. From isoelectric focusing and electrophoretic analyses of about twenty mutants with altered forms of cytoplasmic and mitochondrial malate dehydrogenases[15], the difference in isoelectric point is equivalent to two or more charged amino acid residues.

Other properties of malate dehydrogenase isozymes

Although all of the forementioned properties of the isozymes indicate they are clearly different proteins, analyses of the physico-chemical basis of these differences awaits obtaining the cytoplasmic isozyme in sufficient quantity and purity. Genetical analyse, however, reveal that the isozymes have polypeptide subunits in common[15]. Possible mechanisms of biological modification of genetically common polypeptides in the synthesis of the isozymes are discussed elsewhere[15].

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