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ALLOSTERIC AND MULTIFUNCTIONAL PROPERTIES OF NEUROSPORA MITOCHONDRIAL MALATE DEHYDROGENASE

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SUMMARY

1. The overall forward reactions catalyzed by mitochondrial malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) and aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) of Neurospora apparently proceeds in a concerted mechanism without exchange of the intermediate, oxaloacetate, at equilibrium when glutamate is present in excess.

2. Cofactors of the aminotransferase, pyridoxal phosphate and pyridoxamine phosphate, are non-competitive reversible inhibitors of malate dehydrogenase. L-Aspartate at low concentrations activates malate dehydrogenase and releases pyridoxal phosphate inhibition and at high concentrations serves as a substrate with oxaloacetate as product. Mutation of the structural gene encoding one of the subunits of malate dehydrogenase also causes decrease in specific activity of aspartate dehydrogenase.

3. These and previous studies are discussed in terms of a model of allosteric regulation of a potentially bifunctional enzyme of malate dehydrogenase and aspartate aminotransferase and possible metabolic shunts in aspartate and glutamate synthesis.

INTRODUCTION

Previous investigations of mitochondrial forms of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) and aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) of a number of diverse organisms in several laboratories have indicated an association of these activities of possibly greater significance than their catalysis of consecutive reactions. Since the nature of the pivotal metabolic regulation of the competing fates of the common intermediate, oxaloacetate, in respiration, amino acid synthesis, and gluconeogenesis is poorly understood, we are continuing investigation of the structural, functional and genetic relationships of these enzymes.

The present report describes the modulation of malate dehydrogenase activity by allosteric effectors, pyridoxal phosphate and L-aspartate, the appropriate ligands

of aspartate aminotransferase. In addition, aspartate dehydrogenase activity is found to be apparently associated with malate dehydrogenase. Moreover, the overall forward reaction with malate, NAD^+ and glutamate as substrates appears to be catalyzed in a concerted reaction without exchange of the intermediate, oxaloacetate.

MATERIALS AND METHODS

Chemicals

Pyridoxal phosphate, pyridoxamine phosphate, pyridoxine, α-ketoglutaric acid, L-aspartic acid, L-malic acid, L-glutamic acid, oxaloacetic acid and porcine malate dehydrogenase and aspartate aminotransferase were products of CalBiochem. NADH, NAD^+ (Grade III), $NADP^+$, NADPH (Type II) and 6-benzamido-4-methoxy-M-toluidine chloride (Grade III, Fast Violet B) were products of Sigma Chemical Company. DL-[3-^{14}C]Malate was from Volk Chem. Co. Acetyl pyridine NAD^+ was a product of Pabst Laboratories.

Enzyme preparations and assays

Neurospora wild-type (74A) and a malate mutant (M-20) were cultured and converted to mycelial acetone powder as previously described[1,2]. Mitochondrial malate dehydrogenase was purified 300–500-fold from acetone powder extracts and stored in buffer or the lyophilized state at −20° (refs. 1, 2). Simultaneous purification of malate dehydrogenase and aspartate aminotransferase were as previously described[3,4].

Continuous enzymatic rates of NADH oxidation or NAD^+ reduction were recorded in duplicate in microquartz cuvettes of 1 cm light path in a Gilford Model 2000 recording spectrophotometer at 37° at 340 nm, generally at 0–0.1 full scale absorbancy. First order rates were estimated between 15 and 75 sec after mixing enzyme with reaction mixtures. Pseudo second order kinetics within 15–75 sec were expressed as first order by tangential approximation.

Protein was determined by a macro- or micro biuret method[1].

Preparation of [3-^{14}C]oxaloacetate

3 μmoles of DL-[3-^{14}C]malate (12 μC/μmole) in 0.14 ml water were added to 0.43 ml of 0.1 M glycine–NaOH buffer (pH 10) containing 4.35 μmoles of 3-acetyl pyridine NAD and 0.1 mg crystalline porcine malate dehydrogenase and incubated for 10 min at 37°. From the absorbance at 360 nm and the extinction coefficient of $9.3 \cdot 10^{3}$*, 0.18 μmole of [3-^{14}C]oxaloacetate was formed. The sample was applied on a 26-cm wide strip of Whatman 3 MM paper in 10-μl aliquots and developed by descending chromatography for 17 h in water-saturated butanol–formic acid (95:5, by vol.). The radioactive oxaloacetate was located at the origin with a strip counter ([^{14}C]malate had an R_F of about 0.58). The radioactive oxaloacetate was eluted from the paper by descending capillary action with distilled water at 4° for 24 h. The eluate was stored at −5°. Specific activity was $7.2 \cdot 10^{6}$ counts/min per μmole and the yield was 0.1 μmole. Over 90% of the product was authentic oxaloacetate as judged by enzymatic assay with malate dehydrogenase by fluorimetric measurement of NADH oxidation.

* Pabst Laboratories Handbook, OR-18.

Design of equilibrium exchange experiments

Consider the following reaction equilibrium approached from left to right in the order given:

$$E + \text{L-malate} + \text{L-glutamate} + (\text{NAD}^+ + [3\text{-}^{14}\text{C}]\text{oxaloacetate}) \rightleftharpoons$$

$$E + \text{L-aspartate} + \alpha\text{-ketoglutarate} + \text{NADH} + \text{H}^+$$

where the reaction is initiated by the simultaneous addition of NAD^+ and [3-^{14}C]-oxaloacetate. The time at which equilibrium is reached will be defined as the time of cessation of net NADH formation.

The following conservation conditions define the system with respect to the intermediate oxaloacetate and NADH.

$$(O_f^*)_{t=0} - (O_f^*)_{t=E} = O_b^* \quad (1)$$

$$(O_{Tf})_{t=E} - (O_f^*)_{t=E} = O_f \quad (2)$$

$$(N)_{t=E} - (O_f)_{t=E} = O_b \quad (3)$$

where O and N are concentrations of oxaloacetate and NADH and starred superscripts are radioactive. Subscripts f and b indicate free and bound oxaloacetate. Since the enzyme concentration is infinitesimal with respect to oxaloacetate in the system, "bound" is essentially that oxaloacetate which reacts to form L-aspartate or L-malate. O_{Tf} indicates total free non-radioactive oxaloacetate. The concentrations at the time of initiation of the reaction and at equilibrium are indicated by $t = 0$ and $t = E$, respectively.

Protocol of equilibrium exchange experiments

Exact details are given in the legend of Table IV. The order of addition of reactants in these experiments is important since the half-reactions of malate dehydrogenase and aspartate aminotransferase must be excluded if possible. Enzyme was first incubated with pyridoxal phosphate in buffer. This is reasonable since aspartate aminotransferase alone may require preactivation with its cofactor[5,26]. Then L-glutamate and L-malate were added in large excess and incubated with enzyme and pyridoxal phosphate to saturate the enzymes. The choice of concentrations of L-glutamate and L-malate is dictated by the following considerations. The Michaelis constant of Neurospora aspartate aminotransferase for glutamate (with excess oxaloacetate) at pH 7.4 is 4 mM (ref. 5). We chose a concentration 100 times the Michaelis constant, 0.4 M. The Michaelis constant (malate) of Neurospora malate dehydrogenase is about 1 mM at pH 7.4 with excess NAD^+ (ref. 5). We take 100 times the Michaelis constant, 0.1 M. Finally NAD^+ (2.8 mM, $28 \times K_m$) and limiting radioactive oxaloacetate were added simultaneously to initiate the reaction. The choice of a limiting concentration of labeled oxaloacetate was made from the following considerations. Since the equilibrium constant of the malate dehydrogenase reaction alone is about $1 \cdot 10^{-5}$ at pH 7.4 the concentration of oxaloacetate at equilibrium with the given concentration of L-malate and NAD^+ would be 500 μM (ref. 5). The Michaelis constant of aspartate aminotransferase for oxaloacetate (at saturating glutamate) is 40 μM (ref. 5). Thus malate dehydrogenase is capable of saturating aspartate aminotransferase with [^{12}C]oxaloacetate. We added 2.2 μM (15 000 counts/min) of [^{14}C]oxaloacetate.

After initiating the reaction, the formation of NADH was followed to equilibrium at constant temperature at 340 nm against an appropriate blank control without enzyme. This control was also used later to determine if radioactive oxaloacetate was lost non-enzymatically. The total net absorbance due to NADH was noted and aliquots were removed for colorimetric and radioactive assays of oxaloacetate and analysis of ^{14}C incorporation into aspartate and malate.

Colorimetric assay of oxaloacetate

The method was adapted from the report of BABSON AND PHILLIPS[6] that Fast Violet Blue (6-benzamido-4-methoxy-M-toluidine chloride) forms a specific colored product with oxaloacetate but not with α-ketoglutarate. Samples (0.1 ml) of reaction mixtures, appropriate blanks, and oxaloacetate standards were added to 0.05 ml of an aqueous solution of the diazonium salt (5 mg/ml) at 80°, heated at 80° for 5 min, cooled on an ice-bath, diluted with 0.9 ml of 95% ethanol, and absorbancies at 650 nm were determined. The method detects accurately as little as $4 \cdot 10^{-11}$ moles oxaloacetate per ml. At 0.4–4 nmoles/ml, the respective absorbances are 0.642 and 1.016 and Beer's law is approximated. Suitable blanks of reagents, reaction mixtures without enzyme, and enzyme alone were also analyzed to correct for non-specific color.

Assay of Radioactivity

To an aliquot reacted with dye as above (before dilution with ethanol) was added 0.05 ml saturated lead acetate, 0.1 ml glacial acetic acid, and 0.2 ml ethanol. After storing overnight at 4°, the lead salt of the dye–oxaloacetate complex was collected and washed on a filter paper with water and then transferred to scintillation fluid for counting. Oxaloacetate recovery was about 93% with counting efficiency of about 25% in a Packard scintillation counter.

In the chromatographic system described above in the preparation of [^{14}C]-oxaloacetate, it remains at the origin, the R_F of malate is 0.50 and of L-aspartate 0.68. Unfortunately aspartate and glutamate were not resolved, so specific activities could not be obtained. Attempts to analyze specific activities with an automatic amino acid analyzer with split stream to a radioactive counter were not successful because of the massive quantities of glutamate relative to expected small quantities of aspartate. Therefore, a strip chart counter was employed and the ratios of radioactivity in oxaloacetate, aspartate, and malate were determined by cutting out the recorder tracings and integrating by weighing.

TABLE I

INHIBITION CONSTANTS OF B-6 COENZYMES ON NEUROSPORA MALATE DEHYDROGENASE

Reaction	K_i *(mM)**		
	Pyridoxine	*Pyridoxal phosphate*	*Pyridoxamine phosphate*
Reverse	0.36	0.167	0.076
Forward	1.1	0.71	0.48

* At pH 7.4 in 50 mM phosphate buffer at 37°.

RESULTS

Allosteric effects

Pyridoxal phosphate, pyridoxamine phosphate and pyridoxine are inhibitors of initial rates in either the forward or reverse reaction catalyzed by Neurospora mitochondrial malate dehydrogenase (Table I, Figs. 1, 2). No time-dependent reaction was observed by preincubation of enzyme with inhibitor prior to assay. Inhibition by pyridoxal phosphate and pyridoxamine phosphate was strictly non-competitive

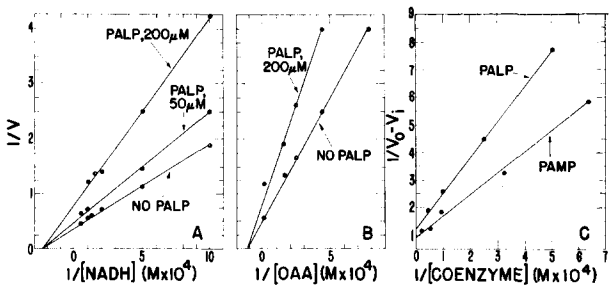

Fig. 1. A. Inhibition of mitochondrial malate dehydrogenase by pyridoxal phosphate (PALP) as a function of NADH concentration. Reverse reaction. Phosphate buffer (pH 7.4, 50 mM); oxaloacetate, (0.6 mM), 37°. B. Inhibition of mitochondrial malate dehydrogenase by pyridoxal phosphate (PALP) as a function of oxaloacetate (OAA) concentration. Conditions as in A. NADH, 0.1 mM. C. Inhibition of mitochondrial malate dehydrogenase by variable concentrations of pyridoxal phosphate (PALP) and pyridoxamine phosphate (PAMP) in the reverse reaction. Conditions as in A and B.

with substrates and the inhibition was reversible by dilution. Moreover, pyridoxamine phosphate was a more effective inhibitor than pyridoxal phosphate (Table I). In addition, inhibition by pyridoxine implies that neither the aldehyde nor phosphate functions are essential. However, the possibility remains that the three inhibitors act *via* different sites on the enzyme.

Pyridoxal phosphate inhibition was reversible by low concentration (0.7 mM) of L-aspartate. Moreover, L-aspartate alone at low concentrations served as an activator of malate dehydrogenase and at high concentrations served as a substrate (Tables II, III).

The differential inhibition of the forward and reverse reaction of malate

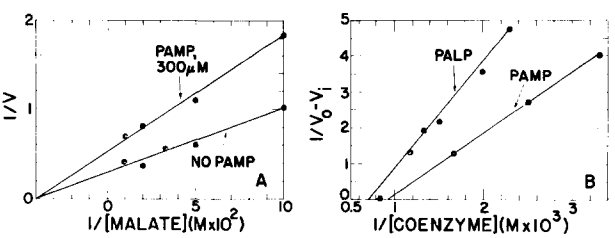

Fig. 2. A. Inhibition of mitochondrial malate dehydrogenase by pyridoxamine phosphate (PAMP) as a function of malate concentration. Forward reaction. Phosphate buffer (pH 7.4, 50 mM); NAD^+ (2.8 mM), 37°. B. Inhibition of mitochondrial malate dehydrogenase by variable concentrations of pyridoxal phosphate (PALP) and pyridoxamine phosphate (PAMP) in the forward reaction. Conditions as in A with 10 mM L-Malate.

TABLE II

ASPARTATE AND MALATE DEHYDROGENASE ACTIVITIES OF NEUROSPORA CELL EXTRACTS

Substrate	*Protein* (mg/ml)*	*$\Delta A_{340\ nm}$/min*	*Specific activity***
L-Aspartate (0.4 M)	0.03	0.012	3.90
	0.05	0.021	4.20
	0.10	0.037	3.58
			Av. 3.89
L-Malate (0.04 M)	0.0025	0.032	124
	0.005	0.065	126
	0.01	0.132	127
			Av. 125

* From acetone powder extracts (see MATERIALS AND METHODS).

** μmoles NADH formed per h per mg protein at 37° in a reaction mixture containing: 50 mM potassium phosphate (pH 8.2), 2 mg NAD^+, substrate and protein in a total volume of 1.0 ml in a cuvette of 1-cm light path.

dehydrogenase by pyridoxal phosphate may be significant for metabolic regulation. Thus the respective inhibition constants of the forward and reverse reactions were 0.71 and 0.167 mM and the nature of the inhibition curves results (at near 0.4 mM pyridoxal phosphate) in the complete inhibition of the initial rates of the reverse reaction with no effect on the forward reaction rate.

Although previous studies[1,7] of Neurospora malate dehydrogenase at pH 8.2 with L-malate indicated nearly normal Michaelis–Menten kinetics with perhaps a slight substrate activation at high concentration, the change in initial rates as a function of L-malate concentration at pH 6.6 reveals three plateaus in the range 0.1–10 mM malate. According to KOSHLAND[8] such kinetic anomalies may indicate the phenomenon of negative cooperativity of an allosteric enzyme.

TABLE III

DEFICIENCIES OF MALATE AND ASPARTATE DEHYDROGENASE ACTIVITIES IN A MALATE MUTANT

Enzyme		*Specific activity****		*Relative specific activity*****	
*Preparation**	*Source***	*Malate dehydrogenase*	*Aspartate dehydrogenase*	*Malate dehydrogenase*	*Aspartate dehydrogenase*
Cell extract	P	125	3.89	1.00	0.031
	M	62	1.98	0.50	0.016
		M/P × 100		50	52
Partially purified	P	1230	70	1.00	0.058
	M	500	48	0.41	0.039
		M/P × 100		41	67

* Extract from acetone powder and partially purified enzymes after $(NH_4)_2SO_4$ and ethanol fractionation.

** P, prototroph; M, malate mutant (M-20).

*** Specific activity by assay conditions of Table II.

**** Relative specific activity malate dehydrogenase (P) = 1.00.

Aspartate dehydrogenase

SHAW AND KOEN[9] first observed that electrophoretically homogeneous mitochondrial malate dehydrogenase of several animals exhibited L-aspartate dehydrogenase activity; however, the mechanism of the reactions was not explored. A possible mechanism is as follows:

$$\text{L-aspartate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NH_4^+ + NADH$$

Neither D-aspartate nor $NADP^+$ were substrates at pH 8.2 or 9.9 for the Neurospora enzyme. In the reverse and forward reaction catalyzed by malate dehydrogenase, no change of initial rates was observed by the addition of NH_4^+ (10 mM) at pH 7.6. Thus, the reversal of the reaction remains to be demonstrated.

Tentative identification and stoichiometry of oxaloacetate as a product of aspartate dehydrogenase was obtained by the following experiment. Malate dehydrogenase was incubated in a reaction mixture containing L-aspartate (80 mM), NAD^+ (2.8 mM) and 50 mM potassium phosphate (pH 7.6) at 37° for 1 h. The change in absorbance at 340 nm relative to a mixture lacking L-aspartate was 0.008 per min and was linear for at least 35 min. The final absorbance of NADH was 0.480 or equivalent to 80 nmoles of oxaloacetate formed. Quantitative colorimetric determination of oxaloacetate indicated that approx. 80 nmoles of oxaloacetate were formed.

TABLE IV

EQUILIBRIUM CONCENTRATIONS OF NADH, [^{12}C]OXALOACETATE, AND [^{14}C]OXALOACETATE IN THE CONCERTED REACTION OF MALATE DEHYDROGENASE AND ASPARTATE AMINOTRANSFERASE OF NEUROSPORA

Time (min)	*Protein (μg/ml)*	*Enzyme Preparation****	*Concentration***						K_1 O_b^*/O_f^*	K_2 O_b/O_f	K_2/K_1
			N	O_{Tf}	O_f^*	O_f	O_b^*	O_b			
0	0	Control	0	2.20							
20	0	Control	0	2.20	2.20						
	720	Step 1	212	4.40	1.28	3.12	0.92	209	0.72	67	93
	190	Step 2	216	4.20	0.53	3.67	1.67	212	3.15	58	18
	20	Step 3	167	4.20	0.75	3.45	1.45	164	1.94	48	25
	0.4	Porcine aspartate amino-transferase	0		0.70	0	1.50	0	2.1		

** The concentration symbols are defined in the text. Units are in μmoles/ml reaction mixture. Enzymes were diluted in a buffer containing sodium phosphate (50 mM, pH 7.0), 0.1% bovine serum albumin, 1 mM 2-mercaptoethanol and 30 μM pyridoxal phosphate at 4°. Diluted enzyme (0.1 ml) was added to 0.3 ml. of 0.1 M potassium phosphate buffer (pH 7.6) containing pyridoxal phosphate (30 nmoles) and incubated for 1 min at 25°. Then 0.4 ml of 1.0 M L-glutamate (pH 7.6) and 0.1 ml of 1.0 M L-malate were added. After incubation for 1 min at 25°, 0.2 ml of a solution of NAD^+ and [3-^{14}C]oxaloacetate were added to initiate the overall reaction. Incubation was at 25° for 20 min. The reaction was followed at 340 nm in a Gilford recording spectrophotometer. The composition of the mixture at time zero was enzyme; L-glutamate, 400 μmoles; L-malate, 100 μmoles; NAD^+, 2.8 μmoles; pyridoxal phosphate, 30 nmoles; phosphate buffer, 52 μmoles; [3-^{14}C]oxaloacetate, 2.2 nmoles, 15 000 counts/min in a total volume of 1.1 ml at a pH of 7.6. Enzyme was omitted from the control mixture. In the porcine aminotransferase assay, L-malate and NAD^+ were omitted.

*** The control was without enzyme. Steps refer to successive stages of simultaneous purification of enzymes, 1, extract; 2, $(NH_4)_2SO_4$; and 3, ethanol.

Some degree of specificity of the association of malate dehydrogenase and aspartate aminotransferase activities is indicated by the data in Table III in which a missense mutation of malate dehydrogenase[2] reduced the specific activity of the enzymes to nearly the same degree.

Concerted reaction of malate dehydrogenase and aspartate aminotransferase

The equilibrium concentrations of NADH, [^{12}C]oxaloacetate, and [^{14}C]oxaloacetate in the concerted forward reaction of malate dehydrogenase and aspartate aminotransferase are summarized in Table IV. Three enzyme preparations at successive stages of purification were employed in addition to controls for non-enzymic reaction and authentic crystalline porcine aspartate aminotransferase. The data from the controls indicate that no detectable non-enzymic reaction occurred and that the preparation of [^{14}C]oxaloacetate is an authentic substrate for porcine aspartate aminotransferase.

The exchange equilibrium constants of labeled and enzymically generated unlabeled oxaloacetate are expressed as the constants, K_1 and K_2. The expectation for independent enzyme reactions is $K_1 = K_2$, whereas for a concerted reaction, the expectation is $K_2 > K_1$. Obviously the latter result is obtained with all three preparations. Decline of the ratio, K_2/K_1, after purification is discussed below.

Ideally, a complete quantitative description of the equilibrium conditions requires specific activities not only of oxaloacetate but also aspartate and malate. Such measurements were technically unsuccessful for reasons described in MATERIALS AND METHODS. Thus a compromise analysis measured the relative proportions of radioactivity in these compounds after chromatography. Here at acid pH, unlike the measurements in Table IV at neutral pH, evidence for non-enzymic conversion of labeled oxaloacetate to aspartate was obtained, as may be expected from the well-known acid catalysis of the formation of aspartate from oxaloacetate and

TABLE V

RELATIVE PROPORTIONS OF RADIOACTIVITY IN INTERMEDIATE, PRODUCT, AND SUBSTRATE IN THE CONCERTED FORWARD REACTION OF MALATE DEHYDROGENASE AND ASPARTATE AMINOTRANSFERASE OF NEUROSPORA

*Sample**	*Relative proportions***				*Enzymically formed radioactive aspartate****
	Oxaloacetate	*Aspartate*	*Malate*	*Total*	
Control	59	23	0	82	0
Step 1	59	24	0	83	1
Step 2	52	25	0	77	2
Step 3	51	22	14	87	0
Porcine aspartate amino transferase	52	35	0	87	12

* As described in Table IV and MATERIALS AND METHODS.

** Estimated after chromatographic separation and strip chart recording of radioactivity (see MATERIALS AND METHODS).

*** After subtraction of non-enzymically formed aspartate in the control. Similar results were obtained in several experiments where the total amount of sample subjected to chromatography was varied. Malate values are corrected for a slight background of about 5 units which is due to streaking of aspartate in chromatography.

glutamate in the presence of pyridoxal phosphate and the acid chromatographic solvent (Table V)[10]. Nevertheless, after correction for non-enzymic reaction, the results are in agreement with the conclusion derived from the experiments in Table IV; that essentially little or no exchange occurred. Moreover, the results demonstrate that essentially no label appeared in either aspartate or malate, whereas porcine aspartate aminotransferase was enzymically active. The appearance of labeled malate with enzyme of purification Step 3 (Table V) and the lower K_2/K_1 values of purified enzyme of Step 2 and Step 3, relative to enzyme in acetone powder extract (Table IV) may reflect a relatively greater instability of aspartate aminotransferase, as previously observed[4].

DISCUSSION

The evidence indicating more than a simple chance association of mitochondrial malate dehydrogenase and aspartate aminotransferase was previously reviewed[3,4,11]. Frequently numerous purification procedures do not separate the two activities. For example, FASELLA *et al.*[12] purified the two enzymes simultaneously 2000-fold from cottonseed without achieving separation finally by gel electrophoresis. Moreover, a 200-fold simultaneous purification from Neurospora was described by MUNKRES[4]. KITTO *et al.*[13] challenged the evidence from purification data and pointed out that: (1) extraordinary conditions allow separation of the two enzymes of both chicken and Neurospora; and (2) chicken cytoplasmic malate dehydrogenase and aspartate aminotransferase exhibit different amino acid compositions. Unfortunately those authors did not examine other properties of the Neurospora mitochondrial enzymes. In the case of the Neurospora mitochondrial enzymes, MUNKRES[4] first demonstrated that a unique procedure would separate the enzymes, but such separated proteins were indistinguishable on the basis of immunoneutralization, peptide mapping and amino acid composition data. Because the aspartate aminotransferase activity was extraordinarily susceptible to irreversible inactivation in the absence of pyridoxal phosphate, in contrast to the behavior of animal aspartate aminotransferase, it was suggested that the two enzymes may be associated with polypeptide subunits in common and that differences in quaternary or secondary structure, perhaps provoked by pyridoxal phosphate, could account for the separation under certain conditions. This view is consistent with the observation of KITTO *et al.*[13] that gel filtration under their conditions yielded molecular weights of 97 000 and 60 000 for Neurospora aminotransferase and dehydrogenase. Since animal mitochondrial aminotransferase can exist[14,15] as active dimer (mol. wt. 120 000) or monomer (mol. wt. 60 000), and mitochondrial malate dehydrogenase exists in a variety of active forms of different molecular weights (refs. 1,16–19 and K. BENVENISTE AND K. D. MUNKRES, unpublished observations) and in view of the observation[3] that sucrose density gradient centrifugation of the Neurospora enzymes indicated the same molecular weight (54 000), it appears that there may be biological and structure–function relationships not recognized by KITTO *et al.*[13], at least in the case of Neurospora.

The genetic evidence is consistent with a model in which the Neurospora enzymes are related by common polypeptides. Thus, analysis of sixty malate assimilation mutants indicated mutation leads to a simultaneous reduction in specific activity of both enzymes[7]. Many of such mutant enzymes also exhibit altered kinetic

and stability properties[2,3,7]. On the basis of amino acid replacement, peptide mapping, and electrophoretic data[2,20], it is concluded that such mutants are missense types with respect to malate dehydrogenase.

Structural studies[21] of purified Neurospora mitochondrial malate dehydrogenase indicated that, under the conditions employed, the protein (mol. wt. 54 000) is a tetramer with sequentially nonidentical monomers of mol. wt. 13 500 in the form $\alpha\alpha\alpha\beta$. Subsequently, tetrameric forms of mitochondrial malate dehydrogenase from other sources have been described[17,22,23]. Although highly purified malate dehydrogenase of Neurospora under some conditions apparently lacks catalytic activity of aspartate aminotransferase, spectrofluorimetric titration data indicate that the enzyme and isolated α-subunit associate with pyridoxal phosphate in a stoichiometric relation of 1:1 with respect to the α-monomer[11]. Isolated β-subunit failed to bind pyridoxal phosphate. Conversely, a specific and unique association of NADH with the β-subunit was demonstrated. In addition spectrofluorimetric and spectrophotometric observations indicate the formation of ternary complexes: D-malate–NAD^+–β and L-aspartate–pyridoxal phosphate–α (K. D. MUNKRES, unpublished results). The foregoing observations suggest that the enzyme may be potentially a bifunctional protein with the active center of the transferase localized on the α-chains and the dehydrogenase being associated with the β-chain. Direct evidence in support of this model was found in the present isotopic competition experiments in equilibrium in the overall forward reaction. With partially purified preparations containing both enzyme activities, added [^{14}C]oxaloacetate did not exchange with enzymatically generated intermediate oxaloacetate and no radioactivity appeared in aspartate or malate.

Since the two half-reactions can be readily demonstrated *in vitro* in preparations with both activities as employed here, it is conceivable that the intracellular concentration of glutamate may serve to determine if the overall forward reaction from malate to aspartate is concerted, a possibility supported by the results of the present experiments where a large excess of glutamate was added. Thus, with low glutamate concentrations, oxaloacetate generated by malate dehydrogenase may be available for other purposes such as respiration or the synthesis of glutamate *via* citrate and the tricarboxylic acid cycle.

The foregoing results lead to the prediction that, as in the case of other bifunctional and multi-enzyme systems[24], the small ligands of one reaction may act as allosteric effectors in the other reaction(s). Such predictions are supported by the results presented here. Pyridoxal phosphate and pyridoxamine phosphate exhibit non-competitive inhibition of malate dehydrogenase activity that is reversible by dilution and low concentrations of L-aspartate. L-Aspartate alone activated malate dehydrogenase at low concentrations and at high concentrations serves as a substrate.

Vitamin B_6 derivatives may play a biochemical role not only in providing a reactive site to a large class of enzymes but also by affecting protein conformation[25]. In special cases of bifunctional and multi-enzyme systems, a B_6 ligand required for the reactive site of one active center may, *via* conformational change, serve as an allosteric effector of other reaction(s) at sterically remote active centers[8,24]. One mechanism of fine metabolic control of the relative rates of the half-reactions in the present multifunctional system could depend upon the effective intracellular concentrations of B_6. Thus, pyridoxal phosphate serves to activate Neurospora mitochondrial

aspartate aminotransferase under some conditions[5,26] and to inhibit mitochondrial malate dehydrogenase. However, there may be a multiplicity of small ligands which ultimately contribute to the regulation of oxaloacetate metabolism, such as the activation of malate dehydrogenase by aspartate, and other modulations by ATP[27–30], CoASAc[29], citrate[31], NH_4^+ (ref. 32), Ca^{2+} (ref. 17), and phospholipid[33]. Ultimately, precise definition of allosteric enzyme regulation, as first emphasized by MONOD *et al.*[34], requires physiological and genetic criteria. Therefore, the results of the present investigation suggest, but do not unequivocally define, B_6 derivatives and aspartate as effectors of mitochondrial malate dehydrogenase. Nevertheless, the present system, unlike many cases of presumed allosteric regulation deduced from *in vitro* studies with animals and higher plants, allows a physiological and genetical approach. For example, we note the feasibility of nutritional assays of malate dehydrogenase and aspartate aminotransferase *in vivo* with an oxaloacetate-requiring mutant[35,36], the classic studies of B_6 biosynthesis with B_6-requiring mutants[37] and B_6 antimetabolites[38], and the existence of mutants with partially defective malate dehydrogenase and aspartate aminotransferase[1,35,36].

As a consequence of the concept of allosteric regulation, it is emerging that not only the state of aggregation but also the specificity of an enzyme may be a function of the nature and concentration of effector ligands[24]. Recognition of this uncertainity principle may serve to clarify some of the uncertainity surrounding the molecular weight of malate dehydrogenase. Molecular weights ranging from 15 000 to 250 000 have been reported. Recent investigations of mitochondrial malate dehydrogenase of Lemna[17], beans[16,18], and porcine heart[19] indicate that a multiplicity of active components of various molecular weight may occur in a given preparation and that the molecular weight distribution may be modified by allosteric effectors. Similarly, recent investigations in our laboratory have revealed conditions yielding an oligomeric series of mitochondrial malate dehydrogenase with molecular weights ranging from 26 000 to at least 250 000 with 54 000 and 67 000 as modal[39]. Moreover, aspartate aminotransferase can apparently exist as active monomer and dimer[14,15]. Hence, in view of the flexibility in state of aggregation of the dehydrogenase and aminotransferase, structures with either or both activities can be readily accommodated. However, it is equally clear from the studies of KAPLAN AND CIOTTI[40] that malate dehydrogenases have evolved and from the genetic studies of Neurospora that even single missense mutations can markedly alter the properties of these enzymes. Thus, views about the allosteric properties and structure–function relationships of malate dehydrogenase and aspartate aminotransferase should be tempered by genetic considerations. Numerous exemplary multifunctional enzymes systems such as tryptophan synthetases[41], fatty acid synthetases[42], pyruvate oxidases[43], serve as cogent precedents.

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