Identification of an NAD(P)⁺-Dependent 'Malic' Enzyme in Small-Intestinal-Mucosal Mitochondria

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An NAD(P)⁺-dependent 'malic' enzyme is shown to be present in mitochondria from small-intestinal mucosa. The intracellular location, activity and regulatory kinetic properties of the enzyme suggest that it participates in the major energy-producing pathway for net oxidation of glutamine-derived tricarboxylic acid-cycle intermediates.

Glutamine, the most abundant plasma amino acid, is an important respiratory fuel of the small-intestinal mucosa (Finch & Hird, 1960; Neptune, 1965; Windmueller & Spaeth, 1978; Watford *et al.*, 1979). There is a net uptake of circulating glutamine by intestine in the rat (Windmueller & Spaeth, 1978), sheep (Wolff *et al.*, 1972), dog (Hills *et al.*, 1967) and man (Felig *et al.*, 1973), and experiments both *in vitro* and *in vivo* indicate that the absorbed glutamine is oxidized to CO₂ (Neptune, 1965; Windmueller & Spaeth, 1978). In order for net glutamine oxidation to occur, the mucosa must contain an enzyme(s) that converts glutamine-derived tricarboxylic acid-cycle intermediates into pyruvate. 'Malic' enzyme (EC 1.1.1.40), oxaloacetate decarboxylase (EC 4.1.1.3), and phosphoenolpyruvate carboxykinase (EC 4.1.1.32) plus pyruvate kinase (EC 2.7.1.40), were listed by Watford *et al.* (1979) as the three possibilities that could form pyruvate from tricarboxylic acid-cycle intermediates in the small intestine. 'Malic' enzyme is strictly NAD⁺-dependent and is localized in the cytosol (see below), and a cytosol oxaloacetate decarboxylase activity has recently been measured (Watford *et al.*, 1979). Both enzymes could contribute to pyruvate formation in the intestine. Phosphoenolpyruvate carboxykinase plus pyruvate kinase are probably not involved, since 3-mercaptopicolinic acid, an inhibitor of phosphoenolpyruvate carboxykinase, had no effect on the rate of alanine formation from glutamine (Hanson & Parsons, 1977; Watford *et al.*, 1979). In the present paper we show that an NAD(P)⁺-dependent 'malic' enzyme is present in small-intestinal mucosa of rat, calf, dog, chicken and human. The enzyme has regulatory kinetic properties, is located in the mitochondria, the site of formation of the glutamine-derived tricarboxylic acid-cycle intermediates, and is sufficiently active to support observed rates of glutamine-dependent pyruvate formation.

Materials and Methods

Male Sprague-Dawley rats (200–250 g) were purchased from Blue Spruce Farms, Altamont, NY,

U.S.A. Beagle dogs were obtained from the Cooperstown Beagle Dog Colony, Cooperstown, NY, U.S.A. Calf (Holstein) small intestine was obtained from a local slaughterhouse. Fumarate, L-malate, nicotinamide and adenine nucleotides, Tris and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffers were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bovine albumin (fraction V) was purchased from Miles Laboratories, Elkhart, IN, U.S.A.

Rat intestinal mucosa was prepared from the entire small intestines of six to eight male rats. Dog and calf mucosa were obtained from sections of duodenum and proximal jejunum. The human specimen was terminal ileum, removed during surgery for carcinoma of the caecum. There was no macroscopic evidence of tumour involvement. Procedures for collection of mucosa, preparation of the homogenate and isolation of the mitochondrial fractions were as described by Pinkus & Windmueller (1977), except that the homogenization medium contained 0.33 M-sucrose, 2 mM-dithioerythritol and 30 mM-Tris/HCl, pH 7.4; 1 g wet wt. of mucosa yielded 0.103 ± 0.004 (mean $\pm$ S.E.M., $n=21$) g of homogenate protein. The yield of twice-washed mitochondrial fraction was 41 ± 5.2 (mean $\pm$ S.E.M., $n=15$) mg of protein/g of homogenate protein. Preparation of the mitochondrial extracts, gel filtration on Sepharose CL-6B columns, and assay of the 'malic' enzyme and malate dehydrogenase activities that were eluted from the column were performed as described by Sauer & Dauchy (1978). A Gilford model 250 spectrophotometer (340 nm) and an Eppendorf photometer (334 nm) were used for the spectrophotometric measurements. Fractions containing NAD(P)⁺-dependent 'malic' enzyme (but no malate dehydrogenase) were pooled and concentrated to about one-tenth the original volume by ultrafiltration at 4°C (PM-10 membrane; Amicon Corp., Lexington, MA, U.S.A.).

The apparent Michaelis constants for malate, NAD⁺ and NADP⁺ were determined at 30°C in cuvettes that contained 50 mM-Tris/HCl, 0.1 mM-

dithioerythritol, 5 mM-fumarate (see below), 5 mM- MnSO_4 , 10 mM-L-malate, either 0.5 mM- NAD^+ or - NADP^+ , and a portion of enzyme, all at pH 7.3, final volume 1 ml. The concentrations of malate and of nicotinamide nucleotides were as above, except when they were variable substrates. The apparent Michaelis constants were derived from double-reciprocal plots of velocity against the malate or nicotinamide nucleotide concentrations. Total consumptions of malate and nicotinamide nucleotide were less than 1% of the initial concentrations, and the corrections suggested by Lee & Wilson (1971) were not made. Plots through the data points were determined by the method of least squares. Stock nicotinamide nucleotide and malate solutions were assayed enzymically (Klingenberg, 1965; Williamson & Corkey, 1969). The apparent Michaelis constants for NAD^+ and NADP^+ were determined fluorimetrically with the

Eppendorf photometer with fluorimetric attachment. Protein was measured by a biuret method (Szarkowska & Klingenberg, 1963), with bovine albumin as standard.

Results

Malate dehydrogenase and NAD^+ -dependent 'malic' enzyme activities interfere when present in the same solution, and the mitochondrial extracts were subjected to gel filtration on Sepharose CL-6B to separate 'malic' enzyme from malate dehydrogenase (Fig. 1). 'Malic' enzyme activity appeared as a single symmetrical peak when either NAD^+ or NADP^+ was the coenzyme, and the positions of the activity peaks were superimposable. Identical results were observed for the rat and calf mucosal mitochondrial extracts. No strictly NADP^+ -dependent

Fig. 1. Elution profile of NAD(P)^+ -dependent 'malic' enzyme and malate dehydrogenase activities in a dog small-intestinal-mucosal mitochondrial extract after gel filtration on Sepharose CL-6B

Mitochondrial extracts, prepared as described in the text, were subjected to gel filtration on 2.5 cm $\times$ 90 cm Sepharose CL-6B columns (130 ml void volume) equilibrated with 0.1 M-KCl/5 mM-Hepes/0.1 mM-EDTA/0.02% NaN_3 /0.5 mM-dithioerythritol (all at pH 7.3); 3 ml fractions were collected and were assayed for malate dehydrogenase and for 'malic' enzyme with either NAD^+ or NADP^+ as coenzyme (see the Materials and Methods section for the assay details). Malate dehydrogenase activity (Δ) and 'malic' enzyme activity with NAD^+ ($\circ$) and NADP^+ ($\square$) are given on separate scales. Total 'malic' enzyme activity (assumed to be 100%) was estimated by adding the activities of the fractions that comprised the 'malic' enzyme peak. These sums were 14195 nmol of NAD^+ reduced/min and 8096 nmol of NADP^+ reduced/min and were divided by 87.8 mg, the total mitochondrial protein from which the extract was derived. NAD(P)^+ -dependent 'malic' enzyme activity for this preparation was 161.7 and 91.9 nmol of NAD^+ and NADP^+ reduced/min per mg of mitochondrial protein respectively. The fractions comprising the peak of the 'malic' enzyme activity were pooled, as indicated by the horizontal arrows. The inset shows malate-saturation curves for the pooled dog NAD(P)^+ -dependent 'malic' enzyme and gives the dependence of activity (nmol of NAD^+ reduced/min; ordinate) on the malate concentration (mM; abscissa) in the cuvette. Measurements were made in the absence of modifiers ($\blacksquare$) or in the presence of 5 mM-fumarate ($\bullet$) or 1 mM-ATP ($\blacktriangle$).

Table 1. Activities and some apparent Michaelis constants of NAD(P)⁺-dependent 'malic' enzyme from small-intestinal-mucosal mitochondria

Activity and apparent Michaelis constants are expressed as means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses. Activity was measured as described in the legend of Fig. 1. The human enzyme was not active with NADP⁺.

Enzyme source	Activity (nmol of NADH or NADPH formed/min per mg)		Apparent Michaelis constants			
			Malate (mM)		NAD ⁺ (μ M)	NADP ⁺ (μ M)
	NAD ⁺	NADP ⁺	With NAD ⁺	With NADP ⁺		
Calf	54.2 $\pm$ 18.3 (3)	24.2 $\pm$ 7.5 (3)	0.93 $\pm$ 0.10 (3)	2.5 $\pm$ 0.52 (3)	64.2 $\pm$ 4.7 (3)	66.7 $\pm$ 9.9 (3)
Dog	151.6 $\pm$ 28.3 (3)	112.2 $\pm$ 35.1 (3)	0.75 $\pm$ 0.08 (3)	2.11 $\pm$ 0.08 (3)	44.3 $\pm$ 7.3 (3)	53.8 $\pm$ 8.7 (3)
Cat	61.6 $\pm$ 10.1 (9)	40.2 $\pm$ 6.7 (9)	0.67 $\pm$ 0.06 (8)	0.85 $\pm$ 0.07 (6)	15.6 $\pm$ 1.8 (5)	17.2 $\pm$ 2.0 (5)
Human	157.0 (1)	—	1.62 (1)	—	57.2 (1)	—

'malic' enzyme (Mandella & Sauer, 1975) was present in intestinal-mucosal mitochondria. Also, when the cytosol fractions (the supernatant fluid obtained after centrifugation of a portion of the homogenate at 105000g for 1 h) was subjected to this gel-filtration procedure, the cytosol isoenzyme of the strictly NADP⁺-dependent 'malic' enzyme was evident, but no NAD(P)⁺-dependent 'malic' enzyme activity was detected. The activities of the NAD(P)⁺-dependent 'malic' enzymes in rat, dog, calf and human small-intestinal-mucosal mitochondria are listed in Table 1. The highest activity was observed in the dog and human. There were marked differences in the relative activity of the enzymes with NAD⁺ and NADP⁺. The calf enzyme was less active with NADP⁺ than either the dog or rat enzymes and the human enzyme was essentially NAD⁺-dependent. The NAD(P)⁺-dependent 'malic' enzyme activity of a single mitochondrial preparation from chicken (White Leghorn) small-intestinal mucosa was 78.1 and 44.1 nmol of NAD⁺ and NADP⁺ reduced/min per mg of mitochondrial protein, respectively. All of the NAD(P)⁺-dependent 'malic' enzymes from intestinal mucosa were active with MgSO₄. For example, for the rat enzyme the ratio of activity with 5 mM-Mn²⁺ to that with 5 mM-Mg²⁺ was 1.47 with NAD⁺ as coenzyme and 1.59 with NADP⁺ as coenzyme.

Fractions were pooled, as indicated by the horizontal arrows in Fig. 1, to yield a 'malic' enzyme preparation that was essentially free of malate dehydrogenase activity. The inset in Fig. 1 shows malate-saturation curves obtained for pooled dog NAD(P)⁺-dependent 'malic' enzyme with NAD⁺ as coenzyme. As with the enzyme from other tissues (Sauer, 1973; Mandella & Sauer, 1975; Sauer & Dauchy, 1978), the activity of the dog mucosal enzyme was increased by fumarate and inhibited by ATP. Similar malate-saturation curves were observed for the rat, calf and human enzymes with both NAD⁺ and NADP⁺, except that the human enzyme was not active with NADP⁺. Apparent Michaelis constants for malate and nicotinamide nucleotides are shown in Table 1

and indicate that the four 'malic' enzymes are quite similar kinetically.

Discussion

Windmueller & Spaeth (1978) have demonstrated *in vivo* that the post-absorptive rat jejunum removed 220 nmol of glutamine/min per g of intestine from the arterial blood. Approx. 55% of the carbon atoms of the absorbed glutamine appeared in the portal blood as CO₂ and 12% as alanine plus lactate, indicating extensive oxidation and decarboxylation of glutamine-derived malate. We found that the mean activity of NAD(P)⁺-dependent 'malic' enzyme measured at 30°C was 61.6 nmol of NADH formed/min per mg of mitochondrial protein in rat intestinal mucosa (Table 1). Each 1 g of intestine yielded 0.6 g of mucosa (Pinkus & Windmueller, 1977), 61.8 mg of homogenate protein and 3.9 mg of mitochondrial protein, assuming 65% recovery of mitochondria (Pinkus & Windmueller, 1977). Since the NADH/pyruvate stoichiometry of the NAD(P)⁺-dependent 'malic' enzyme is 1:1 (Mandella & Sauer, 1975), the rat is capable of producing 240 nmol of pyruvate/min per g of intestine via this enzyme. Thus, even without considering the temperature differences at which the measurements were made, the activity of the NAD(P)⁺-dependent 'malic' enzyme is more than adequate to account for the observed rate of decarboxylation of glutamine-derived malate. It is not possible to tell from these data if catalysis by this enzyme is the preferred route of pyruvate formation rather than catalysis through the cytosol NADP⁺-dependent 'malic' enzyme or oxaloacetate decarboxylase. However, the mitochondrial location and the kinetic properties of the NAD(P)⁺-dependent 'malic' enzyme (stimulation by the malate precursor, fumarate, and inhibition by ATP, the product of oxidative phosphorylation) would appear to fit very well into a pathway designed for catabolism of glutamine as a respiratory fuel.

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