TISSUE SPECIFICITY OF MALATE DEHYDROGENASE* ISOZYMES. KINETIC DISCRIMINATION BY OXALOACETATE AND ITS MONO- AND DIFLUORO ANALOGUES.

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Received December 16, 1965

It was first reported by Davies and Kun (1) that oxaloacetate above 10^{-4}M causes marked substrate inhibition of mitochondrial malate dehydrogenase isolated in pure form from beef heart. Independently, Delbrück, Zebe and Bücher (2) made the same type of observation and also discovered that the cytoplasmic variety of malate dehydrogenase did not show this kinetic anomaly. The degree of substrate inhibition of the mitochondrial enzyme by oxaloacetate was subsequently found to be a function of NADH concentration (3); however, at fixed levels of NADH, substrate inhibition by oxaloacetate clearly distinguishes the two isozymes, since the cytoplasmic enzyme is not inhibited by any concentration of oxaloacetate at any level of NADH. Substitution of one or two H atoms by F in the oxaloacetate molecule profoundly changes the reactivity of the mono- or difluoro analogue towards enzymes for which oxaloacetate is a common substrate (3, 4, 5). While monofluoro oxaloacetate was found to be a potent inhibitor of malate dehydrogenases hitherto tested (3), the difluoro derivative served as a good substrate of both isozymes and exhibited no substrate inhibition towards either mitochondrial or cytoplasmic isozyme (3). Monofluoro oxaloacetate, similar to oxaloacetate, produces substrate inhibition towards the mitochondrial isozyme. The molecular basis of the catalytic differences between isozymes of malate dehydrogenases is unknown. In a broader sense, the biological significance of isozymes is equally obscure. For these reasons, we extended our studies

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to a kinetic comparison of isozymes extracted from various rat tissues. The application of mono- and difluoro oxaloacetates for comparative kinetic studies was of special significance, since these substrate analogues are being used for the study of enzyme regulatory mechanisms in multienzymes derived from various tissues of the rat (6). Two types of observations were made: a) kinetic differences were detected between malate dehydrogenase activities of cytoplasmic and mitochondrial extracts of the same tissue, and b) marked differences in catalytic activities were noticed between homologous enzyme preparations obtained from different tissues. This second observation is of particular interest since it represents a clear experimental example of a tissue specific selective effect of an enzyme inhibitor. Our results show that monofluoro oxaloacetate inhibits the enzymatic reduction of oxaloacetate and difluoro oxaloacetate in liver mitochondria, kidney cytoplasm and kidney mitochondria, but not in the cytoplasm of liver cells. It seems reasonable to predict that complex metabolic responses elicited by enzyme inhibitors (in multienzyme systems or in vivo) must in part be related to a selective recognition of the inhibitors by isozymes possessing different catalytic properties. It is proposed that extension of the type of kinetic analyses exemplified in this paper may contribute to the understanding of the molecular basis of tissue specific physiological responses, well-known to physiologists, yet unexplained in terms of biochemical mechanisms.

EXPERIMENTAL PROCEDURES. Cytoplasmic extracts of rat livers and kidneys were the supernatant fraction of homogenates (in 0.15 M KCl, containing 0.05% EDTA, pH 7.4) obtained by centrifugation at 144,000 x g for 1 hour. Identical kinetic results were obtained with supernatants of sucrose homogenates centrifuged for 1 hour at 144,000 x g (at $0^{\circ}C$). Mitochondrial extracts were prepared from isolated mitochondria, sedimented and washed twice from 0.25 M sucrose (containing 0.05% EDTA, pH 7.4), then disrupted in an ultrasonic disintegrator (cf. 3) in 0.15 M KCl + 0.05% EDTA, pH 7.4. The suspension of disrupted mitochondria was freed from particles by centrifugation (144,000 x g for 1 hour). Mitochondrial extracts obtained by this procedure contained no detectable NADH oxidase. Kinetic assays were carried out by following the disappearance of the absorption band of NADH in a Gilford multiple sample absorbance recorder. Protein was assayed by a colorimetric biuret method (cf. 3). With amounts of extracts employed

for kinetic tests, no oxidation of NADH could be detected in the absence of keto acid substrates.

RESULTS AND DISCUSSION. Kinetic data are summarized in Table I. The substrate behavior of the three keto acids is shown in the columns under V_{max}. These values are extrapolated apparent maximal velocities (obtained at 4×10^{-5}M NADH and directly read from double reciprocal plots) in terms of rates of changes of absorbance at 340 mμ per minute per mg protein at 22^{o}. The amounts of protein per optical test needed for comparable enzymatic rates are shown in the column "Protein/test." Cytoplasmic extract of rat liver reduces all three substrates at rates which are of the same order of magnitude. Monofluoro oxaloacetate, which in all other extracts at concentrations of 5×10^{-5} to 10^{-4}M almost completely inhibits the reduction of both oxaloacetate (competitively) and difluoro oxaloacetate (non-competitively), has no inhibitory effect on cytoplasmic extracts of liver. The rate of re-oxidation of NADH by cytoplasmic extracts of liver in presence of a mixture of fluoro oxaloacetates and oxaloacetate as substrate can be calculated from pre-determined kinetic constants (e.g., according to Thorn, ref. 7). Since these kinetic constants in the case of cytoplasmic extract of rat liver are not sufficiently dissimilar, for practical purposes monofluoro oxaloacetate does not function as an inhibitor in this system. It is striking that in all other extracts the inhibition constant of monofluoro oxaloacetate is almost exactly the same (2 to 7×10^{-6}) with respect to either oxaloacetate or difluoro oxaloacetate, even though the type of inhibition towards oxaloacetate is competitive and towards the difluoro analogue is non-competitive The catalytic differences between malate dehydrogenase activities of cytoplasmic and mitochondrial extracts can be accentuated by expressing the ratios of apparent maximal velocities (Table II) obtained with various keto acid substrates. It is apparent that mitochondrial enzymes tend to resemble each other, although the numerical differences of the ratios OAA/F_1 and OAA/F_2 are significant between various isozymes. Cytoplasmic extracts of kidney differ kinetically from those of liver.

TABLE I

Tissue	Substrate	Cytoplasm				Mitochondrion			
		V_{max}	K_m	K_i (F_1OAA)	Protein / test	V_{max}	K_m	K_i (F_1OAA)	Protein / test
Liver	F_1OAA	2.5	4×10^{-4}	--	10 µg	0.23	5×10^{-5}	--	140 µg
	F_2OAA	4.16	5×10^{-3}	no inhib.	20 µg	6.4	2.5×10^{-3}	7×10^{-6}(nc)	14 µg
	OAA	5.55	3×10^{-5}	no inhib.	10 µg	22.7	2×10^{-5}	2×10^{-6}(c)	5.6 µg
Kidney	F_1OAA	1.33	1.6×10^{-4}	--	75 µg	0.85	1×10^{-4}	--	90 µg
	F_2OAA	6.65	5×10^{-3}	2×10^{-6}(nc)	7.5 µg	17.0	5×10^{-3}	3×10^{-6}(nc)	2.2 µg
	OAA	18.0	3×10^{-5}	2×10^{-6}(c)	7.5 µg	123.0	1×10^{-4}	5×10^{-6}(c)	2.2 µg

Spectrophotometric tests were carried out in 1 ml final volume of reactants, containing 0.05 M phosphate buffer of pH 7.4. Light path = 1 cm. NADH concentrations = 4×10^{-5}M. Initial reaction velocities were read from direct chart recordings, extrapolated to 0 time. Reactions were started by addition of enzyme (10 - 30 µl).

F_1OAA = monofluoro oxaloacetate; F_2OAA = difluoro oxaloacetate; OAA = oxaloacetate.

(c) = competitive; (nc) = non-competitive.

TABLE II

Preparation	V_{max} RATIOS		
	$\frac{OAA}{F_1}$	$\frac{OAA}{F_2}$	$\frac{F_2}{F_1}$
Liver mitochondria	100	3.5	28
Kidney mitochondria	145	7.2	20
Liver cytoplasm	2.2	1.3	1.6
Kidney cytoplasm	14	2.7	5.0

Since in the present studies unpurified extracts of cytochemically characterized cellular fractions were employed, it was of importance to examine the possibility of cross-contamination of extracts by both isozymes, which could have occurred during tissue homogenization. The following arguments were considered: a) Kinetic differences were established by V_{max} ratios obtained with three different substrates on the same preparation. These ratios (Table II) were remarkably constant from preparation to preparation and were very sensitive to artificial contamination (i.e., to addition of cytoplasmic extract to mitochondrial ones or vice versa). The V_{max} obtained by artificial mixtures is identical with that calculated from known kinetic constants of each preparation separately. This type of observation suggests, but does not fully predict, the realness of the observed kinetic differences. b) The differences between mitochondrial and cytoplasmic isozymes is not only quantitative, but qualitative with respect to substrate inhibition by oxaloacetate and monofluoro oxaloacetate. In the following experiment velocities of reactions were determined at 10^{-4} and 10^{-3}M oxaloacetate. The enzyme activity of mitochondrial extracts (expressed as specific activity) was inhibited 50-60% by 10^{-3}M oxaloacetate (at 4×10^{-5}M NADH concentration), as compared to the velocity determined at 10^{-4}M oxaloacetate. This value is identical to that obtained with a homogenous mitochondrial enzyme (cf. 1). When increasing amounts of cytoplasmic extract were added, the substrate inhibition progressively diminished (see Table III). This decrease of substrate inhibition due to contamination with cytoplasmic

TABLE III

Tissue	Mitochondrial extract μg protein	Cytoplasmic extract μg protein	S.A. at 10^{-4}M OAA	S.A. at 10^{-3}M OAA	% Substrate inhibition
Kidney	1.5	--	65	32	51
	--	1.8	11.1	11.1	0
	1.5	1.8	37	22	40
	1.5	7.2	17	13	23.5
Liver	4.7	--	15	7	53
	-	3.0	4.8	4.8	0
	4.7	3.0	10.2	7.2	29
	4.7	9.0	6.9	6.3	8.7

S.A. = specific activity, i.e., μmoles of substrate reduced per mg protein per minute, determined in mitochondrial or cytoplasmic extracts alone or in mixtures as shown in the first two vertical columns.

Experimental conditions were the same as described in legend of Table I.

enzyme strongly suggests that constancy of a maximal substrate inhibition (at a given NADH concentration) in mitochondrial extracts is an intrinsic property of the mitochondrial enzyme. In addition, the magnitude of inhibition by 10^{-3}M oxaloacetate is identical to that observed with the pure mitochondrial enzyme (cf. 1). Therefore, this kinetic parameter actually rules out any significant contamination of mitochondrial extracts by the cytoplasmic isozyme. It is noteworthy that commercially available malate dehydrogenase preparations and various reportedly homogenous preparations of malate dehydrogenase obtained by other workers show varying degrees of substrate inhibition by oxaloacetate. These enzyme proteins were isolated either from whole tissue homogenates or cytochemically less precisely defined starting material (cf. 9).

c) A sensitive test of damage of mitochondria is the appearance of traces of glutamate dehydrogenase activity in the cytoplasmic fraction of homogenates. No glutamate dehydrogenase activity could be detected in cytoplasmic extracts even with undiluted preparations. Since the sensitivity of detection of this enzyme is equal to that of malate dehydrogenase (measured in presence of 10^{-4}M ADP, 10^{-2}M α-ketoglutarate, 0.1 M NH_4^+ and 5 x 10^{-4}M NADH, cf. 8), the preparative cytochemical separation of intact mitochondria can be considered satisfactory.

Molecular properties of pure malate dehydrogenase, obtained from various cytochemically characterized cellular fractions of animal tissues, as related to the observed kinetic differences are under further investigation.

ACKNOWLEDGMENTS. This work was supported by grants of the USPHS (RO1-CA-07955-01 and RO1-HD-01239-09) and of the NSF (GB-3488). We wish to acknowledge travel support from the U.S. Committee on International Exchange of Persons for Dr. Volfin.

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