Studies with Specific Enzyme Inhibitors

XI. Identification of Tissue-Specific Metabolic Organization of Mitochondria by Monofluorocitrate

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SUMMARY

Rates of O_2 uptake of mitochondria vary depending on the nature of added substrate, on assay conditions, and on the tissue from which mitochondria are isolated. Inhibition of aconitase by fluorocitrate influenced O_2 uptake and citrate accumulation in a highly selective manner. Metabolic effectivity of fluorocitrate was dependent on the same variables which determine specific metabolic rates in the absence of the inhibitor. It is proposed that the experimental design applied detects metabolic consequences of tissue-specific control mechanisms.

INTRODUCTION

According to generally accepted biochemical concepts, inhibition of the same enzymic component of ubiquitous multienzyme systems should have identical metabolic consequences. If this assumption is correct, tissue specificity of metabolic effects of enzyme inhibitors should be predictable from catalytic differences which may exist between homologous enzymes isolated from various tissues.

Previous studies carried out with the aid of fluoro oxalacetates showed that marked kinetic differences exist between malate dehydrogenases of cytoplasmic and mitochondrial origin (1). However, kinetic properties of malate dehydrogenases isolated from the same subcellular fractions of various tissues were similar. Despite this similarity, the inhibitory effect of fluoro oxalacetate was quite different on the metabolism of isolated mitochondria prepared from various tissues of the same animal (2), indicating that the metabolic rate limiting role of malate dehydrogenase in various types of mitochondria is not the same. Tissue-specific differences in metabolic rate limiting roles of functionally integrated enzymes are a sensitive measure of specific multienzymic organization within isolated mitochondria and may be considered a biochemical expression of cytodifferentiation (cf. 3).

The present report deals with the experimental detection of variations in the rate-limiting role of aconitase in mitochondria of different tissue origin. It has previously been reported from this laboratory that fluorocitrate formed enzymically from fluoroacetyl-CoA and oxalacetate is the only inhibitory isomer of this fluoroacid which acts preferentially on aconitase and (to a lesser extent) on succinic dehydrogenase (4). The aconitase inhibitory isomer, tentatively identified as the *erythro*-2*S*,3*R* enantiomer (for definition see ref. 5), inhibits aconitase by a complex mechanism consisting of a competitive interaction with citrate (determined by initial rate meas-

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urements) and a noncompetitive, irreversible, and time-dependent interaction with the enzyme protein (cf. 4). The irreversible nature of this inhibition is of particular importance because, on account of this reaction, the aconitase system in various types of mitochondria can be selectively inactivated by fluorocitrate and its rate-limiting role can be determined with great precision.

METHODS

Experimental methods for the determination of initial rates of O_2 uptake (by O_2 electrode) were the same as previously described (2). An additional method, the manometric measurement of O_2 uptake and citrate accumulation (cf. 4), was simultaneously employed. This combination of techniques provided a direct comparison of metabolic rates obtained under significantly different conditions. Uptake of O_2 measured within 2–3 min after addition of substrates is an indicator of metabolism more directly related to activities of primary enzymes acting on added substrates and substrate pairs. On the other hand, under pseudo-steady state conditions (cf. 6) created by the Warburg technique, metabolic rates are followed over a period of 30 min. During this period, O_2 uptake is due to the simultaneous oxidation of various substrates derived from the initially added primary substrate, a condition which itself may alter the rate limiting role of individual enzymic components of the system.

Rates of O_2 uptake and citrate accumulation (determined in the manometric system only) were expressed in the same units (μmoles/g protein/30 mins). Composition of the polarographic system was identical with that previously described (2). The main compartment of Warburg respirometer flasks was composed (in 3 ml final volume) of 150 mM KCl, 25 mM phosphate (pH 7.4), 2.3 mM ADP, 2.5 mM $MgCl_2$, 10 mM substrates and mitochondria (10 mg protein). Gas phase was air, CO_2 was absorbed by KOH, and the temperature of both manometric and polarographic systems was 30°. Citrate analyses were performed by a modified pentabromoacetone procedure suitable for analysis of citrate in presence of fluorocitrate (cf. 4). Crystalline synthetic fluorocitrate cyclohexylammonium salt was prepared as described earlier (cf. 4). The free acid was obtained by ion exchange.

RESULTS

Several types of conclusions can be derived from results summarized in Tables 1 and 2. (a) There is a complicated relationship, exhibiting substrate and tissue-specific variations, between initial rates of O_2 uptake and rates measured under pseudo-steady state conditions. Liver mitochondria metabolize certain substances (glutamate, α-oxoglutarate, succinate, malate plus pyruvate) at a faster rate under initial rate conditions than in the pseudo-steady state system. The oxalacetate-pyruvate substrate couple is metabolized by liver at nearly the same rate in both systems. On the other hand, pyruvate, malate, and oxalacetate are oxidized at a faster rate in the manometric systems (malate and oxalacetate do not raise O_2 uptake above endogenous levels in the polarographic systems). Kidney mitochondria exhibit metabolic behavior opposite to that of liver mitochondria with respect to glutamate as substrate, in agreement with the mechanism proposed earlier (7). Rates of oxidation of malate and oxalacetate by mitochondrial systems of kidney and liver exhibit opposite trends in the polarographic and manometric systems.

There are also marked differences in specific rates of O_2 uptake between various types of mitochondria exposed to the same substrates which cannot be explained simply by differences in enzymic composition (e.g., α-oxoglutarate is oxidized four times faster by kidney mitochondria than by liver, although oxoglutarate dehydrogenase content is of the same order of magnitude in extracts of both mitochondria. Similarly, the capacity of the electron transfer system between succinate and O_2 is not very much different in these two types of mitochondria).

Comparing various substrates of brain

TABLE 1

Effect of fluorocitrate in presence of glutamate, α-oxoglutarate, pyruvate, and succinate

Mitochondrial source and experimental conditions[a]	Glutamate			α-Oxoglutarate			Pyruvate			Succinate		
	O_2'	O_2''	C	O_2'	O_2''	C	O_2'	O_2''	C	O_2'	O_2''	C
Liver												
Endogenous	120	—	—	—	—	—	—	—	—	—	—	—
+ S	480	252	0	460	330	0	60	132	130	10,000	497	0
+ S + F-Citr.	270	371	79	170	263	73	60	186	130	6,000	552	76
Difference (%)	−44	+47	∞	−63	−20	∞	0	+40	0	−40	+10	∞
Kidney												
Endogenous	270	—	—	—	—	—	—	—	—	—	—	—
+ S	350	620	5	1740	855	25	0	60	0	15,000	1900	24
+ S + F-Citr.	75	365	50	340	870	100	0	10	0	12,000	1700	210
Difference (%)	−80	−41	1000	−80	0	+400	0	−84	0	−20	−10	+900
Brain												
Endogenous	80	—	—	—	—	—	—	—	—	—	—	—
+ S	120	450	0	160	284	0	130	162	0	320	450	5
+ S + F-Citr.	35	240	26	40	250	0	50	99	0	20	300	60
Difference (%)	−71	−47	∞	−75	(−8)	0	−61	−39	0	−94	−33	+1200
Heart												
Endogenous	700	—	—	—	—	—	—	—	—	—	—	—
+ S	1300	1033	0	1300	1148	0	100	81	0	600	1348	0
+ S + F-Citr.	1300	1082	0	1300	1227	0	100	34	0	600	694	70
Difference (%)	0	0	0	0	0	0	0	−58	0	0	−49	∞

[a] Abbreviations: Endogenous = no added substrate (0 = added substrate does not raise O_2 uptake). O_2' = initial rates (polarography). O_2'' = rates measured in the Warburg apparatus. S = rates in presence of substrates. S + F-Citr. = rates in presence of substrates $+6 \times 10^{-4}$ M fluorocitrate. C = accumulation of citrate (in μmoles/1 g protein/30 min).

mitochondria, the malate-pyruvate substrate couple is oxidized at the highest specific rates, which are the same under both initial rate and pseudo-steady state conditions. Heart mitochondria behave qualitatively similarly to brain mitochondria with respect to maximal specific rates measured in the polarographic system with the malate-pyruvate couple. However, the rate of O_2 uptake in presence of heart mitochondria metabolizing malate-pyruvate couple is only one-half in the manometric system of that determined in the polarographic system. There is a relatively low rate of succinate oxidation by heart mitochondria measured under initial rate conditions. This rate is much greater in the manometric system than in the polarographic one. It is well known that heart mitochondria contain perhaps the most active succinoxidase system (8); yet under initial rate conditions liver and kidney mitochondria oxidize succinate at rates at least 10 times greater than heart mitochondria. Under pseudo-steady state conditions succinate oxidation by kidney and heart mitochondria is nearly the same. Heart mitochondria oxidize glutamate and α-oxoglutarate at nearly the same rate in both experimental systems, but exhibit much lower rates in the manometric system when malate, oxalacetate, malate plus pyruvate, or oxalacetate plus pyruvate is the added substrate.

(b) Comparison of rates of citrate accumulation without added fluorocitrate demonstrates readily visible metabolic differences between various types of mitochondria. Liver mitochondria synthesize excess citrate only in the presence of pyruvate and malate as single added substrates and, to a lesser extent, from malate plus

TABLE 2
Effect of fluorocitrate in presence of malate, oxalacetate, and substrate couples

Mitochondrial source and experimental conditions[a]	Malate			Oxalacetate			Malate + pyruvate			Oxalacetate + pyruvate		
	O_2'	O_2''	C	O_2'	O_2''	C	O_2'	O_2''	C	O_2'	O_2''	C
Liver												
Endogenous	120	—	—	—	—	—	—	—	—	—	—	—
+ S	0	185	126	0	117	0	480	266	86	230	209	50
+ S + F-Citr.	0	185	170	0	20	139	300	283	200	230	177	116
Difference (%)	0	0	+35	0	−83	∞	−38	0	+130	0	−15	+130
Kidney												
Endogenous	270	—	—	—	—	—	—	—	—	—	—	—
+ S	790	560	50	565	280	40	1740	910	200	890	470	20
+ S + F-Citr.	365	370	250	0	0	200	1740	775	410	0	85	350
Difference (%)	−54	−34	+500	−100	−100	+500	0	−15	+100	−100	−82	+1750
Brain												
Endogenous	80	—	—	—	—	—	—	—	—	—	—	—
+ S	160	150	10	80	116	9	500	540	110	240	390	40
+ S + F-Citr.	60	150	80	0	69	62	500	400	325	0	100	85
Difference (%)	−62	0	+800	−100	−30	+700	0	−26	+200	−100	−75	+100
Heart												
Endogenous	700	—	—	—	—	—	—	—	—	—	—	—
+ S	1100	558	0	1300	122	0	2300	1413	40	1800	543	70
+ S + F-Citr.	600	285	60	1300	180	75	2300	963	160	1800	286	100
Difference (%)	−54	−50	∞	0	+48	∞	0	−31	+400	0	−47	+43

[a] Abbreviations: Endogenous = no added substrate (0 = added substrate does not raise O_2 uptake). O_2' = initial rates (polarography). O_2'' = rates measured in the Warburg apparatus. S = rates in presence of substrates. S + F-Citr. = rates in presence of substrates $+6 \times 10^{-4}$ M fluorocitrate. C = accumulation of citrate (in μmoles/1 g protein/30 min).

pyruvate and oxalacetate plus pyruvate substrate couples. There is a smaller but more general net synthesis of citrate from various substrates (except from pyruvate) by kidney mitochondria, reaching a maximal specific rate in presence of malate plus pyruvate substrate couple. Brain mitochondria also synthesize citrate at maximal rates from the malate-pyruvate substrate couple, but accumulate no citrate from glutamate, α-oxoglutarate or pyruvate as added substrate. Low rates of citrate accumulation by heart mitochondria are in sharp contrast to high rates of O_2 uptake, particularly because there are large quantities of citrate-synthesizing enzyme in this tissue (9). Only malate plus pyruvate and oxalacetate plus pyruvate substrate couples yield significant amounts of net citrate accumulation in heart mitochondria. It should be mentioned that in each case citrate was determined in the total volume of incubation mixtures; thus the sum of extra- and intramitochondrial citrate was analyzed.

(c) Respiration after inhibition of aconitase by fluorocitrate is the measure of the metabolic capacity of potential bypass

mechanisms in various mitochondria. There is no universal direct relationship between the inhibitory effect of fluorocitrate on O_2 uptake and its effect on rates of citrate accumulation. This paradoxical phenomenon is particularly noticeable in the case of liver mitochondria with glutamate, pyruvate, succinate, malate, and pyruvate plus malate as substrates (whereas in the manometric system O_2 uptake actually increases in some instances in presence of fluorocitrate). Similar effects of fluorocitrate were recorded with kidney mitochondria oxidizing α-oxoglutarate or succinate. Fluorocitrate augmented citrate accumulation 4-fold (with α-oxoglutarate as substrate) or 9-fold (in presence of succinate) without markedly diminishing O_2 uptake under pseudo-steady state conditions. Oxidation of α-oxoglutarate by kidney mitochondria under initial rate conditions is strongly inhibited by fluorocitrate, but this effect vanishes with time, indicating some compensatory rearrangement in the metabolic organization of the system.

It is of interest to note that in absence of fluorocitrate certain substrates in certain types of mitochondria always yield a net accumulation of citrate (e.g., in liver, pyruvate and malate), while the same substrate (i.e., pyruvate) in kidney, brain, or heart mitochondria causes no net synthesis of citrate, even in presence of fluorocitrate.

DISCUSSION

The experimental model applied defines the problem of tissue specific metabolic organization of isolated mitochondria in the following terms: (a) Rates of metabolism of well-known substrates markedly differ in mitochondria isolated from various tissues. Furthermore, these large differences in reaction rates show time-dependent variations (compare initial rates with those measured under pseudo-steady state). (b) Net accumulation of citrate from certain substrates is dependent on the tissue origin of mitochondria. (c) Inhibition of the aconitase system by fluorocitrate does not cause a universal cessation of mitochondrial metabolism. The effects of fluorocitrate on O_2 uptake and citrate accumulation depend entirely on the mitochondrial type and on the substrate presented to mitochondria.

This metabolic picture is incompatible with the operation of an obligatory consecutive multienzymic system which is controlled by identical factors in all types of mitochondria. As also shown previously (2), the present results reinforce the conclusion that inhibition of one segment of the metabolic system does not abolish the oxidation of various substrates, as it should if compulsory sequential metabolic organization of individual enzymes existed. Mitochondria are more correctly described in terms of enzymic organization by a branched or network system, capable of providing alternative pathways for a given substrate. Specific metabolic organization of various types of mitochondria is noticeable through the predominance of one among several possible pathways for a given substrate. Various products, not only CO_2 and H_2O, accumulate. The nature and quantity of products formed from the same precursors may be different, depending on the tissue origin of mitochondria.

Exposure of mitochondria to various substrates simulates physiological (i.e., nutritional) variations on a mitochondrial level. No attempts were made at this time to determine the enzyme chemical (or other) mechanisms responsible for the complex observations recorded here and earlier (cf. 2). The purpose of these experiments was to design experimental models capable of detecting metabolic specificity of isolated mitochondria. Further studies concerned with the molecular mechanisms responsible for metabolic differences are dependent on availability of these models. It is of interest that the inhibitory effect of fluorocitrate on aconitase was generally assumed to result in cessation of major metabolic activities of mitochondria (cf. 10). This assumption prevailed even though unexplained large variations of citrate accumulation in various tissues (cf. 10) were found after fluoroacetate poisoning. Metabolic specificity defined in terms of specific multienzymatic organization obviates the dilemma posed

by assumed universal biochemical mechanisms and known specific physiological functions of various tissues.

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