Biochimica et Biophysica Acta, 370 (1974) 564–572

BBA 67359

CITRATE AS A REGULATOR OF ACETYL-CoA METABOLISM IN LIVER MITOCHONDRIA

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(Received May 6th, 1974)

SUMMARY

1 The reversibility of citrate synthesis and the effects of citrate and fluorocitrate on citrate synthesis in whole mitochondria have been investigated

2 Cleavage of citrate to oxaloacetate (trapped as malate) and acetyl-CoA (trapped as acetylcarnitine) in whole liver mitochondria can be demonstrated The maximum rate of this reversed reaction is at most 1/40 of the maximum rate of citrate synthesis

3 Citrate and fluorocitrate are competitive inhibitors with respect to oxaloacetate of purified pig heart synthase The K_i for both compounds was found to be approx 1 5 mM

4 Both citrate and fluorocitrate inhibit citrate synthesis and increase ketogenesis in whole liver mitochondria They probably act as competitive inhibitors with respect to oxaloacetate, since malate counteracts this inhibition

5 For kinetic reasons it is concluded that the citrate synthesis reaction probably never approaches equilibrium in the intact tissue The importance of citrate in the regulation of citrate and ketone body formation in the liver is discussed

INTRODUCTION

Since citrate synthase (EC 4 1 3 7) represents the enzyme admitting acetyl groups into the citric acid cycle, the kinetic properties of this enzyme have been widely investigated Numerous inhibitors have been found and suggested as physiological regulators Such regulators may be of particular importance in the liver where a lowered rate of citrate synthesis will increase ketogenesis

It has also been suggested that citrate synthase is an equilibrium enzyme and that the acetyl-CoA/CoA ratio of mitochondria can be calculated from the citrate/oxaloacetate ratio and the equilibrium constant [1]

Recently we have observed that fluorocitrate and citrate inhibit citrate synthesis and increase ketogenesis in liver mitochondria [2] These findings may be explained by the fact that citrate is a competitive inhibitor with respect to oxaloacetate

Abbreviation HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

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in citrate synthesis [3, 4], and/or by a displacement of equilibrium In both cases a higher acetyl-CoA/CoA ratio and increased ketogenesis will result

In the present paper we have checked the reversibility of citrate synthesis in liver mitochondria and the effect of citrate on citrate synthesis and ketogenesis from different acetyl-CoA precursors The results are discussed in relation to the properties of citrate synthase

MATERIALS AND METHODS

Malate dehydrogenase (EC 1 1 1 37), pig heart citrate synthase (EC 4 1 3 7), carnitine acetyltransferase (EC 2 3 1 7), citrate lyase (EC 4 1 3 6), β-hydroxybutyrate dehydrogenase (EC 1 1 1 30), glutamate dehydrogenase (EC 1 4 1 2), NAD, NADH, CoA and rotenone were all obtained from Sigma Chemical Co , St Louis, Mo , U S A Fluorocitrate, barium salt, was obtained from Calbiochem It was converted to the sodium salt by addition of excess Na_2SO_4 prior to use

(−)Carnitine and (−)acetylcarnitine were gifts from Otsuka Pharmaceutical Factory, Tokushima, Japan (−)Palmitycarnitine was prepared according to Bremer [5]

Mouse liver mitochondria were prepared by homogenizing the livers (usually five livers) in approx 10 vol of 0 25 M sucrose in a Potter–Elvehjem homogenizer with a Teflon pestle The cell debris and nuclei were sedimented at 800 × *g* for 5 min, and the mitochondria at 8000 × *g* for 10 min The mitochondria were washed once in sucrose and finally suspended in 0 15 M KCl, usually to a concentration of approx 20 mg of protein per ml Rat liver mitochondria were prepared by the same procedure but were washed twice with sucrose Mitochondrial incubations were stopped by addition of 1/4 vol of 2 M $HClO_4$ After removal of the protein by centrifugation the supernatant was neutralized with 2 M KOH

All metabolites were measured enzymatically, coupled to appearance or disappearance of NADH Malate was measured with malate dehydrogenase at pH 9 with approx 0 3 M hydrazine as trapping agent for oxaloacetate Citrate was measured with citrate lyase coupled to malate dehydrogenase Oxoglutarate was measured by conversion to glutamate by glutamate dehydrogenase Acetoacetate and β-hydroxybutyrate were measured with β-hydroxybutyrate dehydrogenase, the former in 0 1 M phosphate buffer at pH 7 5, the latter in 0 7 M glycine buffer at pH 9, with approx 0 3 M hydrazine as trapping agent for acetoacetate Acetylcarnitine was measured by following spectrophotometrically the formation of NADH in the presence of carnitine acetyltransferase, malate dehydrogenase, citrate synthase, malate, NAD and catalytic amounts of CoA The carnitine acetyltransferase was added last since this enzyme also contained some citrate synthase

Kinetic experiments on citrate synthase were performed on purified pig heart enzyme The backward reaction was followed by measuring NADH disappearance in the presence of excess malate dehydrogenase, carnitine acetyltransferase and (−)carnitine (5 mM) Carnitine and carnitine acetyltransferase were added to remove acetyl-CoA formed in the reaction In this way the reaction was rectilinear virtually until all of the NADH had been oxidized

The forward reaction was followed by measuring the formation of NADH in a system consisting of limiting amounts of citrate synthase (80 munits), excess malic

dehydrogenase (25 units), carnitine acetyltransferase (0 33 unit), acetylcarnitine (5 mM), CoA (0 2 mM), NAD (5 mM), NADH (0 2 mM), and variable amounts of malate (0 5–20 mM) NADH was added to "buffer" the system, i e , an increase in NADH (measured with a Unicam SP1800 ultraviolet spectrophotometer fitted with a Unicam AR25 Recorder) did not change the NADH/NAD ratio and the oxaloacetate concentration appreciably, and the reaction was, therefore, rectilinear for a much longer time than when no NADH is added from the beginning Also, larger absolute changes in NADH therefore could be measured, and the "buffering" of the system made it unnecessary to use fluorometers to measure initial, small changes in NADH Also, the rate of NADH formation in the presence of added NADH is a true measure of the rate of citrate synthesis and, under otherwise identical conditions, is as much as nearly twice as fast as the rate obtained (apparent) when no NADH is present initially (For mathematical treatment see Shepherd and Garland [6])

RESULTS

Citrate cleavage in mitochondria

Since rat liver mitochondria contain a relatively low level of carnitine acetyltransferase [7], mouse liver was used for these experiments However, with the extremely slow citrate cleavage taking place, rat liver also has a sufficiently high level of the enzyme

Table I shows that it is possible to demonstrate formation of malate and acetylcarnitine from citrate in liver mitochondria The reaction was stimulated both by addition of carnitine and by rotenone, the former by removing the acetyl-CoA formed, and the latter by inhibiting the oxidation of NADH which is needed to reduce oxaloacetate to malate The reaction is very slow, however, and to demonstrate the effect of carnitine on malate formation it was necessary to have an efficient block of the forward reactions of the citric acid cycle Rotenone alone was not sufficient, but sodium arsenite was Fluorocitrate and malonate also were inefficient Even with arsenite the stoichiometry of the experiments was relatively poor More malate than acetylcarnitine was always formed, and some malate was formed in the absence of carnitine as acetyl acceptor Since citrate was required also for this malate formation,

TABLE I

CITRATE CLEAVAGE IN MOUSE LIVER MITOCHONDRIA

Complete system mitochondria (15 5 mg of protein) were incubated with imidazole–HCl buffer (pH 6 5) 33 mM, KCl, approx 0 1 M, sodium arsenite, 2 mM, rotenone, 40 μg, citrate 9 mM, and (−) carnitine, 4 5 mM Total volume was 2 3 ml, the incubation time was 20 min and the temperature was 37 °C

	Malate formed (μmoles)	Acetylcarnitine formed (μmoles)
Complete system	0 30	0 23
No citrate	0 04	0 075
No carnitine	0 14	0 01
No rotenone	0 09	0 075

TABLE II

EFFECT OF pH ON CITRATE CLEAVAGE IN MOUSE LIVER MITOCHONDRIA

Mitochondria (7 5 mg of protein) were incubated with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 15 mM phosphate (pH adjusted as shown with KOH), approx 85 mM KCl, 2 mM sodium arsenite, 20 μg rotenone, 0 1 mM dinitrophenol, and 10 mM citrate (−)Carnitine was added as shown Total volume was 1 ml and incubation time was 20 min The temperature was 37 °C

	Malate formed (μmoles)		Acetylcarnitine formed (μmoles)
pH	− carnitine	+ carnitine	+ carnitine
6 5	0 09	0 15	0 11
7 0	0 065	0 10	0 07
7 5	0 06	0 075	0 05

it may have been formed via a small leak in the arsenite block or, more likely, by removal of the acetyl-CoA formed by reactions other than acetylcarnitine formation (e g ketogenesis, or hydrolysis) Other additions such as dinitrophenol and phosphate had no effect on the system

Table II shows that the cleavage is more rapid at acid pH At pH 7 5 the effect of carnitine was barely detectable This effect of pH is in agreement with the properties of citrate synthase The backward reaction of the enzyme has an optimum at pH 6 1 , whereas the forward reaction has its optimum above pH 8 [8]

In Table III the rates of the forward and the backward reactions are compared Under the conditions used (pH 6 5) the forward reaction with pyruvate and malate as substrates was 70 times faster than acetylcarnitine formation from citrate and about 40 times faster than malate formation (A small amount of malate may have been formed from endogenous substrates, see Table I) At higher pH the ratios were even greater since the rate of cleavage of citrate is diminished (Table II)

The backward reaction in whole mitochondria evidently depends not only on citrate synthase, but also on malic dehydrogenase and carnitine acetyl transferase

TABLE III

RELATIVE RATES OF CITRATE SYNTHESIS AND CITRATE CLEAVAGE IN MOUSE LIVER MITOCHONDRIA

Mitochondria (13 mg of protein) were incubated with 22 mM imidazole buffer (pH 6 5 or 7 5) and approx 100 mM KCl Forward reaction pyruvate, 4 5 mM, malate, 4 5 mM, fluorocitrate, 100 μM, dinitrophenol, 0 1 mM Backward reaction citrate, 9 mM, carnitine, 4 5 mM, sodium arsenite, 2 mM, rotenone, 40 μg Total volume was 2 3 ml The incubation time was 10–40 min and the temperature was 37 °C

	pH	Malate formed (μmoles)		Acetylcarnitine formed (μmoles)		Citrate formed (μmoles)	
		20 min	40 min	20 min	40 min	10 min	20 min
Backward reaction	6 5	0 20	0 37	0 11	0 28		
Forward reaction	6 5	—	—	—	—	3 1	7 5
Forward reaction	7 5	—	—	—	—	3 9	7 3

Malic dehydrogenase is present in huge excess in mitochondria Carnitine acetyltransferase probably is also present in excess in the cleavage of citrate since much more rapid formation of acetylcarnitine has been observed in liver mitochondria, e g with pyruvate as substrate [9] It is likely, therefore, that citrate synthase limited the reaction and that it ran near its V The observed increase in rate resulting from lowering the pH supports this conclusion, since the backward reaction has a pH optimum near pH 6 with purified citrate synthase [8]

Since the backward reaction was so slow, no further kinetic characterization of the reaction in intact mitochondria was attempted

The forward reaction was nearly the same at pH 6 5 and pH 7 5 It is likely, therefore, that it was limited by puryvate dehydrogenase and not by citrate synthase, which has a pH optimum above pH 8

The effect of citrate and fluorocitrate on the forward reaction

Table IV shows that β-oxidation, i e the total acetyl group formation from

TABLE IV

EFFECTS OF FLUOROCITRATE AND CITRATE ON CITRATE FORMATION IN RAT LIVER MITOCHONDRIA

Rat liver mitochondria (10 mg of protein) were incubated with 20 mM HEPES buffer (pH 7 0), approx 100 mM KCl, 0 1 mM dinitrophenol, and 1 mM malate The following additions were done as shown (−)palmitylcarnitine, 0 125 mM, pyruvate, 1 mM, (−)acetylcarnitine, 1 mM, sodium arsenite, 2 mM, fluorocitrate, 0 1 mM, citrate, 1 mM Total volume was 2 5 ml The incubation time was 4 min and the temperature was 37 °C

Acetyl-CoA precursor	Inhibitor	Citrate formed (μmoles)	α-Ketoglutarate formed (μmoles)	Acetoacetate formed (μmoles)
Palmitylcarnitine	Arsenite	0 14	0 51	0 13
Palmitylcarnitine	Fluorocitrate	0 52	–	0 28
Palmitylcarnitine	Fluorocitrate + citrate	0 33	–	0 34
Acetylcarnitine	Fluorocitrate	0 38		Trace
Acetylcarnitine	Fluorocitrate + citrate	0 09	–	Trace
Pyruvate	Fluorocitrate	0 45	–	Trace
Pyruvate	Fluorocitrate + citrate	0 20	–	0 05
None	Fluorocitrate	0 17	–	0
None	Fluorocitrate + citrate	0	–	Trace

palmityl carnitine (citrate + α-ketoglutarate + 2 × acetoacetate) was unaffected by fluorocitrate and citrate, but less citrate and more ketone bodies were formed Thus, the sum of citrate and α-ketoglutarate formed from palmitylcarnitine and malate in the presence of arsenite was greater than the citrate formed in the presence of fluorocitrate (see also Fig 2) A further decrease in citrate and an increase in acetoacetate was found upon addition of citrate in addition to fluorocitrate

Citrate formation from pyruvate or acetylcarnitine again was depressed by

citrate, but now there was no corresponding increase in acetoacetate formation This indicates that both the pyruvate dehydrogenase and carnitine acetyl transferase were inhibited by accumulated acetyl CoA In another experiment (not shown) citrate strongly decreased the disappearance of both acetylcarnitine and malate in uncoupled mitochondria Altogether, the results indicate that citrate most likely inhibits primarily the utilization of acetyl-CoA, and only secondarily its formation To correlate feedback inhibition by citrate on citrate synthesis in intact mitochondria with the properties of citrate synthase, kinetic experiments on pure pig heart citrate synthase were performed Recent studies have shown that citrate synthase from different species and from different animal organs are very similar, although the kinetic constants may vary to some extent [4] With pig heart synthase we found the K_m for citrate in the backward reaction to be 1–1 5 mM at pH 7 Matsuoka and Srere [4] found a K_m of 0 16 mM at pH 6 1 and 3 mM at pH 8 1 for the rat liver enzyme We have also measured the ratio between the V of the forward and the backward reactions for the pig heart enzyme Ratios of about 200, 300 and 400 were found at pH 6 5, 7 0 and 7 5, respectively

Smith and Williamson [3], found that citrate is a competitive inhibitor with respect to oxaloacetate in the forward reaction [11] They found the K_i to be 1 6 mM at pH 7 4 Fig 1 confirms this finding and shows in addition that fluorocitrate simi-

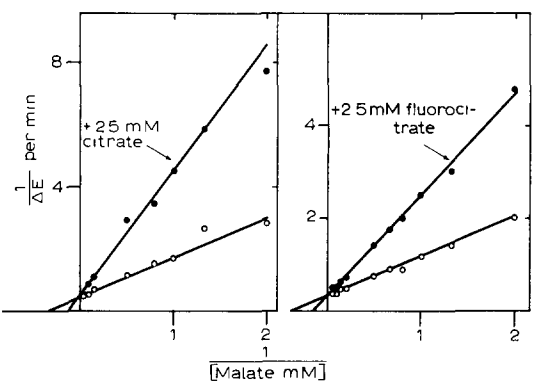

Fig 1 Citrate and fluorocitrate as competitive inhibitors of citrate synthase The reaction mixture contained pig heart citrate synthase, approx 80 munits, carnitine acetyltransferase, 0 35 unit, malic dehydrogenase 25 units, acetylcarnitine, 5 mM, NAD, 5 mM, NADH, approx 0 2 mM, CoA, 0 2 mM, imidazole–HCl buffer, pH 7 0, 35 mM, malate, varying, 0 5–20 mM, and where indicated, citrate or fluorocitrate, 2 5 mM The reaction was started by the addition of citrate synthase and followed in a Unicam SP1800 ultraviolet spectrophotometer with a SP1805 Programme Controller Total volume was 1 ml and the temperature was 23 °C (room temperature)

larly is a competitive inhibitor with respect to oxaloacetate From the drawn lines in Fig 1, an apparent K_m of 2 5 mM for malate was calculated for both experiments, and from these values and the NADH/NAD^+ ratios used in the reaction mixtures the K_m for oxaloacetate was calculated to be 1 8 and 2 5 μM in the two experiments, respectively For citrate a K_i of 1 25 mM for fluorocitrate a K_i of approx 1 5 mM were calculated Thus, fluorocitrate is nearly as efficient as citrate as a competitive inhibitor of citrate synthesis

The ketogenic effect of fluorocitrate was counteracted by malate (Fig 2) This interaction is competitive in nature, since a higher concentration of malate was required to suppress ketogenesis when the concentration of fluorocitrate was elevated A similar experiment was performed at pH 6 6 and almost identical curves were obtained, although the maximum rate of ketogenesis was less than half the rate at pH 7 4

We have also tested the ketogenic effect of fluorocitrate (0 1 mM) in different energy states of rat liver mitochondria With palmitylcarnitine and 1 mM malate in the medium, fluorocitrate approximately doubled ketone body formation in State 4 (about 85% β-hydroxybutyrate and 15% acetoacetate) In State 3 (excess ADP) ketone body formation was tripled by fluorocitrate (30% hydroxybutyrate and 70% acetoacetate) Thus, the ketogenic effect is not limited to the low-energy or uncoupled state of the mitochondria

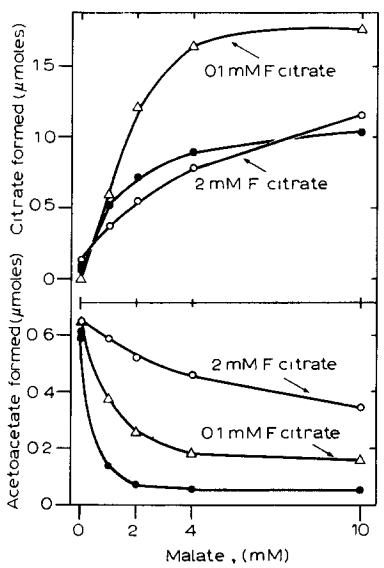

Fig 2 The effect of fluorocitrate on citrate synthesis and ketogenesis in rat liver mitochondria Rat liver mitochondria (3 4 mg of protein) were incubated with imidazole–HCl buffer, pH 7 4, 30 mM, bovine serum albumin (fatty acid free), 0 4%, carbonyl cyanide-*m*-chlorophenylhydrazone, 10 nmoles, (−)palmitylcarnitine, 0 31 μmole, malate, varying, 0–10 mM, KCl, approx 0 07 M Total volume was 2 5 ml The incubation time was 10 min and the temperature was 37 °C ●—●, no fluorocitrate, △—△, 0 1 mM fluorocitrate, ○—○, 2 mM fluorocitrate

Fig 2 also shows that most of the citrate formed in the absence of fluorocitrate evidently was oxidized, since less citrate accumulated in the absence of fluorocitrate in spite of the fact that ketogenesis was more suppressed by malate Since more citrate accumulated with a low than a high concentration of fluorocitrate, it is likely that the ketogenic effect of a low fluorocitrate concentration is due mainly to the accumulated citrate This is in agreement with our previous observation that citrate addition alone can be ketogenic in coupled mitochondria

DISCUSSION

The results here reported for whole mitochondria are in good agreement with the properties of isolated citrate synthase

According to the Haldane relationship the ratio between the V (forward) and the V (backward) is determined by the expression

$$v_f/v_b = K_{eq} \frac{K_1 K_2}{K_3 K_4}$$

where K_{eq} is the equilibrium constant for the reaction, K_1 is the K_m for oxaloacetate, K_2 the K_m for acetyl-CoA, K_3 the K_m for citrate in the backward reaction, and K_4 the K_m for CoA in the backward reaction From the kinetic constants reported by Matsuoka and Srere [4] for rat liver citrate synthase, the v_f/v_b can be calculated $v_f/v_b = 2300$ at pH 8 1

Since v_f decreases and v_b increases at lower pH, the ratio is lower at physiological pH With pig heart citrate synthase we found v_f/v_b to be approx 400 at pH 7 5 In intact mitochondria we found the ratio to be at least 40 at pH 6 5

Since the V of the backward reaction is very slow, it is evident that the rate of the backward reaction must be negligible under all conditions The forward reaction must approach this low value before the reaction can approach equilibrium Most likely, therefore, the citrate synthase must deviate appreciably from equilibrium under all physiological conditions, and to calculate the mitochondrial acetyl-CoA/CoA ratio from the oxaloacetate/citrate ratio and the equilibrium constant [1] seems of doubtful validity

This conclusion is supported by calculation of the mass–action ratio for the combined malic dehydrogenase–citrate synthase in heart mitochondria by Williamson et al [10] This mass–action ratio deviated by two orders of magnitude from the equilibrium constant at pH 7 4

The feedback inhibition by citrate shows that the oxaloacetate/citrate ratio (rather than the oxaloacetate concentration) is important for the rate of citrate formation This might be unexpected since the K_m for oxaloacetate is very low ($< 5\ \mu M$) and the K_i for citrate is relatively much higher (1–4 mM) However, the concentration of oxaloacetate in the mitochondria is believed to be less than its low K_m value, and the concentration of citrate in mitochondria in vivo has been calculated to be well above its K_i concentration [11] Studies in vitro have also shown that citrate can accumulate in the mitochondrial matrix under certain conditions [12] Thus, a regulatory effect of citrate is possible, as shown by the inhibition of citrate synthesis and stimulation of ketogenesis by fluorocitrate and citrate in our experiments The ketogenic effect of low concentrations of fluorocitrate most likely is due mainly to accumulated citrate since the ketogenic effect is relatively strong even at very low concentrations Thus the fluorocitrate formed from 40 nmoles of fluoroacetylcarnitine was ketogenic in liver mitochondria [2]

Regulation via the other substrate/product pair (acetyl-CoA/CoA) is similarly possible However, the acetyl-CoA concentration in mitochondria probably approaches the mM range which is many times its K_m (10–20 μM), and since the K_i for CoA is much higher (70 μM) [3] it seems less likely that the acetyl-CoA/CoA ratio influences the rate of citrate synthesis to the same extent as does the oxaloacetate/

citrate ratio Similarly, the K_i for succinyl-CoA is relatively high [3], and high succinyl-CoA/CoA ratios are required to inhibit citrate synthesis appreciably

The citrate concentration in liver has not been found to vary to any great extent However, the distribution of citrate between cytoplasm and mitochondria has been calculated to vary [11] Such a variable distribution may be due to the recently reported inhibition of the citrate transporter in the mitochondria by palmityl-CoA [13]

ACKNOWLEDGEMENTS

This work was supported by the Norwegian Research Council for Science and the Humanities, USPHS Grants AM13939, AA00289 and HL04219, the American Heart Association (Indiana Affiliate), and the Grace M Showalter Residuary Trust J B is a recipient of a Fulbright–Hayes travel grant

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