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ASSOCIATION OF ATP CITRATE LYASE WITH MITOCHONDRIA

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SUMMARY: (1) The association of ATP citrate lyase with mitochondria was studied with isolated rat hepatocytes and mitochondria. (2) When hepatocytes were treated with digitonin, about 25% of the lyase activity was released like a mitochondrial enzyme. (3) The effect of temperature on release of lyase from hepatocytes was different from that on the release of other cytosolic or mitochondrial enzymes. (4) The fraction of total hepatic lyase in mitochondrial preparations made with exogenous $MgCl_2$ was 30 times greater than that for a cytosolic marker enzyme, phosphoglycerate kinase. (5) Lyase substrates enhanced the release of the enzyme both from hepatocytes and from isolated mitochondria. (6) The metabolic significance of association of ATP citrate lyase with mitochondria is discussed. (7) Data obtained in the course of these experiments indicate that less than 3% of adenylate kinase is cytosolic.

In eukaryotic cells, ATP citrate lyase (EC 4.1.3.8) catalyzes the first step in the synthesis of fatty acids from citrate. One of its substrates, citrate, is produced in the mitochondria and transported to the cytosol by a specific tricarboxylate carrier (1). CoA, another of its substrates, has been reported to be located "almost exclusively" (2) in mitochondria. In contrast, the enzyme is generally considered to be located in the cytosol (3). However, in a previous study (4), using a rapid method for quantifying the cellular distribution of enzymes, we found that a fraction of rat liver ATP citrate lyase is in a non-cytosolic compartment. That method involves treatment of isolated rat hepatocytes with digitonin and comparison of the release of a given enzyme with the release of marker enzymes for the cytosol, lactate dehydrogenase (EC 1.1.1.27), the mitochondrial intermembrane space, adenylate kinase (EC 2.7.4.3), and the mitochondrial matrix, citrate synthase (EC 4.1.3.7). We have now applied that method and conducted experiments with isolated mitochondria to evaluate the influence of dietary state, substrates and temperature on the association of ATP citrate lyase with mitochondria.

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MATERIALS AND METHODS: Isolated hepatocytes were prepared by the procedure of Berry and Friend (5), modified as described by Cornell et al. (6), from male Wistar rats (170-210 g) that had been starved 2 days or rats (200-240 g) that were starved 2 days and then fed a fat-free diet (TD 70257, Teklad Test Diets, Madison) for 2 days (refed rats). Kyro EOB was obtained from Dr. D.H. Hughes, Procter and Gamble Co., Cincinnati, OH). Digitonin was prepared and hepatocytes were fractionated as described by Janski and Cornell (4). Mitochondria were isolated from livers of refed rats according to Johnson and Lardy (7). After homogenization and centrifugation (600 x g) to sediment heavy cell debris, the supernatant, containing mitochondria in 0.25 M sucrose (pH 7.4), was divided into two equal portions. One portion was made 10 mM in exogenous $MgCl_2$ and the other contained no added $MgCl_2$. The mitochondria were sedimented by centrifugation (8,500 x g), and were washed 3 times with 0.25 M sucrose (pH 7.4). For mitochondria with exogenous $MgCl_2$, the wash buffers also included 10 mM $MgCl_2$. $MgCl_2$ was not added until after homogenization of liver in 0.25 M sucrose (pH 7.4) and centrifugation (660 x g) because inclusion of $MgCl_2$ during homogenization caused mitochondria to sediment at 600 x g. Enzymes were assayed at 38°C. ATP citrate lyase was measured according to Cottam and Srere (8), except 10 mM citrate and 110 µM CoA were used. Citrate synthase (9), phosphoglycerate kinase (10), fumarase (11) and fatty acid synthetase (spectrophotometric assay) (12) were assayed by published procedures. Lactate dehydrogenase (10), adenylate kinase (10) and aspartate aminotransferase (13) were assayed with modifications (4) of the published procedures. Total cellular activities of enzymes were determined as described in a previous report (4). Free magnesium concentrations were calculated as described by Veloso et al. (14).

RESULTS AND DISCUSSION: The results in Table I indicate that the release of ATP citrate lyase is affected by the dietary state of the animal used for hepatocyte preparation, the temperature of medium used to fractionate hepatocytes, and the presence of Kyro EOB, a nonionic detergent, in the fractionation medium. When the fractionation is performed as in our previous study (4), i.e., at 1°C and with Kyro EOB to accelerate the release of cytosolic markers, only 76% of the lyase in cells from starved rats is released like lactate dehydrogenase, but essentially all of the lyase behaves like a cytosolic enzyme when the cells are from refed rats. As the temperature of the fractionation medium is increased, the amount of lyase released like lactate dehydrogenase decreases, especially with hepatocytes from starved rats. The effect of Kyro EOB is seen only with cells from refed rats fractionated in a medium at 1°C; in the absence of the detergent, a portion of lyase is released differently from lactate dehydrogenase, and the patterns of release at different temperatures more closely resemble those seen with cells from starved rats. It is important to note that total ATP citrate lyase

TABLE I: Effect of Temperature and Kyro EOB on Release of ATP Citrate Lyase Relative to Lactate Dehydrogenase[a]

Rat Used in Preparation of Hepatocytes	Addition to Fractionation Medium		Temperature of Medium (°C)	Time of Exposure to Medium (sec)	Release of ATP Citrate Lyase Relative to Lactate Dehydrogenase
	Digitonin (mg/ml)	Kyro EOB (%,v/v)			
Starved	4.0	0	1	15	0.79
Starved	4.0	0.2	1	7	0.76
Starved	4.0	0	10	7	0.65
Starved	4.0	0.2	10	5	0.65
Starved	0.4	0	23	7	0.46
Starved	0.4	0.2	23	5	0.50
Refed	4.0	0	1	15	0.82
Refed	4.0	0.2	1	7	0.97
Refed	4.0	0	10	7	0.79
Refed	4.0	0.2	10	5	0.84
Refed	4.0	0	16	5	0.73
Refed	0.4	0	23	7	0.74
Refed	0.4	0.2	23	5	0.76

[a] Hepatocytes (0.16-0.20 grams wet weight per ml) were preincubated at 38°C in Krebs-Henseleit medium plus bovine serum albumin (2.5%). When hepatocytes were isolated from the liver of a refed rat, 10 mM glucose was also included in the medium. Fractionation of 0.2 ml of the hepatocyte suspension was accomplished by exposure to 1.0 ml fractionation medium (0.25 M sucrose, 20 mM 3-morpholinepropanesulfonic acid, 3 mM EDTA, pH 7.0), containing the indicated additions and at the indicated temperature, using methods described previously (4). The time of exposure to the medium was selected to cause 80-90% release of lactate dehydrogenase. The data are given as the ratio of the percentage release of ATP citrate lyase relative to the percentage release of lactate dehydrogenase.

activities in hepatocytes from starved and refed rats, respectively, are 1.3 and 30 μmol/min/gram wet weight of cells when assayed at 38°C. Thus, while the percentage of non-cytosolic lyase seen at 23°C with cells from starved rats is about twice that for cells from refed rats, the amount of non-cytosolic lyase is about 12 times greater in the refed rat hepatocytes. In other experiments (results not shown) we have found that the release of lyase relative to lactate dehydrogenase was not altered by increasing the temperature from 23°C to 36°C, and variations in digitonin and Kyro EOB concentrations had no effect at 23°C.

To see whether the effect of temperature on the release of ATP citrate lyase (Table I) is a characteristic of that enzyme, the cytosolic percentages

TABLE II: Cytosolic Fractions of Enzymes in Hepatocytes from Refed Rats[a]

Enzyme	Percentage of Enzyme in Cytosol	
	Temperature of Fractionation Medium	
	23°C	1°C
Lactate Dehydrogenase	100	100
Phosphoglycerate Kinase	102 ± 1	103 ± 1
Pyruvate Kinase	100 ± 1	100 ± 1
Fatty Acid Synthetase	101 ± 1	98.9 ± 1.0
ATP Citrate Lyase	78.0 ± 1.2	97.0 ± 1.6
Fumarase	34.7 ± 1.1	30.0 ± 0.8
Aspartate Aminotransferase	15.4 ± 0.4	21.0 ± 0.6
Adenylate Kinase	2.87 ± 0.09	9.09 ± 0.31
Citrate Synthase	0	0

[a] Hepatocytes from livers of refed rats were fractionated using conditions described in the legend of Table I. Fractionation medium at 23°C contained 0.4 mg digitonin per ml and that at 1°C contained 4.0 mg digitonin per ml and 0.2 % (v/v) Kyro EOB. For all enzymes except adenylate kinase the percentage of enzyme in the cytosol was calculated from 10 different experiments in which the time of exposure was different for each experiment. At 23°C, the exposure times used were 3, 4, 5, 6, 7, 8, 9, 10, 12 and 15 sec, and at 1°C they were 3, 4, 5, 6, 8, 10, 12, 15, 20 and 25 sec. Using these exposure times, release of lactate dehydrogenase varies from 40% to 100%. Calculation of cytosolic percentages involves the assumption that release of lactate dehydrogenase and citrate synthase represent release of soluble components in the cytosolic and mitochondrial matrix spaces, respectively. This calculation is described elsewhere (4). In the case of adenylate kinase, only the first 5 exposure times were used to calculate cytosolic enzyme because the ratio of release of adenylate kinase to lactate dehydrogenase is constant only for the first 5 time points and increases rapidly thereafter. This is presumably due to leakage of adenylate kinase from the intermembrane space when hepatocytes are exposed for longer time intervals. The values given are the means ± standard errors from the different experiments.

of other enzymes were determined at 1°C and 23°C. As shown in Table II, the cellular distributions relative to lactate dehydrogenase of three cytosolic enzymes (phosphoglycerate kinase, pyruvate kinase and fatty acid synthetase) and two enzymes found both in the cytosol and the mitochondrial matrix (fumarase and aspartate aminotransferase) are not affected by temperature. The cytosolic percentage of the marker for the mitochondrial intermembrane space, adenylate kinase, was less at 23°C than at 1°C. This difference may reflect simply a difference in leakage from the intermembrane space because, in the case of adenylate kinase, the calculations include no correction for leakage. Thus, 2.87% is the maximum amount of adenylate kinase that is cytosolic. The

non-cytosolic fraction of ATP citrate lyase probably is not in either the matrix or intermembrane space of mitochondria because at 1°C only small fractions of the enzyme markers for these compartments are released like lactate dehydrogenase, but all of the lyase is released like lactate dehydrogenase.

Hexokinase is a well-known example of a cytosolic enzyme which can bind to mitochondria; the bound enzyme is released by its substrates, but Mg^{2+} both stabilizes the binding and prevents the release induced by ATP (15). Similarly, the substrates for ATP citrate lyase accelerate the release of the non-cytosolic fraction of the lyase (Table III). The maximum effect on release of lyase was achieved with 5 μM CoA, 1.0 mM citrate or 1.0 mM ATP. Addition of $MgCl_2$, an effector of the lyase reaction, did not change the amount of non-releasable lyase, but it did block or decrease the effects of

TABLE III: Effects of Substrates or Products on Release of ATP Citrate Lyase from Hepatocytes[a]

Substrates or Products in Fractionation Medium	Percentage of ATP Citrate Lyase Remaining in Hepatocytes After Fractionation	
	$MgCl_2$ in Fractionation Medium	
	None	10 mM
None	24 (26)	23 (28)
CoA (20 μM)	2 (4)	7 (5)
Acetyl CoA (20 μM)	9	10
Citrate (2 mM)	5 (7)	29 (30)
Oxaloacetate (2 mM)	9	20
ATP (2 mM)	3 (9)	22 (22)
ADP (2 mM)	7	17

[a]Hepatocytes from livers of refed rats were fractionated using conditions described in the legend of Table I. Fractionation medium was at 23°C and contained 0.4 mg digitonin per ml and the indicated concentrations of substrates or products. Hepatocytes were exposed 20 sec to medium, during which time, 95-100% of lactate dehydrogenase was released. None of the additions to the fractionation medium affected release of lactate dehydrogenase. The experiments were performed with and without 10 mM $MgCl_2$ in the fractionation medium. Values in parenthesis are from duplicate experiments with a different hepatocyte preparation.

citrate, oxaloacetate, ADP and ATP. In contrast, $MgCl_2$ did not alter the effects of CoA and acetyl CoA. When $MgCl_2$ was present, the free Mg^{2+} concentration in the fractionation medium ranged from 4.7 mM to 6.3 mM, and, under the conditions described in Table III, citrate, oxaloacetate, ATP and ADP would be complexed with Mg^{2+}. Thus, it appears that only the free forms of these substrates are effective in releasing ATP citrate lyase from hepatocytes.

In order to study the association and release of ATP citrate lyase more directly, experiments were conducted with isolated mitochondria as described by Table IV. Because Mg^{2+} affects the substrate-induced release of lyase from hepatocytes (Table III), mitochondria were isolated in the absence and presence of $MgCl_2$. The isolation procedure involves centrifugation of the homogenate and 3 washes of the mitochondria; yet, when 10 mM $MgCl_2$ was present, the isolated mitochondria contained 2.2% of the total hepatic ATP citrate lyase, while mitochondria prepared without added Mg^{2+} contained only 0.2% of the cellular lyase activity. In contrast, these mitochondria contained much less of a cytosolic marker enzyme, phosphoglycerate kinase: 0.07% of the hepatic content when $MgCl_2$ was added and 0.02% when $MgCl_2$ was absent. The values given above for the lyase content in mitochondrial preparations do not reflect losses of mitochondria during the isolation procedure. Using the recovery of total cellular citrate synthase as an index, we estimate that the recovery of mitochondria was 52% when $MgCl_2$ was present and 25% when $MgCl_2$ was absent. Thus, if all the mitochondria had been recovered, the lyase content would have been 4.2% of the cellular activity for preparations with $MgCl_2$ and 0.8% for those without added $MgCl_2$. These values suggest a role for Mg^{2+} in stabilizing the association of ATP citrate lyase with mitochondria. In a similar way, Green et al. (16) previously concluded that magnesium helps to stabilize the association of some glycolytic enzymes with erythrocyte membranes. Results shown in Table IV are also consistent with a stabilizing role for Mg^{2+}; when mitochondria isolated in the absence of Mg^{2+} were washed an additional time, 25% of the residual ATP citrate lyase was released. The

TABLE IV: Effects of Substrates on Release of ATP Citrate Lyase from Isolated Mitochondria[a]

Additions to Wash Medium	Percentage Release of Bound ATP Citrate Lyase	
	$MgCl_2$ in Mitochondrial Preparation	
	None	10 mM
None	25	16
$MgCl_2$ (10 mM)		6.2
CoA (20 μM)	53	
CoA (20 μM) + $MgCl_2$ (10 mM)		100
Citrate (2 mM)	82	
Citrate (2 mM) + $MgCl_2$ (10 mM)		22
ATP (2 mM)	85	
ATP (2 mM) + $MgCl_2$ (10 mM)		34
CoA (20 μM), Citrate (2 mM), ATP (2 mM) — no $MgCl_2$	98	
CoA (20 μM), Citrate (2 mM), ATP (2 mM) — $MgCl_2$ (10 mM)		91

[a]Mitochondria from refed rats were isolated with or without $MgCl_2$ present as described in the text and were stored on ice until used. Mitochondria isolated with $MgCl_2$ present were suspended at a concentration of 45 mg protein per ml in 0.25 M sucrose, pH 7.4, containing 10 mM $MgCl_2$, and mitochondria isolated without $MgCl_2$ present were suspended at a concentration of 27 mg protein per ml 0.25 M sucrose, pH 7.4 (wash medium). After sedimentation of mitochondria (0.5 ml of suspension) by centrifugation at 10,000 x g at room temperature and removal of the supernatant, the mitochondria were washed twice at room temperature with 0.5 ml of wash medium, containing the indicated additions, by resuspension and then sedimentation of the mitochondria. The two washings were assayed separately and values for percentage of lyase released are the sum of the two washings. For each wash condition described a separate 0.5 ml portion of the mitochondrial suspension was used. The total amount of ATP citrate lyase bound to mitochondria was determined in washes and extracts after exhaustively washing mitochondria with wash medium containing all three substrates and then sonicating the mitochondria.

effects of substrates on release of lyase from mitochondria are similar to those seen with hepatocytes. In the presence of excess Mg^{2+}, citrate and ATP are less effective in releasing lyase activity; but CoA was not less effective in the presence of excess Mg^{2+}.

As indicated above in the discussion of Table III, it appears unlikely that the non-cytosolic ATP citrate lyase is located either in the mitochondrial matrix or intermembrane space. This implies that the enzyme is associ-

ated with one of the mitochondrial membranes. As with the much-studied binding of hexokinase to mitochondria (15), the metabolic significance of ATP citrate lyase associated with mitochondria remains to be determined. The hepatic biosynthesis of fatty acids utilizes citrate, produced within mitochondria, and CoA, which is thought to be highly concentrated in the mitochondrial matrix (2) and very low in the cytosol. There is no known mechanism for transport of CoA into or out of mitochondria. Thus, for efficient production of cytosolic acetyl CoA, association of ATP citrate lyase with mitochondrial membranes may be advantageous.

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