BBA 65872

THE INTRACELLULAR AND INTRAMITOCHONDRIAL DISTRIBUTION OF MALONYL-CoA DECARBOXYLASE AND PROPIONYL-CoA CARBOXYLASE IN RAT LIVER

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(Received November 5th, 1968)

SUMMARY

1. Malonyl-CoA decarboxylase (EC 4.1.1.9) and propionyl-CoA carboxylase (EC 6.4.1.3) are mitochondrial enzymes.

2. The distribution of malonyl-CoA decarboxylase in fractions obtained by treatment of rat-liver mitochondria with digitonin follows the same pattern as 'matrix' enzymes such as malate dehydrogenase and glutamate dehydrogenase.

3. Propionyl-CoA carboxylase is also a 'matrix' enzyme but is much more loosely bound, if at all, to the inner side of the inner membrane.

INTRODUCTION

It has long been known that malonate can be oxidized by yeast, moulds, bacteria and animals (see ref. 1). The mechanism of this oxidation remained obscure until in 1938 KREBS *et al.*[2] found that the first step in the metabolism of malonate is dependent upon the presence of O_2. They considered the possibilities that O_2 may cause an oxidation of malonate before it is decarboxylated or that it may bring about an activation of malonate, for example by a phosphorylative mechanism.

This last proposal was shown to be correct by HAYAISHI[3,4] and WOLFE and coworkers[5–7], who demonstrated that in preparations from *Pseudomonas fluorescens* malonate is activated with ATP *plus* CoASH and the malonyl-CoA decarboxylated to acetyl-CoA. The same pathway has since been shown to be present in rat-kidney homogenate[8], tissue slices of rat kidney, liver, heart and diaphragm[9], and rat-kidney mitochondria[9].

The localization of malonyl-CoA decarboxylase (EC 4.1.1.9) in the cell has not been determined. Extensively purified preparations of fatty acid synthetase isolated from the supernatant fraction of various cells are often contaminated with this enzyme[10–13]. Malonyl-CoA decarboxylase present in preparations of yeast fatty acid synthetase has been partially purified[14]. LORCH *et al.*[15] found the enzyme in rat-liver

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microsomes but did not examine the mitochondria. As already mentioned, NAKADA *et al.*[9] found that rat-kidney mitochondria are able to decarboxylate malonate.

The pathway of propionate metabolism in animal tissue has been studied by several investigators[16–23]. Since the demonstration by LARDY AND ADLER[17] that acetone extracts of liver mitochondria are able to metabolize propionate, these mitochondria have been used as the source for purifying propionyl-CoA carboxylase[24–27] (EC 6.4.1.3). The intracellular localization of propionyl-CoA carboxylase has, however, never been thoroughly investigated with the aid of marker enzymes.

In the present study the intracellular and intramitochondrial localization of malonyl-CoA decarboxylase and propionyl-CoA carboxylase has been studied in rat liver.

METHODS

Fractionation of rat liver

A rat liver was fractionated essentially according to DE DUVE *et al.*[28] into a nuclear fraction (N) and a "cytoplasmic extract" fraction (E). The E fraction was further separated into a heavy mitochondrial fraction (M), a light mitochondrial fraction (L), a microsomal fraction (P) and a particle-free supernatant (S). The fat and connective tissue were removed from the liver, and a Potter–Elvehjem homogenizer was used with a very tight-fitting teflon pestle. The fractionation medium was 0.25 M sucrose. The homogenate was filtered through two layers of fine-mesh nylon cloth[29]. The M fraction consisted of the sediment and the loosely packed material above the pellet[30]. Fractions N, M and L were washed 3 times and fraction P once.

Fractionation of rat-liver mitochondria

Mitochondria were prepared from the livers of fasted rats by the method of HOGEBOOM[31], exactly as described by MYERS AND SLATER[32]. The mitochondria were twice washed in 0.25 M sucrose, and fractionated with digitonin according to SCHNAITMAN *et al.*[33], using 1.26 mg digitonin per 10 mg protein. The suspension was separated into a 10-min, 9500 $\times$ g_{max} pellet (1), a 10-min, 40 000 $\times$ g_{max} pellet (2), a 60-min, 230 000 $\times$ g_{max} pellet (3), and a 60-min, 230 000 $\times$ g_{max} supernatant (4). The 230 000 $\times$ g_{max} was chosen instead of 144 000 $\times$ g_{max} (ref. 33) because this usually considerably lowered the contribution of outer-membrane components in the final supernatant.

Enzyme assays

The subfractions were stored in 2-ml portions at about $-19°$, and thawed and tested within 3 days. Prior to testing, 1 mM glutathione (pH 7.2) was added, and the fractions were subjected to ultrasonic vibrations in the cold for 2 periods of 1 min in an M.S.E. sonic disintegrator (output 20 kHz). This was sufficient maximally to activate latent enzymes in frozen and thawed mitochondria. The assays were carried out at 25°, except those for acid phosphatase and glucose-6-phosphatase which were assayed at 37° (ref. 28).

Malate dehydrogenase (EC 1.1.1.37), glutamate dehydrogenase (EC 1.4.1.2), cytochrome *c* oxidase (EC 1.9.3.1), rotenone-insensitive NADH–cytochrome *c* reductase, aspartate aminotransferase (EC 2.6.1.1), adenylate kinase (EC 2.6.1.1), acid phosphatase (EC 3.1.3.2) and glucose-6-phosphatase (EC 3.1.3.9) were assayed as de-

scribed in refs. 34 (see also ref. 35), 36, 33, 37, 38, 34, 28 and 28, respectively, with the following modifications: (1) 1.5 μM rotenone was present in the assays of malate dehydrogenase and aspartate aminotransferase; (2) the medium used for the assay of glutamate dehydrogenase contained 25 mM potassium phosphate buffer (pH 7.5), 5 mM NH_4Cl, 0.1 mM NADH, 0.9 μM antimycin and 10 mM α-oxoglutarate.

Malonyl-CoA decarboxylase was assayed in Warburg vessels. The main compartment contained in a final volume of 0.5 ml, 40 mM potassium phosphate buffer (pH 7.0) and 3 μM rotenone. The reaction was started by the addition of 0.62 mM [1,3-$^{14}C_2$]-malonyl-CoA (47 nC). After 15 min the reaction was stopped by the addition of 0.05 ml 2.5 M H_2SO_4 from the side-arm. The centre well contained 0.01 ml 1 M hyamine hydroxide in methanol on a strip of Whatman no. 1 paper edged with paraffin wax[39]. After 1 h of shaking the filter paper was placed in a liquid-scintillation vessel and 10 ml toluene with 40 mg 2,5-diphenyloxazole and 1 mg 1,4-bis-(5-phenyloxazolyl-2)-benzene *plus* 0.5 ml methanol were added.

The assay of propionyl-CoA carboxylase (see ref. 40) was carried out in liquid-scintillation vessels in a metabolic shaker. The medium (volume 0.5 ml) contained 66.5 mM Tris–HCl (pH 8.5), 10.4 mM $KH^{14}CO_3$ (100 nC), 2.66 mM ATP, 3 mM $MgCl_2$, 100 mM KCl and 2.5 mM 2-mercaptoethanol. The reaction was started by adding 0.61 mM propionyl-CoA. After 15 min the reaction was stopped by the addition of 0.05 ml 2.5 M H_2SO_4. After 1 h the vessels were gassed with N_2 and 12.5 ml scintillation liquid was added (6.67 ml toluene containing the fluors, 3.33 ml Triton X-100 and 2.5 ml ethanol[41]). After standing at 4° the fluid was mixed and counted.

All enzyme activities are expressed in units of μmoles substrate metabolized per min.

Protein was determined immediately after each fractionation by the biuret method[42].

Cytochrome *c* was prepared from beef heart[43], and malonyl-CoA and propionyl-CoA by the thiophenol method[44].

CoASH was obtained from Sigma and the other cofactors and enzymes from Boehringer.

RESULTS

The intracellular distribution of malonyl-CoA decarboxylase and propionyl-CoA carboxylase

Table I and Fig. 1 show that malonyl-CoA decarboxylase is a mitochondrial enzyme, and not a microsomal one as proposed by LORCH *et al.*[15]. Propionyl-CoA carboxylase seems to have a bimodal distribution. When corrected for cross contamination, the mitochondrial fraction contained 75% and the supernatant 25% of the total activity. The activity of acetyl-CoA carboxylase (EC 6.4.1.2) in the supernatant was too low at pH 8.5 (*cf.* ref. 45) to account for this propionyl-CoA carboxylation. This activity is possibly an artefact of the preparation, since propionyl-CoA carboxylase is easily released from damaged mitochondria (see below).

The intramitochondrial distribution of malonyl-CoA decarboxylase and propionyl-CoA carboxylase

Table II and Fig. 2 show that malonyl-CoA decarboxylase follows the pattern

TABLE I

THE INTRACELLULAR DISTRIBUTION OF MALONYL-CoA DECARBOXYLASE AND PROPIONYL-CoA CARBOXYLASE

As marker enzymes were chosen cytochrome oxidase for the mitochondria, glucose-6-phosphatase for the microsomes, and acid phosphatase for the lysosomes[28]. E is cytoplasmic extract; for N, M, L, P, and S see legend to Fig. 1.

	Abs. values	*Percentage values*						
	E+N	*E+N*	*N*	*M*	*L*	*P*	*S*	*Recovery*
Protein (mg)	996	100	19.6	19.2	1.9	26.2	29.5	96.5
Cytochrome *c* oxidase	353	100	34.2	49.1	2.3	6.7	0.0	92.2
Glucose-6-phosphatase	121	100	5.3	4.1	3.2	77.8	1.6	91.9
Acid phosphatase	46.6	100	5.3	24.6	30.1	18.2	10.4	88.6
Malonyl-CoA decarboxylase	4.16	100	35.5	44.0	2.5	2.1	4.3	88.3
Propionyl-CoA carboxylase	4.19	100	30.8	36.9	2.4	0.2	25.1	95.4

of the 'matrix' enzymes malate dehydrogenase and glutamate dehydrogenase (see refs. 33, 34, 37, 46–52).

The partition of aspartate aminotransferase and propionyl-CoA carboxylase over the four subfractions is different. Judging from the percentage activity in Fraction 4, aspartate aminotransferase is much more tightly bound to the inner membrane, as already found by SCHNAITMAN AND GREENAWALT[46], and propionyl-CoA carboxylase is much less tightly bound, if at all. The high activity of propionyl-CoA carboxylase in the 60-min, 230 000 × g_{max} precipitate is due to the high sedimentation coefficient of the enzyme[53]. In a similar experiment where the outer-membrane components were sedimented at 60-min, 140 000 × g_{max}, the propionyl-CoA carboxylase activity in the sediment was much lower.

Because of the ease with which it is detached from the inner membrane, the distribution of propionyl-CoA decarboxylase in the four fractions obtained by digitonin fractionation resembles that of adenylate kinase, which is present in the inter-mem-

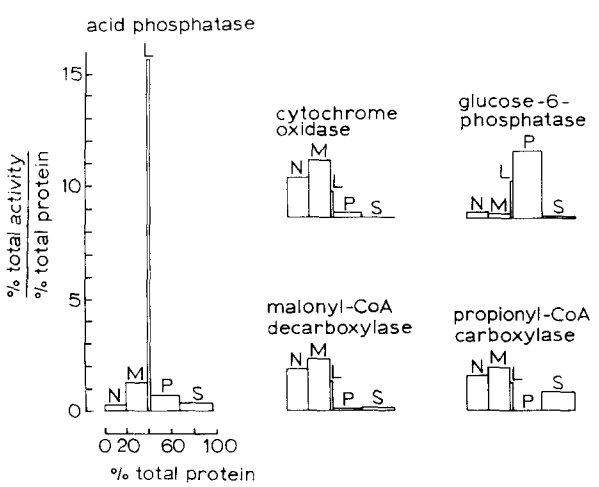

Fig. 1. The intracellular distribution of malonyl-CoA decarboxylase, propionyl-CoA carboxylase and marker enzymes. Fraction N is the nuclear fraction, M is the heavy mitochondrial fraction, L is the light mitochondrial fraction, P is the microsomal fraction and S is the particle-free supernatant.

TABLE II

THE INTRAMITOCHONDRIAL DISTRIBUTION OF MALONYL-CoA DECARBOXYLASE, PROPIONYL-CoA CARBOXYLASE, ASPARTATE AMINOTRANSFERASE, AND MARKER ENZYMES

Cytochrome oxidase was used as marker for the inner membrane[33,37,50-52,66], rotenone-insensitive NADH–cytochrome *c* reductase for the outer membrane[37,52], adenylate kinase for intermembrane space[34,46-48], and acid phosphatase for the lysosomes[28]. Mit. = mitochondria. Fraction 1 is the 10-min, 9500 × g_{max} pellet, Fraction 2 the 10-min, 40 000 × g_{max} pellet, Fraction 3 the 60-min, 230 000 × g_{max} pellet and Fraction 4 the 60-min, 230 000 × g_{max} supernatant.

	Abs. values	*Percentage values*					
			Fraction				
	Mit.	*Mit.*	*1*	*2*	*3*	*4*	*Recovery*
Protein (mg)	410	100	38.8	32.5	5.0	15.8	92.2
Cytochrome *c* oxidase	468	100	50.0	48.3	4.2	0.0	102.5
Aspartate aminotransferase	446	100	52.6	38.3	5.8	4.2	100.9
Malate dehydrogenase	531	100	45.7	33.6	2.9	11.2	93.5
Malonyl-CoA decarboxylase	5.8	100	34.2	26.5	2.7	21.0	84.2
Glutamate dehydrogenase	188	100	42.8	28.7	1.1	24.5	97.1
Propionyl-CoA carboxylase	7.3	100	11.2	9.3	6.8	34.9	62.3
Adenylate kinase	139**	100	0.8	1.3	0.5	40.0	42.6
Acid phosphatase	27.5	100	2.6	7.5	12.6	58.7	81.5
Rotenone-insensitive NADH–cytochrome *c* reductase	92.9	100	9.5	16.2	53.9	1.9	81.5

* After treatment with digitonin.
** ATP formed.

brane space[34,46-48]. However, it differs from the latter in that appreciable activity is found in Fractions 1 and 2 (and 3, but this is due to sedimentation of the liberated enzyme). Moreover, the activity of propionyl-CoA carboxylase in intact mitochondria is latent in that it is greatly increased by sonication, as was also found to be the case with glutamate dehydrogenase (*cf.* ref. 54) and malonyl-CoA decarboxylase (Table III).

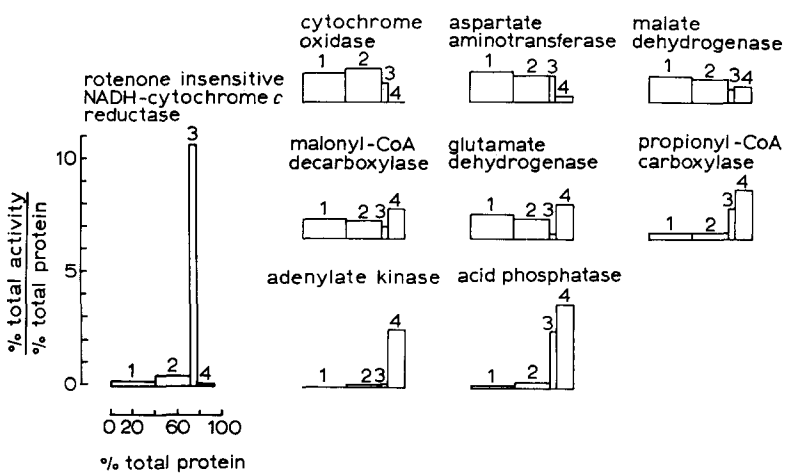

Fig. 2. The intramitochondrial distribution of malonyl-CoA decarboxylase, propionyl-CoA carboxylase, aspartate aminotransferase and marker enzymes. Fraction 1 is the 10-min, 9500 × g_{max} pellet, Fraction 2 the 10-min, 40 000 × g_{max} pellet, Fraction 3 the 60-min, 230 000 × g_{max} pellet and Fraction 4 the 60-min, 230 000 × g_{max} supernatant.

TABLE III

THE EFFECT OF SONICATION ON THE ACTIVITY OF GLUTAMATE DEHYDROGENASE, MALONYL-CoA DECARBOXYLASE AND PROPIONYL-CoA CARBOXYLASE

The mitochondria were washed twice and sonicated 4 times for 1 min.

	Specific activity (munits/mg protein)		*Stimulation factor*
	Intact mitochondria	*Sonicated mitochondria*	
Glutamate dehydrogenase	29.2	193	6.6
Malonyl-CoA decarboxylase	1.28	9.70	7.5
Propionyl-CoA carboxylase	2.36	18.8	8.0

Since propionyl-CoA would be expected to pass through the outer membrane (*cf.* ref. 55), it must be concluded that, in the intact mitochondria, the carboxylase lies within the inner membrane. Also in contrast to the behaviour of adenylate kinase[47,48], propionyl-CoA carboxylase is not extracted from mitochondria by 0.1 M phosphate buffer (pH 7.2). Thus, our data do not support the suggestion of CIMAN AND SILIPRANDI[56] that the carboxylase is localized on both sides of the mitochondrial membrane.

The low recoveries of propionyl-CoA carboxylase and adenylate kinase are probably due to release of lysosomal enzymes (*e.g.*, peptidases) by the digitonin treatment, and thawing and sonicating of the samples prior to the assay. The specific activity of acid phosphatase, a lysosomal enzyme, is the highest in the 60-min, 230 000 × g_{max} supernatant. Fig. 3 shows that the greater the percentage found in this fraction, the lower the overall recovery of a particular enzyme.

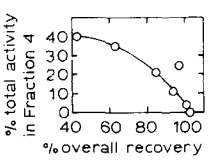

Fig. 3. Relationship between percentage of an enzyme found in the final supernatant after digitonin fractionation and the overall recovery of the enzyme. The data of Table II are plotted with the exception of the outer-membrane enzyme (rotenone-insensitive NADH–cytochrome *c* reductase) and the lysosomal enzyme (acid phosphatase).

DISCUSSION

According to the enzyme distribution illustrated in Fig. 2, enzymes present in mitochondria stripped of their outer membrane differ with respect to the tightness with which they are bound to the inner membrane in the presence of digitonin. In descending order come cytochrome *c* oxidase, aspartate aminotransferase, malate dehydrogenase, malonyl-CoA decarboxylase = glutamate dehydrogenase, and propionyl-CoA carboxylase.

The question arises of the physiological role of malonyl-CoA decarboxylase in

mammalian tissue. Malonyl-CoA inhibits many enzymes such as acetyl-CoA carboxylase[57] and pyruvate carboxylase[58] and it can regulate the chain length of the fatty acids synthesized by the supernatant fatty acid synthetase[59–61]. The function of malonyl-CoA decarboxylase could be to regulate the intracellular level of malonyl-CoA[62]. However, since rat heart contains little if any supernatant fatty acid synthetase (E. M. WIT-PEETERS, unpublished observations) or pyruvate carboxylase (EC 6.4.1.1.)[63,64], whereas the malonyl-CoA decarboxylase activity is quite high (about one third of the activity of rat-liver mitochondria), it seems likely that there is another metabolic role for the enzyme.

Together with the malonate-activating enzyme, malonyl-CoA decarboxylase could play a role in the detoxication of malonate. However malonate is seldom encountered in the cell, except in some plants[65].

A more likely function of malonyl-CoA decarboxylase is to decarboxylate malonyl-CoA formed by carboxylation of acetyl-CoA. Although acetyl-CoA carboxylase is absent from mitochondria[45], acetyl-CoA is slowly carboxylated by propionyl-CoA carboxylase[26,63,45], now shown to be exclusively a mitochondrial enzyme.

ACKNOWLEDGEMENTS

The author wishes to thank Professor E. C. SLATER for his advice and encouragement and Mrs. E. M. WIT-PEETERS for her advice and collaboration in some of the experiments. The aid of Miss A. LÜTJENS and Mr. J. BAKKER, and the technical assistance of Miss G. KLAASSEN are gratefully acknowledged. The present investigations have been carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.).

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