THE SUBMITOCHONDRIAL DISTRIBUTION OF THE FATTY ACID OXIDIZING SYSTEM IN RAT LIVER MITOCHONDRIA

Diana S. Beattie

Veterans Administration Hospital, Leech Farm Road, and the Pharmacology Department, University of Pittsburgh School of Pharmacy, Pittsburgh, Pennsylvania

Received December 5, 1967

The complete oxidation of fatty acids to CO_2 and H_2O in mitochondria requires, in addition to the enzymes of β-oxidation, the enzymes of the citric acid cycle and the electron transport system. Recent studies on the structural organization of the mitochondrion have demonstrated that the inner membrane-matrix fraction is the location of the latter two enzyme systems (Schnaitman et al., 1967; Schnaitman and Greenawalt, 1967; Sottacosa et al., 1967; Parsons et al., 1967). The location of the enzymes of fatty acid oxidation in the mitochondrion in relationship to these auxiliary systems is thus of some interest. The outer membrane of beef heart mitochondria has been reported as the site of fatty acid oxidation (Allmann et al., 1966a) while Yates et al., (1966) have suggested that the enzymes of β-oxidation may be closely associated with the cristal membranes.

The present communication demonstrates that the enzymes of β-oxidation are located in the inner membrane-matrix fraction of rat liver mitochondria fractionated by the digitonin method of Schnaitman et al., (1967). In addition, fatty acid oxidation in the inner membrane-matrix fraction is stimulated by carnitine to the same extent as that of intact mitochondria (Fritz and Yue, 1963) suggesting that the inner membrane is the carnitine "barrier" (Chappelle and Crofts, 1965).

MATERIALS AND METHODS

Rat liver mitochondria were prepared in 0.25 M sucrose by the method of Schneider (1957) and washed four times with 0.25 M sucrose adjusted to pH 7.4 with Tris. The final mitochondrial suspension was treated with a 2% digitonin solution containing BSA (0.5 - 1.0 mg/ml) such that the final concentration of digitonin was 1.1 mg/10 mg of mitochondrial protein and separated into three fractions; inner membrane plus matrix, outer membrane and a soluble fraction as described by Schnaitman et al.(1967). The completeness of the separation was monitored by the enzyme markers; succinic dehydrogenase and isocitric dehydrogenase for the inner membrane-matrix fraction and rotenone-insensitive NADH-cytochrome c reductase for the outer membrane (Schnaitman and Greenawalt, 1967; Sottacosa et al., 1967).

Fatty acid oxidation was measured spectrophotometrically by the formazan method of Mii and Green (1954) as modified by Allmann et al. (1966a). The assay mixture (0.8 ml) contained 20 μmoles of histidine, pH 7.0, 2.5 μmoles of methylene blue, 2.0 μmoles of L-malate, 1.0 μmole of ATP, 0.3 μmoles of NAD, 4.0 μmoles of 2, 3, 5-triphenyl tetrazolium, substrate and enzyme. The substrate tested was either palmitate (20 mμmoles) or palmityl-CoA (30 mμmoles). Cofactors were added as described in the legends to the tables. The reaction mixture was incubated for 15 min at 37° in an atmosphere of nitrogen. Fatty acid oxidation was also measured as $^{14}CO_2$ production from 1-(^{14}C) palmitate as previously described (Beattie and Basford, 1965).

Nucleotides, palmityl-CoA and digitonin were obtained from Sigma; methylene blue, 2, 3, 5-triphenyl tetrazolium, palmitic acid, and carnitine from Mann; coenzyme A from P-L Biochemicals; and (^{14}C) palmitate from New England Nuclear.

RESULTS AND DISCUSSION

Table I shows the distribution of the enzymes of fatty acid oxidation in rat liver

mitochondria assayed in the presence of carnitine. Over 90% of the total mitochondrial activity was present in the inner membrane-matrix fraction with either palmitate or palmityl-CoA as substrate. This fraction also contained 78% of the succinic dehydrogenase, a marker for the inner membrane, 77% of the isocitric dehydrogenase, a marker for the matrix, and 11% of the rotenone-insensitive NADH-cytochrome c reductase, a marker for the outer membrane.

TABLE I

Oxidation of Palmitate and Palmityl-CoA in Isolated Mitochondrial Fractions

	Palmitate		Palmityl-CoA	
Fraction:	Sp. Act.[a]	% Recovery	Sp. Act.[a]	% Recovery
Mitochondria	11.0	100	17.5	100
Inner Membrane-Matrix	17.2	95	24.2	93
Outer Membrane	13.9	5.1	-	-
Soluble	0	0	-	-

a. Activities are expressed as mumoles of formazan formed / min/ mg protein. Palmitate was assayed (see methods) in the presence of 25 μg of CoA and 2 μmoles of carnitine; palmityl-CoA in the presence of 2 μmoles of carnitine; assays were performed in duplicate and the control value obtained in the absence of substrate was subtracted from all values.

The specific activity obtained with the inner membrane-matrix fraction was 1.6 times that of the intact mitochondria with both substrates, a figure consistent with the recovery of 65% of the total mitochondrial protein in this fraction. A 25% increase in specific activity was observed in the outer membrane fraction; however, this represented only 5% of the total activity. The outer membrane also contained 12% of the succinic dehydrogenase activity representing an extent of contamination by the inner membrane more

than sufficient to account for the fatty acid oxidation activity observed in this fraction. No fatty acid oxidation activity was observed in the soluble fraction, although 23% of the matrix enzyme, isocitric dehydrogenase, was present. The enzymes of β-oxidation thus appear more tightly bound to the inner-membrane-matrix fraction obtained by the digitonin method than either succinic dehydrogenase or isocitric dehydrogenase.

The addition of carnitine approximately doubled palmitate oxidation in the inner membrane-matrix fraction (Table II) using two methods of assay. A greater carnitine stimulation was observed when exogenous CoA was present, although added CoA did not enhance fatty acid oxidation in the absence of carnitine and in some cases was inhibitory. Carnitine also stimulated the oxidation of palmityl-CoA. These results are consistent

TABLE II

Effect of carnitine on Palmitate Oxidation in Mitochondria and the Inner Membrane-Matrix Fraction

	Mitochondria	Inner Membrane-Matrix	
Conditions:	Sp. Act.[a]	Sp. Act.[a]	Sp. Act.[b]
Palmitate	8.5	15.3	1980
+ carnitine	16.3	26.4	3735
+ CoA			1955
+ CoA + carnitine			4970
Palmityl-CoA		10.9	
+ carnitine		16.0	

a. Activities expressed as mumoles of formazan produced/min/mg protein. Where indicated 2.0 μmoles of carnitine and 25 μg of CoA were added.
b. Activity expressed as cpm/mg protein at 37° for 30 min. Assay medium contained in a final volume of 2.0 ml; 1.0 μmole ATP, 10 mμmoles malate, 50 μmoles Tris, pH 7.4, 10 μmoles $MgCl_2$, 1.0 μmole K phosphate, pH 7.4, 100 μg cytochrome c, 0.05 μc of 1-(^{14}C) palmitate and 0.154 M KCl.

with the demonstration that carnitine palmityltransferase is associated with the inner membrane of rat liver mitochondria (Norum et al., 1966; Yates and Garland, 1966) and the conclusion that the enzymes of β-oxidation are within a compartment inside the carnitine barrier (Chapelle and Crofts, 1965).

The location of the enzymes of fatty acid oxidation in the inner membrane-matrix fraction of rat liver mitochondria does not agree with the results of Allmann et al.(1966a), who concluded that the outer membrane was the site of the β-oxidation enzymes in beef heart mitochondria fractionated by the phospholipase method of Allmann et al.(1966b). One interpretation of this difference is that heart and liver mitochondria may differ in the location of the fatty acid oxidation system; however, this does not seem likely, since Parsons et al. (1967) have concluded that the outer membrane fraction obtained by the phospholipase method is contaminated with soluble enzymes associated with the inner membrane or matrix.

The complete oxidation of palmitate by the inner membrane-matrix fraction indicates that acyl-CoA synthetase is also present in this fraction. Norum et al. (1966) suggested that acyl-CoA synthetase is located in the outer membrane of rat liver mitochondria; however, in their method of fractionation, involving sonication, over 50% of the synthetase activity was lost. This raises the possibility that this enzyme may be less tightly bound to the inner membrane than the other enzymes of β-oxidation and that it may not be the limiting enzyme, since 95% of the total fatty acid oxidation activity of the intact mitochondria was recovered in the inner membrane-matrix fraction.

Acknowledgment: This work was supported, in part, by Grant HD 02361 from the National Institutes of Health.

REFERENCES

Allmann, D. W., Galzigna, L., McCaman, R. E., and Green, D. E. Arch. Biochem. Biophys. 117, 413 (1966a).

Allmann, D. W., Bachmann, E., and Green, D. E. Arch. Biochem. Biophys. 115, 165 (1966b).
Beattie, D. S. and Basford, R. E. J. Neurochem. 12, 103 (1965).
Chappelle, J. B. and Crofts, A. R. Biochem. J. 95, 707, (1965).
Fritz, I. B. and Yue, K. T. N. Lipid Research 4, 279 (1963).
Mii, S. and Green, D. E. Biochim. Biophys. Acta 13, 425 (1954).
Norum, K., Farstad, M., and Bremer, J. Biochem. Biophys. Res. Comm. 24, 797 (1966).
Parsons, D. F., Williams G. R., Thompson, W., Wilson, D. F., and Chance, B. (1967). In Symposium on Mitochondrial Structure and Compartmentation, Tager, J. M., Papa, S., Quagliariella, E., and Slater, E. C., Ed., Bari, Italy, Adriatica Editrice (in press).
Schnaitman, C. A. and Greenawalt, J. W. J. Cell Biol. 35, 122A (1967).
Schnaitman, C. A., Erwin, V., and Greenawalt, J. J. Cell Biol. 32, 719 (1967).
Schneider, W. C. In Manometric Techniques, Umbreit, W. W., Burris, R., and Stauffer, J. F., Ed., Burgess, Minneapolis. (1957).
Sottocasa, G., Kuylenstierna, B., and Ernster, L. J. Cell Biol. 32, 415 (1967).
Yates, D. and Garland, P. Biochem. Biophys. Res. Comm. 23, 460 (1966).
Yates, D., Shepherd, D., and Garland, P. Nature 209, 1213 (1966).