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Characterisation of a novel enzyme of human fatty acid β-oxidation: a matrix-associated, mitochondrial 2-enoyl-CoA hydratase

Sandra Jackson*[1], Jochen Schaefer*, Bruce Middleton‡ and Douglass M. Turnbull*

*Division of Clinical Neuroscience, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K.

‡Department of Biochemistry, University of Nottingham Medical School, Nottingham NG7 2UH, U.K.

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Summary We present evidence for the existence of a previously unrecognised enzyme of mitochondrial β-oxidation in man. This enzyme, which is situated in the mitochondrial matrix and has medium- and long-chain 2-enoyl-CoA hydratase activity, was identified by studying tissues from a patient with a severe deficiency of the trifunctional protein of mitochondrial β-oxidation. The novel enzyme is present in all tissues studied - heart, liver and cultured skin fibroblasts - but is particularly active in liver. Together with other recently identified enzymes of mitochondrial β-oxidation, the existence of this new enzyme suggests that two mitochondrial β-oxidation enzyme systems exist in man: one associated with the mitochondrial membrane, responsible for the β-oxidation of long-chain fatty acids and one in the mitochondrial matrix, responsible for the oxidation of medium- and short-chain fatty acids.

The mitochondrial β-oxidation of saturated fatty acids proceeds by a repeated sequence of four reactions, catalysed in turn by the acyl-CoA dehydrogenases, the 2-enoyl-CoA hydratases, the 3-hydroxyacyl-CoA dehydrogenases and the 3-ketoacyl-CoA thiolases. Until recently it was assumed that all of the enzymes involved in this process had been identified and that, with the exception of long-chain 3-hydroxyacyl-CoA dehydrogenase (1) and long-chain 2-enoyl-CoA hydratase (2), all were located in the mitochondrial matrix. However, two additional enzymes of mitochondrial β-oxidation have now been identified, a very long-chain acyl-CoA dehydrogenase (3) and a trifunctional enzyme of β-oxidation (4-6), both of which are located in the mitochondrial inner membrane and are involved in the oxidation of long-chain fatty acids. The trifunctional enzyme catalyzes the 2-enoyl-CoA hydration, 3-hydroxyacyl-CoA dehydrogenation and 3-ketoacyl-CoA thiolysis of long- and medium-chain acyl-CoA esters, but is inactive towards short-chain (C_4) substrates. It is composed of two non-identical subunits: a large 78 kDa (α-) subunit and a smaller 50 kDa (β-) subunit which are present in equimolar amounts. The α-subunit catalyses the 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, while the β-subunit catalyses the 3-ketoacyl-CoA thiolase activity (7, 8). The long-chain 2-enoyl-CoA

[1] To whom correspondence should be addressed. Fax: +44-191-2228553.

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hydratase partially purified from pig heart mitochondria by Schulz (2) was subsequently shown to be part of the trifunctional protein (6).

To date, only two 2-enoyl-CoA hydratases have been identified in mammalian mitochondria : the 2-enoyl-CoA hydratase component of the trifunctional protein and a matrix associated, short-chain 2-enoyl-CoA hydratase (EC 4.2.1.17) (9-11). The latter enzyme is most active towards short-chain (C_4) substrate, but does have some activity towards longer-chain length substrates. However, this activity rapidly decreases with increasing chain length of the substrate (11). Our earlier studies in mitochondria isolated from various tissues from a patient with trifunctional protein deficiency showed that despite very low activity of long-chain 3-hydroxyacyl-CoA dehydrogenase and long-chain 3-ketoacyl-CoA thiolase, there was considerable residual 2-enoyl-CoA hydratase activity with 2,3-tetradecenoyl-CoA as substrate. (12). We now present further studies in this patient which demonstrate the presence of a third, matrix-associated 2-enoyl-CoA hydratase in both human liver and cardiac mitochondria. The discovery of yet another enzyme involved in mitochondrial β-oxidation has important implications for our understanding of the organisation of this pathway.

Materials and Methods

Materials. C_{4-16} acyl-chlorides and tetrahydrofuran were obtained from Aldrich (Dorset, UK). Coenzyme A, ammonium persulphate, goat anti-rabbit IgG (whole molecule) peroxidase conjugate, electrophoresis grade acrylamide, N,N,N'N-tetramethylethylenediamine and polyoxyethylene-sorbiton monolaurate were all from Sigma (Dorset, UK). Acyl-CoA oxidase (Arthrobacter species) was from Boehringer Mannheim UK (Sussex, UK). All other chemicals were from Merck Ltd (Dorset, UK). ECL Western blotting detection system was from Amersham International plc (Amersham, UK).

Generation of antibodies. The human trifunctional protein was purified from infant liver mitochondria as previously described (5) and antibodies were generated in rabbit to the individual subunits. Antibodies were also raised in rabbit to bovine liver short-chain 2-enoyl-CoA hydratase (Sigma), to pig heart short-chain 3-hydroxyacyl-CoA dehydrogenase (Sigma) and to purified pig liver medium-chain acyl-CoA dehydrogenase (13).

Preparation of mitochondrial fractions. Mitochondrial fractions from muscle, heart and liver were prepared from controls and from a patient with trifunctional protein deficiency (12).
Immunoblotting. Mitochondrial proteins and purified human trifunctional protein (0.05μg) were separated by SDS-PAGE (14) and transferred to nitrocellulose (15). The immunoreactive 78kDa subunit and 50kDa subunit proteins were then detected with an ECL Western blotting detection system (Amersham). The immune complex was then stripped from the nitrocellulose by incubation in 62.5 mM Tris, pH 6.7, 100mM mercaptoethanol, 2.5% SDS for 1 hour at 70°C and the nitrocellulose was reprobed with anti-medium-chain acyl-CoA dehydrogenase (MCAD) antibody.

Measurement of enzyme activity. 2-Enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities were measured spectrophotometrically as previously described (16). 3-Ketoacyl-CoA esters were synthesised as previously described (16).

Immunoprecipitation of short-chain 2-enoyl-CoA hydratase. The short-chain 2-enoyl-CoA hydratase was immunoprecipitated from sonicated, detergent-solubilised mitochondrial extracts as previously described for the short-chain hydroxyacyl-CoA dehydrogenase (12).

Subfractionation of mitochondria and measurement of enzyme activity in membrane and matrix fractions. Liver mitochondria from the patient and two controls were diluted and sonicated as described previously (12), then a known volume of this suspension was centrifuged at 100,000g_{av} for 1 hour at 4°C. The high speed pellet (membrane fraction) was

resuspended by homogenisation in the original volume of starting buffer and Triton X-100 (ratio of 1:1(w/w) Triton X-100 to protein) was added to both the high speed pellet and high speed supernatant. After 30 minutes on ice, both fractions were centrifuged at 11,600g_{av} for 10 minutes at 4°C. The two low speed supernatants thus obtained were divided into four and then incubated on ice for 2 hours either with or without the addition of antibody generated to short-chain 2-enoyl-CoA hydratase, or with or without antibody generated to short-chain 3-hydroxyacyl-CoA dehydrogenase. Antibody was added in sufficient concentration to ensure maximum immunoprecipitation of the short-chain specific enzymes as previously determined. Following centrifugation at 11,600g_{av} for 10 minutes at 4°C, 3-hydroxyacyl-CoA dehydrogenase activity was determined using C_4 and C_{16} substrate and 2-enoyl-CoA hydratase activity was measured using C_4, C_{10}, C_{12} and C_{14} substrate in the relevant fractions.

Results

Immunoreactive trifunctional enzyme protein. In comparison to controls there was a virtual absence of both subunits in every tissue studied from the patient (Figure 1). However, comparable amounts of immunoreactive medium-chain acyl-CoA dehydrogenase protein were detected in the patient and controls when the immunoblots were stripped and reprobed with antibody to this protein.

2-Enoyl-CoA hydratase activity in cardiac and liver mitochondria. Measurement of the 2-enoyl-CoA hydratase activity in cardiac and liver mitochondria from the patient revealed that despite a virtual absence of immunoreactive trifunctional protein in these tissues there is still considerable residual activity with medium- and long-chain substrates (Table 1). This residual activity is much more pronounced in liver, the activity with each substrate being similar in mitochondria from the patient and control subjects in this tissue. Even after immunoprecipitation of the short-chain enzyme, which removes any overlapping 2-enoyl-CoA hydratase activity due to this enzyme, the activity with all 2-enoyl-CoA esters of chain-length C_4-C_{16} is similar in liver mitochondria from the patient and controls (Table 1). This demonstrates that the high residual hydratase activity in this tissue is not due to the short-chain enzyme and strongly suggests the existence of a third, highly active 2-enoyl-CoA hydratase in liver mitochondria. Similar results were obtained in cardiac mitochondria after immunoprecipitation of the short-chain 2-enoyl-CoA hydratase (Table 1). However, the residual activity in cardiac mitochondria from the patient is considerably less than that in controls. This indicates that the contribution of the trifunctional protein 2-enoyl-CoA hydratase to the total 2-enoyl-CoA hydratase activity is greater in this tissue than in liver.

Chain-length specificity of 2-enoyl-CoA hydratases in human liver and cardiac mitochondria. Any residual 2-enoyl-CoA hydratase activity measured in mitochondria from the patient after immunoprecipitation of the short-chain enzyme must be due to the novel enzyme. In liver mitochondria this enzyme is most active with medium-chain substrates (Figure 2A), hence it seems appropriate to name it medium chain 2-enoyl-CoA hydratase. The substrate profile of the short-chain enzyme can be deduced from the activity lost from mitochondria following immunoprecipitation of this enzyme. Thus, human short-chain 2-enoyl-CoA hydratase is active with all substrates from C_4 to C_{16}, although by far the greatest activity is with the C_4 substrate (Table 1, Figure 2B). The trifunctional enzyme is also active with all 2-enoyl-CoA substrates from C_6 to C_{16} with maximum activity towards the C_{10} substrate (Figure 2A).

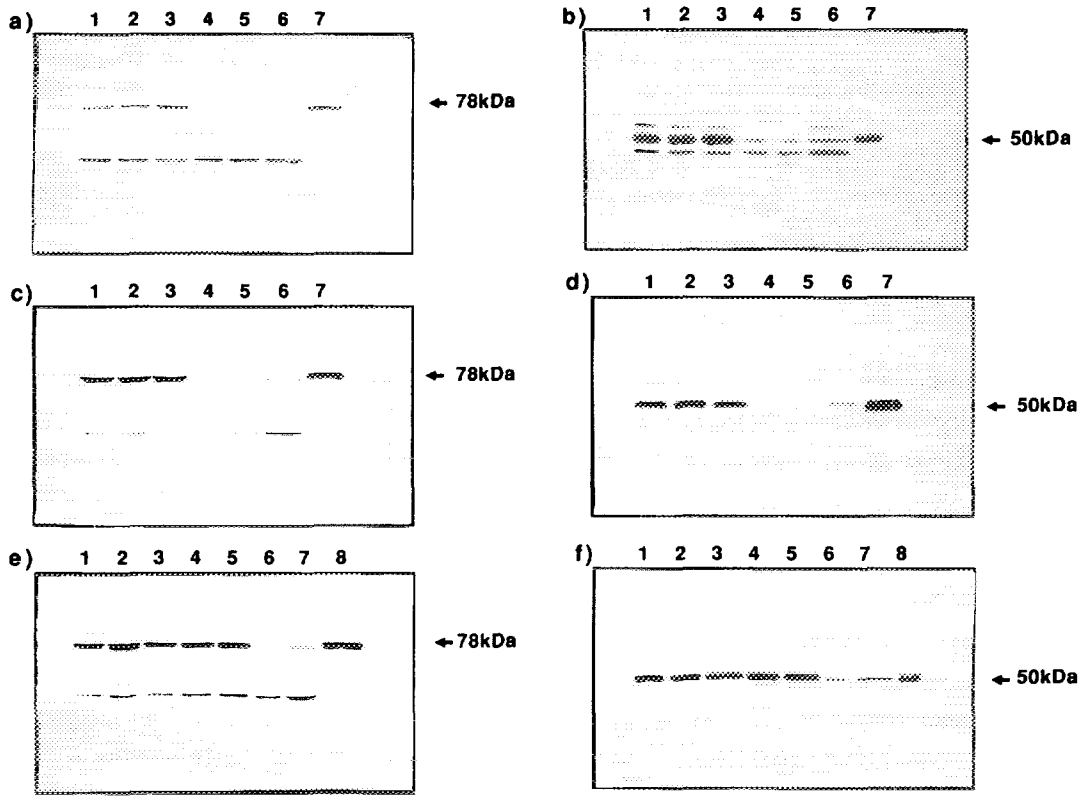

Figure 1. Immunoblot analysis of the 78kDa and 50kDa subunits of the mitochondrial trifunctional enzyme of b-oxidation in mitochondria isolated from various human tissues.

a) Liver mitochondria. Lanes 1-3, controls and lanes 4 and 5, patient (11.6 μg protein); lane 6, patient (23.2 μg protein); lane 7, 0.05 μg purified human liver trifunctional enzyme. **b)** As for a) except: lanes 1-5, 7.2 μg protein; lane 6, 14.4 μg protein. **c)** Cardiac mitochondria. Lanes 1-3, controls and lanes 4 and 5, patient (10 μg protein); lane 6, patient (20 μg protein); lane 7, 0.05 μg purified human trifunctional enzyme. **d)** as for c) except: lanes 1-5, 4 μg protein; lane 6, 8 μg protein. **e)** Muscle mitochondria. Lanes 1-5, controls (11.6 μg protein); lanes 6 and 7, patient (11.6 and 23.2 μg protein respectively); lane 8, 0.05 μg of purified human trifunctional enzyme. **f)** As for e) except: lanes 1-6, 4 μg protein; lane 7, 8 μg protein. Immunoblots a), c) and e) were probed with antibody to the 78 kDa subunit; b), d) and f) were probed with antibody to the 50 kDa subunit.

Intramitochondrial location of medium-chain 2-enoyl-CoA hydratase. Liver mitochondria from the patient and two control subjects were sonicated and centrifuged as described. To ensure that adequate separation into matrix and membrane fractions was achieved, the activity of short-chain 3-hydroxyacyl-CoA dehydrogenase (matrix-associated) and the long-chain 3-hydroxyacyl-CoA dehydrogenase component of the trifunctional protein (membrane-associated) were measured. The long-chain 3-hydroxyacyl-CoA dehydrogenase activity in the two fractions was determined after immunoprecipitation of the short-chain 3-hydroxyacyl-CoA dehydrogenase, as the short-chain enzyme has considerable overlapping activity towards the long-chain substrate and would confuse the results (12). In mitochondria from the two controls, virtually all of the short-chain 3-hydroxyacyl-CoA dehydrogenase activity was in the matrix fraction (supernatant), whereas nearly all of the long-chain activity was in the membrane fraction

Table 1 2-Enoyl-CoA hydratase activity in normal and deficient human liver and cardiac mitochondria

Chain length of substrate	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}
Liver							
No immunoprecipitation							
Control - mean ± SD (n=3)	14.54±1.8	5.40±1.3	5.20±1.0	4.54±0.5	2.73±0.1	1.66±0.1	1.0±0.04
Patient	11.69	4.61	4.31	3.38	1.98	1.26	0.73
After immunoprecipitation of short-chain 2-enoyl-CoA hydratase							
Control - mean ± SD (n=3)	0.93±0.4	2.33±0.6	2.73±0.7	2.65±0.4	2.05±0.3	1.22±0.1	0.72±0.03
Patient	0.95	2.34	2.35	1.74	1.57	1.03	0.52
Patient's activity as a percentage of control mean	102	100	86.1	65.7	76.6	84.4	71.2
Cardiac							
No immunoprecipitation							
Control 1 and 2	3.18, 3.02	2.04, 2.02	2.00, 2.08	2.67, 2.61	1.51, 1.53	1.56, 1.65	0.73, 0.85
Patient	2.41	0.90	0.79	0.89	0.55	0.41	0.26
After immunoprecipitation of short-chain 2-enoyl-CoA hydratase							
Control 1 and 2	0.21, 0.15	1.39, 1.49	1.42, 1.46	2.34, 2.20	1.32, 1.33	1.49, 1.60	0.73, 0.84
Patient	0.19	0.27	0.33	0.49	0.44	0.35	0.26
Patient's activity as a percentage of control mean	105.6	18.8	22.9	21.6	33.3	22.7	33.1

Values are μmol 2-enoyl-CoA hydrated min^{-1} mg $protein^{-1}$ unless expressed as a percentage.

(pellet) (Table 2). Likewise, in the patient virtually all of the the short-chain 3-hydroxyacyl-CoA dehydrogenase activity was in the soluble fraction, whereas no long-chain 3-hydroxyacyl-CoA dehydrogenase activity was detected in either fraction.

The short-chain 2-enoyl-CoA hydratase activity was present predominantly in the matrix fraction in mitochondria from both the patient and controls. The activity with medium- and long-chain substrates was measured after immunoprecipitating the short-chain enzyme in order to remove activity towards these substrates due this enzyme. In control mitochondria, 2-enoyl-CoA hydratase activity with medium-chain and long-chain substrates was fairly evenly divided between the matrix and membrane fractions (Table 2). This is in contrast to the findings with the long-chain 3-hydroxyacyl-CoA dehydrogenase activity which was only found in the membrane fraction. Since the long-chain 2-enoyl-CoA hydratase component of the trifunctional enzyme is associated with the long-chain 3-hydroxyacyl-CoA dehydrogenase component of the trifunctional protein, and hence with the membrane fraction, the additional medium- and long-chain 2-enoyl-CoA hydratase activity in the matrix fraction must be due to the novel enzyme. In liver homogenate from the patient a much higher proportion of the activity with all three substrates was found in the matrix fraction when compared with controls (Table 2). This again confirms that the newly identified medium-chain 2-enoyl-CoA hydratase is predominantly in the matrix fraction.

Discussion

Existence of a medium-chain 2-enoyl-CoA hydratase in humans. Immunoprecipitation of the short-chain 2-enoyl-CoA hydratase in liver and cardiac mitochondria from a patient with a severe deficiency of immunoreactive trifunctional protein failed to completely remove all residual 2-enoyl-CoA hydratase activity. In addition, mitochondrial subfractionation combined with immunoprecipitation of the short-chain enzyme demonstrated that this residual 2-

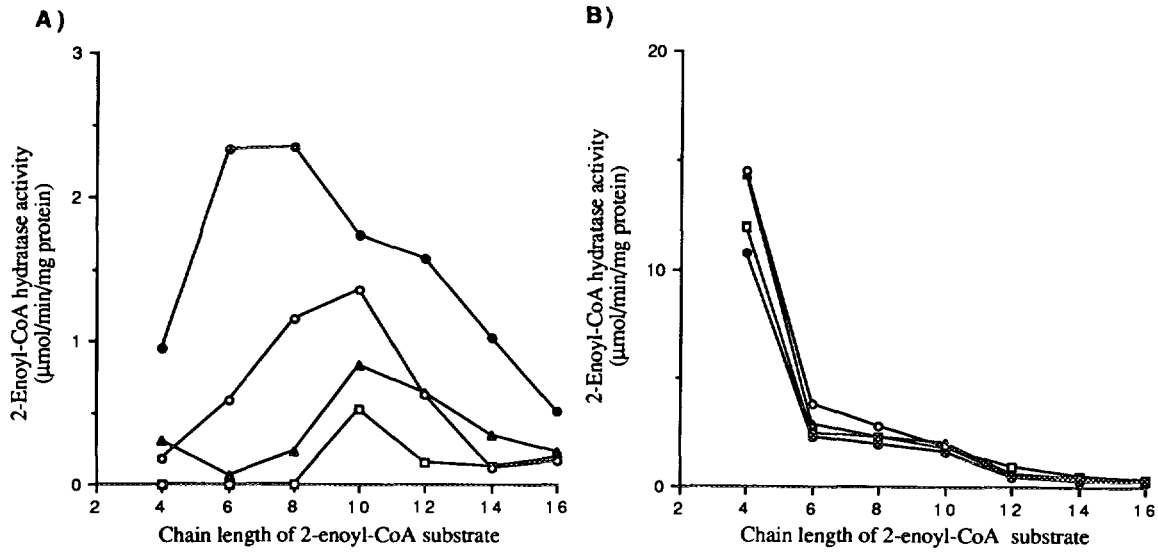

Figure 2. Substrate profile of the three liver mitochondrial 2-enoyl-CoA hydratases.
A) Trifunctional enzyme 2-enoyl-CoA hydratase activity in three controls (—□— ; —▲— ; —○—). The activity shown is the difference in 2-enoyl-CoA hydratase activity between the patient and each of the controls after immunoprecipitation of the short-chain 2-enoyl-CoA hydratase.

Novel medium-chain 2-enoyl-CoA hydratase (—●—) activity. The activity shown is the residual 2-enoyl-CoA hydratase activity in the patient after immunoprecipitation of the short-chain 2-enoyl-CoA hydratase.

B) The short-chain 2-enoyl-CoA hydratase activity in liver mitochondria from three controls (—□— ; —▲— ; —○—) and patient 1 (—●—) with substrates of different chain length. The activity shown is the difference between the 2-enoyl-CoA hydratase activity measured before and after immunoprecipitation of the short-chain 2-enoyl-CoA hydratase.

enoyl-CoA hydratase activity is matrix-associated and partitions independently of the trifunctional enzyme.This demonstrates the existence of a third mitochondrial 2-enoyl-CoA hydratase in human cardiac and liver. We have performed similar studies in cultured skin fibroblasts from the patient and controls and have demonstrated the presence of the novel enzyme in this tissue as well.

Preliminary studies in pig liver mitochondria indicate that the third 2-enoyl-CoA hydratase is also present in pig. A mitochondrial matrix fraction was prepared and applied to an anion-exchange column. Two peaks of activity were obtained. The second peak was shown to be due to short-chain 2-enoyl-CoA hydratase (crotonase) and a small amount of residual trifunctional

Table 2 Fractionation of 2-enoyl-CoA hydratase activity in human liver mitochondria

	3-Hydroxyacyl-CoA dehydrogenase activity				Short-chain 2-enoyl-CoA hydratase activity		2-Enoyl-CoA hydratase activity after immunoprecipitation of the short-chain enzyme					
	short-chain		long-chain		C_4 as substrate		C_{10} as substrate		C_{12} as substrate		C_{14} as substrate	
	Matrix fraction	Membrane fraction	Matrix fraction	Membrane fraction	Matrix fraction	Membrane fraction	Matrix fraction	Membrane fraction	Matrix fraction	Membrane fraction	Matrix fraction	Membrane fraction
Control 1	97.1	2.9	22.8	77.2	96.4	3.6	39.6	60.4	48.6	51.4	42.2	57.8
	97.2	2.9	17.3	82.7	95.0	5.0	ND	ND	49.2	50.8	ND	ND
	98.2	1.8	18.4	81.6	95.7	4.3	ND	ND	49.7	50.3	ND	ND
Control 2	96.5	3.5	18.8	81.2	95.3	4.7	48.3	51.7	57.9	42.1	53.6	46.4
Patient	97.8	2.2	-	-	95.1	4.9	63.6	36.4	73.5	26.5	70.6	29.4

Results are expressed as % of total combined activity in the high speed pellet and supernatant. ND is not done.
Short-chain HOAD activity was measured using C_4 as substrate. Long-chain HOAD activity was measured using C_{16} as substrate after immunoprecipitation of the short-chain enzyme.

protein. The first peak was applied to a cation-exchange column. A peak of 2-enoyl-CoA hydratase activity was obtained which was shown by gel electrophoresis to be free of both the short-chain enzyme and the trifunctional protein, confirming the presence of the novel enzyme in this tissue.

The novel enzyme is active with medium and long-chain substrates. Although the substrate profile of the newly identified enzyme is similar to that of the long-chain 2-enoyl-CoA hydratase component of the trifunctional protein, the former protein is matrix associated whilst the latter is membrane associated. The existence of a third 2-enoyl-CoA hydratase which is matrix-associated, would allow the complete β-oxidation of medium- and short-chain fatty acids in the mitochondrial matrix. This suggests that two β-oxidation systems exist in mitochondria. In one system long-chain fatty acids are activated to their CoA-ester at the cytoplasmic surface of the outer mitochondrial membrane, enter mitochondria via the carnitine dependent transport system and are β-oxidised to their medium-chain CoA derivatives via the concerted action of the inner membrane associated enzymes (very long-chain acyl-CoA dehydrogenase and the trifunctional protein). In the other system medium and short-chain fatty acids enter the mitochondria directly, are activated to their CoA-esters in the matrix and are β-oxidised (along with the chain-shortened products of the membrane-bound long-chain fatty acid oxidation) by the combined activities of the matrix enzymes.

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