The mitochondrial trifunctional protein: centre of a $\beta$-oxidation metabolon?

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Abstract

The trifunctional enzyme comprises three consecutive steps in the mitochondrial $\beta$-oxidation of long-chain acyl-CoA esters: 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. Deficiencies in either 3-hydroxyacyl-CoA dehydrogenase activity, or all three activities, are important causes of human disease. The dehydrogenase and thiolase have a requirement for NAD$^+$ and CoA respectively, whose levels are conserved within the mitochondrion and thus provide possible means for control and regulation of $\beta$-oxidation. Using analysis of the intact CoA ester intermediates produced by the complex, we have examined the sensitivity of the complex to NAD$^+$/NADH and acetyl-CoA. We consider the evidence for channelling within the trifunctional protein and propose a model for a $\beta$-oxidation ‘metabolon’.

Introduction

The mitochondrial trifunctional protein of $\beta$-oxidation was purified to homogeneity after the

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identification of patients deficient in one or more activities of the protein [1-4]. The enzyme comprises three of the four activities required for β-oxidation of long-chain acyl-CoA esters: long-chain 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase; only a long-chain acyl-CoA dehydrogenase is lacking. The protein consists of two subunits, with 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities on the α-subunit, and 3-ketoacyl-CoA thiolase on the β-subunit. There is a requirement for NAD⁺ in the reaction catalysed by 3-hydroxyacyl-CoA dehydrogenase, and for CoA in the reaction catalysed by 3-ketoacyl-CoA thiolase (Figure 1). The genes for both rat and human α- and β-subunits have subsequently been cloned, allowing molecular analysis of defects and the identity of the enzyme catalytic domains and their relationship with other enzymes of β-oxidation to be defined [5,6].

**Human trifunctional protein deficiency**

Deficiencies in enzyme activities of the trifunctional protein have been recognized as important causes of hypoketotic hypoglycaemia in the neonatal period. Defects in the trifunctional protein fall into two groups: patients with an isolated defect in 3-hydroxyacyl-CoA dehydrogenase and those with a deficiency of all three activities and absence of immunoreactive protein [7]. Many cases of isolated 3-hydroxyacyl-CoA dehydrogenase activity are caused by a G1528C point mutation, which results in a glutamate-to-glutamine change in the α-subunit [8]. This position is within the putative active site and is conserved in other 3-hydroxyacyl-CoA dehydrogenases. Patients in the second group have been found to have either deletions in the α-subunit cDNA or point mutations in the β-subunit [9,10]. As these mutations were found to result in greatly decreased stability of both subunits, it appears that formation of a complex is important. In support of this, the individual subunits are unstable when expressed heterologously [11]. It is also of particular interest that the genes for the α- and β-subunits are adjacent to each other, but head-to-head, on chromosome 2p23, and are probably transcribed from the same bidirectional promoter region [12]; hence expression and induction of the two subunits may be co-ordinated.

There are several particularly interesting features of trifunctional protein deficiency which may depend on subtle differences between, and molecular properties of, mutant proteins. The first of
Figure 2

Annotated sequences of (top panel) α- and (bottom panel) β-subunits of the trifunctional protein

The amino-acid sequences are those of the full-length precursors of the human trifunctional protein (TFP) [5]. Domain structures are from [5], signatures are from PROSITE [30], mutations are those described by [8–10] and active-site residues calculated by homologies with short-chain 3-hydroxyacyl-CoA dehydrogenase and crotonase [31]. ECH, 2-enoyl-CoA hydratase; HOAD, 3-hydroxyacyl-CoA dehydrogenase.
these is the association between trifunctional protein deficiency and acute fatty liver of pregnancy. Mothers carrying foetuses with 3-hydroxyacyl-CoA dehydrogenase deficiency, especially those with a particular point mutation in the α-subunit, are at risk of developing the life-threatening condition of acute fatty liver of pregnancy [8,13]. Most β-oxidation defects present with symptoms in various tissues, especially those with a high requirement for β-oxidation (i.e. muscle, heart and liver) [14]; a striking observation in trifunctional protein deficiency is the presence of ophthalmological abnormalities in many patients [15], a finding which is not present in patients with defects in either medium-chain acyl-CoA dehydrogenase or carnitine palmitoyltransferase II, the other common β-oxidation defects. A further unusual finding in patients with deficiencies in the trifunctional protein is the presence of lactic acidosis [7]. This is more typical of a respiratory chain defect and has been associated with a decrease in complex-I activity in skeletal-muscle mitochondria [16]. It has been hypothesized that this lactic acidosis is due to the accumulation of toxic acyl-CoA esters in patients defective in the trifunctional protein [16,17].

Catalytic properties of the enzyme
The α-subunit of the enzyme comprises the 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities of the enzyme and the β-subunit comprises the 3-ketoacyl-CoA thiolase activity of the enzyme. The sequences of the two subunits, with catalytic features and known point mutations, are indicated in Figure 2. In addition, the α-subunit has significant homology with Δ⁹,Δ⁷-enoyl-CoA isomerase between residues 47 and 178 [5]. However, Δ⁹,Δ⁷-enoyl-CoA isomerase activity has not been detected in trifunctional protein isolated from human liver [2], pig heart [18] or rat [6]. The Δ⁹,Δ⁷-enoyl-CoA isomerase active site is similar to that of 2-enoyl-CoA hydratases [19] and several other multifunctional β-oxidation enzymes have both activities at the same active site [20]. The reason for the lack of Δ⁹,Δ⁷-enoyl-CoA isomerase activity when long-chain Δ⁹,Δ⁷-enoyl-CoA isomerase activity is necessary for the β-oxidation of long-chain polyunsaturated fatty acids is not known. The structural features of the active sites of the isomerase/hydratase family that confer either hydratase or isomerase activity or both on an enzyme are being investigated [21]. The substrate chain-length specificities of the three component enzyme activities are highest with substrates of chain length between 12 and 16 carbon atoms and virtually absent with 4-carbon-atom substrates [1,2]. However, short-chain 3-hydroxyacyl-CoA dehydrogenase, short-chain 2-enoyl-CoA hydratase and medium-chain 3-ketoacyl-CoA thiolase each have some activity towards long-chain CoA esters so that there is some residual activity with long-chain substrates in patients with trifunctional protein deficiency even in the absence of immunoreactive protein [4].

Co-factor requirement
As the overall reaction of the trifunctional protein requires NAD⁺ and CoA, both of which are moieties conserved within the mitochondrion, turnover of NAD⁺/NADH (by complex I of the respiratory chain) and CoA/acetyl-CoA (by citrate synthase, the 3-hydroxy-3-methylglutaryl-CoA cycle or carnitine acetyl transferase) provide possible intramitochondrial controls over β-oxidation flux. We have investigated the sensitivity of the isolated trifunctional protein to NAD⁺/NADH (by using a pyruvate/lactate/lactate dehydrogenase system to set the ratio NAD⁺:NADH) and CoA/acetyl-CoA (similarly using carnitine/acetyl carnitine/acetyl carnitine acetyl transferase to set the CoA:acetyl-CoA ratio) and found that the trifunctional protein was not very sensitive to NAD⁺/NADH but was more sensitive to CoA/acetyl-CoA [22]. However, we have suggested that there is a pool of NAD⁺/NADH that is channelled between complex I and the trifunctional protein so that turnover of this pool may be important in controlling β-oxidation flux in the intact mitochondrion [23,24].

Channelling within the trifunctional protein
Although 3-ketoacyl-CoA esters are readily detected as intermediates of peroxisomal β-oxidation [25], we have not detected their presence as intermediates of mitochondrial β-oxidation in any tissue studied [26]. Even in patients with profound deficiencies of 3-ketoacyl-CoA thiolase as part of a deficiency of the trifunctional protein, there is no accumulation of 3-ketoacyl-CoA esters [4,27]. This could reflect channelling of 3-ketoacyl-CoA esters between the active sites of the 3-hydroxyacyl-CoA dehydrogenase (on the α-subunit) and the 3-ketoacyl-CoA thiolase (on the β-subunit). Yao and Schulz [28] have indeed provided evidence for channelling of 3-ketoacyl-CoA on the basis of a discrepancy between the expected flux through the complex (calculated from the kinetic
properties of the individual enzymes and the observed concentration of 3-ketoacyl-CoA esters) and the observed flux, which is very much faster. We have also found evidence for channelling between these two active sites on the basis of differential effects of detergents on the 3-ketoacyl-CoA thiolase and the flux through the whole complex (S. Eaton, B. Middleton and K. Bartlett, unpublished work). When intact rat heart mitochondria are incubated with a novel inhibitor of long-chain 3-ketoacyl-CoA thiolase activity, which does not directly inhibit long-chain 3-hydroxyacyl-CoA dehydrogenase activity (when measured under conditions that detect only this step), 3-ketoacyl-CoA esters do not accumulate, whereas 3-hydroxyacyl-CoA esters do accumulate to a great extent (S. Eaton, T. Bursby, B. Middleton and K. Bartlett, unpublished work), providing a correlate to both the observations in the isolated trifunctional protein and in patients deficient in 3-ketoacyl-CoA thiolase activity. We are at present attempting to elucidate a structural basis for this channelling. So far the presence or absence of channelling between the 2-enoyl-CoA hydratase active site and the 3-hydroxyacyl-CoA dehydrogenase has not been determined.

A model for a β-oxidation metabolon

Given the presence of three of the four activities required for the β-oxidation of long-chain saturated fatty acids in a single membrane-associated protein (although the domains of the trifunctional protein that bind to the membrane are unknown), and the membrane association of very-long-chain acyl-CoA dehydrogenase (VLCAD) [29], complex I and electron-transfer flavoprotein (ETF):ubiquinone oxidoreductase, the presence of a β-oxidation ‘metabolon’ associated with the inner membrane seems possible. One possible such model is shown in Figure 3; in this model, the trifunctional protein is the centre of a metabolon which has its own pools of NAD+/NADH and ETF/ETFH₂, receives 2-enoyl-CoA from VLCAD, passes NADH $+H^+$ directly to complex

**Figure 3**

A model for mammalian mitochondrial β-oxidation

ACD, acyl-CoA dehydrogenase; CPT, carnitine palmitoyltransferase; 2-ECH, 2-enoyl-CoA hydratase; 3-HOAD, 3-hydroxyacyl-CoA dehydrogenase; 3-KAT, 3-ketoacyl-CoA thiolase; ETF, oxidized electron transfer flavoprotein; ETFH, reduced electron transfer flavoprotein; ETF:QO, electron-transfer flavoprotein:ubiquinone oxidoreductase; complex I, NADH:ubiquinone (UQ) oxidoreductase. From [26] with permission.
I and returns chain-shortened acyl-CoA to VLCAD, unless the chain length has decreased far enough for the CoA ester to become a better substrate for the matrix ‘soluble’ β-oxidation system. Acyl-CoA esters could be transferred between active sites either by channelling or by ‘surface crawling’ along the matrix-facing side of the inner mitochondrial membrane due to their hydrophobic nature. However, the precise biology of such a metabolon remains unknown.

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References

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The carnitine acyltransferases: modulators of acyl-CoA-dependent reactions

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Abstract
Carnitine and carnitine acyltransferases were thought to be merely a mechanism for the rapid transfer of activated long-chain fatty acids into the mitochondrion for β-oxidation [1], until enzymologists came along. By kinetic, physical and localization studies, eight different mammalian carnitine acyltransferases have been characterized (reviewed in [2,3]). Of these, five have been cloned and sequenced. The carnitine:acylcarnitine exchange carrier, first characterized in mitochondria...