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Mutations at the flavin binding site of ETF:QO yield a MADD-like severe phenotype in *Drosophila*

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ABSTRACT

Following a screening on EMS-induced *Drosophila* mutants defective for formation and morphogenesis of epithelial cells, we have identified three lethal mutants defective for the production of embryonic cuticle. The mutants are allelic to the *CG12140* gene, the fly homologue of electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO). In humans, inherited defects in this inner membrane protein account for multiple acyl-CoA dehydrogenase deficiency (MADD), a metabolic disease of β-oxidation, with a broad range of clinical phenotypes, varying from embryonic lethal to mild forms. The three mutant alleles carried distinct missense mutations in ETF:QO (G65E, A68V and S104F) and maternal mutant embryos for ETF:QO showed lethal morphogenetic defects and a significant induction of apoptosis following germ-band elongation. This phenotype is accompanied by an embryonic accumulation of short- and medium-chain acylcarnitines (C4, C8 and C12) as well as long-chain acylcarnitines (C14 and C16:1), whose elevation is also found in severe MADD forms in humans under intense metabolic decompensation. In agreement the ETF:QO activity in the mutant embryos is markedly decreased in relation to wild type activity. Amino acid sequence analysis and structural mapping into a molecular model of ETF:QO show that all mutations map at FAD interacting residues, two of which at the nucleotide-binding Rossmann fold. This structural domain is composed by a β-strand connected by a short loop to an α-helix, and its perturbation results in impaired cofactor association via structural destabilisation and consequently enzymatic inactivation. This work thus pinpoints the molecular origins of a severe MADD-like phenotype in the fruit fly and establishes the proof of concept concerning the suitability of this organism as a potential model organism for MADD.

1. Introduction

Multiple acyl-CoA dehydrogenase deficiency (MADD, OMIM: 231680), also known as glutaric aciduria type II, is an inherited disorder of the mitochondrial fatty acid β-oxidation (FAO) which results from defects in either electron transfer flavoprotein (ETF) or in electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO). These two proteins constitute a metabolic hub through which electrons shuttled by at least 11 acyl-CoA dehydrogenases (ACDHs), resulting from fatty acid oxidation, amino acid and choline catabolism [1], are transferred to the mitochondrial respiratory chain via the quinone pool. ETF is a dimer of 30 kDa (ETFα) and 28 kDa (ETFβ) subunits held by a structural AMP and containing a FAD which oxidises the different ACDHs shuttling electrons to ETF:QO, its redox partner. ETF:QO is a 64 kDa monomer which binds to the inner mitochondrial membrane possibly via an amphipatic helix, and it contains different cofactors ([4Fe–4S], FAD, ubiquinone) organised in distinct structural domains. Genetic lesions in any of the genes encoding for these proteins, frequently point mutations, yield rather distinct clinical phenotypes. These range from extremely severe forms resulting in death at the embryonic (type I) or neonatal stage (type II), to mild forms (type III) which can be controlled through diet and in some cases riboflavin [2], carnitine and CoQ10 supplementation. Nevertheless, even these 'mild' cases are frequently life-threatening,

Abbreviations: FAO, fatty acid β-oxidation; MADD, multiple acyl-CoA dehydrogenase deficiency; ETF:QO, electron transfer flavoprotein:ubiquinone oxidoreductase; ETF, electron transfer flavoprotein; CoA, coenzyme A; MCAD, medium chain acyl-CoA dehydrogenase; SCAD, short chain acyl-CoA dehydrogenase; EMS, ethyl methanesulfonate; FAD, flavin adenine dinucleotide

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for example in children as a result of metabolic decompensation during fever and sugar depletion.

The functional failure in ETF and ETF:QO variants carrying missense mutations has multiple origins, from defects in protein biogenesis, folding and stability, to catalytic impairment due to a perturbation of the cofactors. Among these, FAD plays a pivotal role, not only in ETF [3,4] and ETF:QO, but also in all acyl-CoA dehydrogenases which also harbour this organic cofactor [5]. In fact, the flavin moiety is determinant for protein structure and function: catalytically, it is very versatile as its oxidation–reduction properties can be fine-tuned through interactions with the polypeptide chain at the binding pockets [6,7], and the many structures available also show the important structural role it plays in the protein three-dimensional organisation [8–13]. This cross-talk is clearly illustrated in the Rossmann fold, a nucleotide binding structural domain also present in ETF:QO, which comprises a β-strand connected by a short loop to an α-helix, and includes an expanded sequence motif ($V/IxGx_{1-2}GxxGxxxG/A$) that affords both FAD binding and stabilisation of the secondary structure elements involved [14]. Small changes in the FAD interacting residues may change the redox potential of the flavin, its affinity towards the protein, or ultimately disrupt binding, with consequent enzymatic inactivation and structural disruption. Moreover, the relevance of flavins as cofactors also results from the fact that they are able to carry out both one and two electron transfer reactions [2].

In spite of a growing understanding of the biochemical details of defective processes in β-oxidation, as well as an increasing expansion of the genotypic and phenotypic characteristics of patients suffering from MADD, the fact is that cellular studies have been mostly limited to patient fibroblasts. However, recent studies have opened the perspective of implementing organism models for MADD by addressing the effects of defects in ETF:QO in zebrafish and in the nematode *Caenorhabditis elegans* [15,16]. These have been the cases of the study on the zebrafish mutant *xav* which corresponds to a non-sense mutation that results in an inactivating truncation of ETF:QO [15], and in the nematode, two mutant alleles of *let-721* which correspond to deleterious point mutations in ETF:QO [16]. These studies exemplify how model organisms can contribute to a broader biological understanding of processes affected in MADD—for example the zebrafish study elicited that neurogenesis is affected through the PPARG–ERK pathway and the nematode work puts in evidence that ETF:QO is expressed under complex transcriptional control.

In this work we report the genotypic, phenotypic and biochemical characteristics of a severe MADD-like phenotype in the fruit fly *Drosophila melanogaster*, establishing the proof of principle concerning the suitability of this organism as a potential model not only for MADD but also for inherited fatty acid disorders in general. Our focus on this study has been to correlate developmental defects of *Drosophila* mutants with specific alterations at the level of protein structure and stability. We have established that three independent mutant alleles, corresponding to three distinct point mutations in ETF:QO, are lethal as a result of a specific knock-down of FAD binding by direct disruption of the cofactor binding motif within the nucleotide binding Rossmann fold. This study thus provides a direct molecular rationale for the effect of specific mutations at the cellular and structural levels, and offers a platform to test in *Drosophila* a range of MADD-mutations yielding diverse clinical phenotypes, from severe to milder forms of the disease.

2. Materials and methods

2.1. Fly work

All *ETF:QO* alleles were isolated from a previously reported maternal screen [17]. All flies were raised at 25 °C using standard techniques. Riboflavin supplementation of the medium was also tested from 0.2 to 5 mM. To generate *ETF:QO* mutant clones (negative for nuclear GFP label; nGFP*minus*), females y, w, hsFLP; FRT42B nGFP/CyO hshid were crossed with males w; FRT42B, *ETF:QO*/CyO. The offspring was heat-shocked two times for 1 h at 37 °C during second and third *instar* larvae. To generate homozygous clones of *ETF:QO* in wing imaginal discs, female y, w, hsFLP; FRT42B nGFP/CyO hshid flies were crossed with w; FRT42B, *ETF:QO*/CyO males. The offspring was heat shocked for 1 h at 37 °C, 48 h after a 24-hour egg collection, corresponding to second *instar* larvae. Wing discs were dissected from crawling third *instar* larvae. Maternal mutant embryos for *ETF:QO* were obtained using the FLP/FRT*ovoD* system [18]. w; FRT42B *ETF:QO*/CyO virgins were crossed to y, w, hsFLP; FRT42B *ovoD*/CyO hshid males at 25 °C and the progeny was heat-shocked two times at 37 °C for 1 h during second and third *instar*.

2.2. Mapping and cloning of ETF:QO alleles

Complementation Group 1 mutant alleles were mapped using the Bloomington 2R deficiency kit to chromosome region 46C. By a candidate gene approach we concluded that all three different alleles of Group 1 (A56-12, B42-1 and B43-36) were most likely mutant alleles of *ETF:QO* as they failed to complement a lethal P-element (*w[1118]; PBac{w[+mC]=WH}CG12140[f05640]*) associated with the *ETF:QO* gene *locus*. Molecular characterisation of the isolated *ETF:QO* alleles identified different point mutations within the coding region of this gene. All mutations were independently sequenced three times from different PCR reactions and we used a mutant allele isolated in the same screen but from a different complementation group to identify single-nucleotide polymorphisms present in the original mutagenised stock.

2.3. Immunostainings

Third *instar* wing imaginal disc fixation and staining were performed using standard procedures [19]. For maternal phenotypic analysis, embryos were fixed and stained using standard procedures [17]. The oogenesis phenotypic analysis was performed with tissue dissected from 2 or 3 days old females where mutant clones were induced by heat-shock at second *instar* larvae stages, and fixed in phosphate buffered saline (PBS) with 4% formaldehyde for 20 min. Primary antibodies used were anti-Armadillo N2 7A1 mouse at 1:20 (Developmental Studies Hybridoma Bank (DSHB)), anti-Cleaved Caspase-3 rabbit at 1:500 (Cell Signaling, 9661S), anti-Neurotactin mouse clone BP106 at 1:133 (DSHB) and anti-pTyr mouse at 1:1000 (Cell Signaling, 9411). Secondary antibodies were Cy3- and Cy5-conjugated at 1:1000 (Jackson ImmunoResearch Laboratories, West Grove, PA). For F-Actin staining, Phaloidin-Rhodamine was used at 1:200 (Sigma) (stock concentration 1 $mg.ml^{-1}$) with 5 min incubation. Ovaries and embryos were mounted in fluorescent mounting medium (DakoCytomation, Inc.) and wing discs were mounted in Vectashield (Vector Laboratories). Samples were visualised with a Leica SP5 confocal microscope.

2.4. Acylcarnitine analysis

For each assay, approximately 20 mg of biological samples was used. The thawed embryos, which had been harvested 0–6 h after egg laying and stored in 20% Glycerol at −80 °C, were centrifuged at 12,000×*g* for 10 min to remove glycerol. Embryos were then resuspended in 200 µl of sucrose buffer (200 mM sucrose, 10 mM MOPS pH 7.2, 0.1 mM EDTA) and centrifuged again. Sucrose buffer was removed and the embryos were washed two more times before being homogenised with a pestle in sucrose buffer containing 0.5 mM of phenylmethylsulphonylfluoride (PMSF) (Roth). The solutions were sonicated during 30 s at 10% intensity 2 times, with an interval of 1 min, before being submitted to a speed vacuum in order to decrease the volume to approximately 50 µl. The embryo proteins were quantified by the Bradford method, and the same amount of protein was applied in the Whatman 903® filter paper and dried overnight away from light. Acylcarnitine analysis by MS/MS spectrometry was carried out as