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Cofactors and metabolites as potential stabilizers of mitochondrial acyl-CoA dehydrogenases

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ABSTRACT

Protein misfolding is a hallmark of a number of metabolic diseases, in which fatty acid oxidation defects are included. The latter result from genetic deficiencies in transport proteins and enzymes of the mitochondrial β-oxidation, and milder disease conditions frequently result from conformational destabilization and decreased enzymatic function of the affected proteins. Small molecules which have the ability to raise the functional levels of the affected protein above a certain disease threshold are thus valuable tools for effective drug design. In this work we have investigated the effect of mitochondrial cofactors and metabolites as potential stabilizers in two β-oxidation acyl-CoA dehydrogenases: short chain acyl-CoA dehydrogenase and the medium chain acyl-CoA dehydrogenase as well as glutaryl-CoA dehydrogenase, which is involved in lysine and tryptophan metabolism. We found that near physiological concentrations (low micromolar) of FAD resulted in a spectacular enhancement of the thermal stabilities of these enzymes and prevented enzymatic activity loss during a 1 h incubation at 40 °C. A clear effect of the respective substrate, which was additive to that of the FAD effect, was also observed for short- and medium-chain acyl-CoA dehydrogenase but not for glutaryl-CoA dehydrogenase. In conclusion, riboflavin may be beneficial during feverish crises in patients with short- and medium-chain acyl-CoA dehydrogenase as well as in glutaryl-CoA dehydrogenase deficiencies, and treatment with substrate analogs to butyryl- and octanoyl-CoAs could theoretically enhance enzyme activity for some enzyme proteins with inherited folding difficulties.

1. Introduction

Acyl-CoA dehydrogenases (ACDH) constitute a protein family that in humans comprises 11 mitochondrial proteins. These proteins are involved in fatty acid oxidation and amino acid metabolism and comprise the short (SCAD), medium (MCAD), long (LCAD) and very-long (VLCAD) chain acyl-CoA dehydrogenases, ACAD9, ACAD10, ACAD11, isovaleryl-CoA dehydrogenase (IVD), short/branched chain acyl-CoA dehydrogenase (SBCAD), isobutyryl-CoA dehydrogenase (IBDH) and glutaryl-CoA dehydrogenase (GCD). These enzymes all share high sequence similarity, but differ in their substrate specificities. The electrons gained in the respective dehydrogenation reactions by all ACDH are transferred to the respiratory chain through electron transfer flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QO) [1–3]. These proteins share a common structural fold with two or four subunits forming homodimers or homotetramers which invariably harbor a FAD moiety as catalytic redox cofactor [4].

Genetic disorders in mitochondrial acyl-CoA dehydrogenases involved in fatty acid oxidation (FAO) and amino acid metabolism are among the most common metabolic disorders. For example, MCAD deficiency is by far the most frequent disorder of this group with a frequency of 1:15,000 in Caucasian populations [5–7]. Newborn screening for many of these disorders is currently performed in many countries. Patients show a wide spectrum of symptoms, which in many cases are triggered by metabolic stress (high flux through the respective pathway) or pathophysiological conditions (e.g. fever), leading to further destabilization of mutant proteins with residual enzyme activity. Treatment regimes aiming at flux reduction by avoiding respective metabolites and their precursors in the diet have proved beneficial [8–9]. As many disease associated gene variations in the genes giving rise to variant proteins are of the missense type [10–11] the pathogenesis is in many cases resulting from defective folding [12]. As FAD has been shown to function as a chaperone [13–14], treatment with riboflavin, the precursor of FAD may theoretically increase the folding efficiency of mutant proteins with residual activity [15–16]. However, although riboflavin

Abbreviations: ACDH, acyl-CoA dehydrogenases; SCAD, short chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; GCD, glutaryl-CoA dehydrogenase; FAD, flavin adenine dinucleotide; DSF, differential scanning fluorimetry; T_m, midpoint of thermal unfolding

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treatment has been sporadically tried in acyl-CoA dehydrogenase deficiencies [8,17–19], no conclusive molecular studies addressing the mechanistic and structural basis of these effects on the dehydrogenase enzymes have been performed.

In the current work we have investigated the effects of addition of FAD, riboflavin or ACDH substrates and substrate analogs to study in detail the effects of these compounds on the *in vitro* structural stability of the two fatty acid oxidation ACDH, short-chain acyl-CoA dehydrogenase (SCAD) and medium-chain acyl-CoA dehydrogenase (MCAD) and on glutaryl-CoA dehydrogenase (GCD), which is involved in amino acid metabolism. Our study can provide the basis for evidence-based mechanistic treatment strategies as it establishes the proof of principle for conformational and kinetic stabilization by cofactor and substrates in these proteins. Thus, treatment with substrate analogs to butyryl- and octanoyl-CoAs could theoretically enhance enzyme activity for some enzymes with inherited folding difficulties.

2. Materials and methods

2.1. Chemicals

All reagents were of the highest purity grade commercially available. Fatty acid substrates, FAD and riboflavin were purchased from Sigma. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from VWR International.

2.2. ACDH expression and purification

Plasmids with MCAD, SCAD or GCD cDNA lacking the sequence encoding the mitochondrial transit peptide, and supplied with an N-terminal ATG start codon and a C-terminal his-tag (6xhis + 1xgln) were constructed in the same arrangement in a pBluescriptKS(−) (Stratagene) derived vector as described for the MCAD plasmid pWt described [20]. *Escherichia coli* JM109 cells (Promega) were transformed using standard procedures, and were grown in TB-medium (12 g Bacto tryptone, 24 g yeast extract, 9.4 g dipotassium phosphate, 2.2 g monopotassium phosphate and 2 ml glycerol per liter) supplemented with 100 $\mu g.ml^{-1}$ ampicillin at 30 °C in a shaking incubator until OD_{532} of 0.5 was reached. The cells were then induced overnight with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) [21]. Cells were harvested by centrifugation, resuspended in 10 mM hepes, 20% ethylene glycol at pH 7.8, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) in the presence of DNase (PVL) and FAD, and disrupted in a French press. The soluble fraction was subjected to a His-binding resin, 5 ml His-Trap HP (GE Healthcare), equilibrated in 10 mM hepes, 20% ethylene glycol, 200 mM NaCl, 10 mM imidazole and 0.5 mM PMSF at pH 7.8 (buffer A). The column was washed with five volumes of buffer A, and bound proteins were eluted by a linear gradient ranging from 10 to 500 mM imidazole, in buffer A. SCAD eluted as pure proteins at ~220 mM imidazole and MCAD and GCD eluted at ~160 mM imidazole. The purity of the pooled enzyme fractions was confirmed by SDS/PAGE. Pure fractions with 2.5 fold excess FAD were fast-frozen using liquid nitrogen and stored at −80 °C.

2.3. Biochemical and enzymatic assays

UV/visible spectra and enzymatic activities were measured using a Shimadzu UVPC-1601 spectrometer with cell stirring. Before each experiment FAD excess added to buffers as a preservative was removed by extensive washing using ultra filtration/dilution. Protein concentration was determined by the Bradford assay and FAD content was measured by the absorbance at 450 nm. All experiments were performed with pure proteins containing full occupancy of FAD site. Final buffer for protein assays was 10 mM hepes pH 7.8. The acyl-CoA dehydrogenase enzymatic activity was measured at 30 °C monitoring 2,6-dichlorophenolindophenol (DCPIP) reduction at 600 nm, in an assay with phenazine methosulfate and butyryl-CoA, or octanoyl-CoA or glutaryl-CoA, respectively to SCAD, MCAD or GCD [22].

2.4. Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) was used to determine the melting temperatures (T_m) of the proteins under different conditions. This method is based on the use of the fluorophore Sypro Orange which becomes fluorescent upon binding to hydrophobic protein patches that get exposed upon thermal unfolding [23], being very useful to determine protein stability in a variety of conditions [24]. Briefly, different buffers or compound solution was distributed into PCR plates (Bio-Rad). Prior to measurements, protein solution (1.2 μM) with Sypro Orange 5× (Invitrogen) was added to each well. The plates were sealed with optical quality sealing tape (Bio-Rad) and run in an iCycler iQ Real-Time PCR instrument (Bio-Rad) using excitation filter from 530 to 560 nm and emission filter from 575 to 595. Temperature range used was from 20 to 90 °C, with increments of 2 $°C.min^{-1}$. Raw data were exported to a spreadsheet, background corrected and the thermal denaturation curves obtained from which the midpoint transitions (melting temperatures, T_m) have been determined for up to 96 different conditions in each assay. See Fig. S1 for a representative plot of melting curves determined by DSF.

2.5. Functional studies under thermal stress

To study the effect of compounds on the biological activity of the model enzymes, activity was measured after heat stress. Briefly, ACDHs (1.2 μM) were incubated for 1 h at 40 °C in the presence of the selected compounds: substrates (20 μM) and/or FAD (20 μM). Protein solution was added to the compound solution right before incubation at 40 °C. Control samples in the absence of the compounds were also prepared and treated in the same conditions. The enzymatic activity for each condition was determined immediately after mixing (time 0) and then after 1 h of incubation as described in the biochemical and enzymatic assay section. No protein precipitation was observed.

3. Results

3.1. Effect of acyl-CoA substrates on ACDH stability under physiological conditions

Cofactors, substrates and inhibitors are among small molecules which are known to have the potential to exert a direct stabilizing action over a protein fold by recovering misfolded conformations for example via nucleation effects which restore native interactions and/or promote the correct oligomeric state. Using differential scanning fluorescence (DSF) we have investigated *in vitro* the effect of diverse fatty acid substrates and related molecules on the stability of three ACDH: glutaryl-CoA dehydrogenase (GCD), short chain acyl-CoA dehydrogenase (SCAD) and the medium chain acyl-CoA dehydrogenase (MCAD). In order to mimic conditions such as those found in mitochondria, we have used near physiological substrate concentrations [25], which were always present in assays at low micromolar concentrations (from 0.8 to 100 μM). With this approach, we seek also to simulate conditions of mitochondrial ACDH dysfunction, in which substrate levels and metabolites are increased. For the purpose, we have screened the effect of butyryl-CoA, octanoyl-CoA, glutaryl-CoA, palmitoyl-CoA, acetyl-CoA and CoA, on the protein thermal stabilities and the melting temperatures (T_m) were measured at increasing concentrations (Fig. 1 and Supplemental Fig. S1, Table S1). The results obtained showed that preferred substrates (butyryl-CoA and octanoyl-CoA, respectively) of SCAD and MCAD