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ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · DECEMBER 2011

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

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ARTICLE INFO

Article history:
Received 15 July 2011
Received in revised form 14 September 2011
Accepted 15 September 2011
Available online 24 September 2011

Keywords:
Fatty acid oxidation
Acyl-CoA dehydrogenase
Chemical chaperone
Differential scanning fluorimetry
Substrate
Thermofluor

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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doi:10.1016/j.bbadis.2011.09.009

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

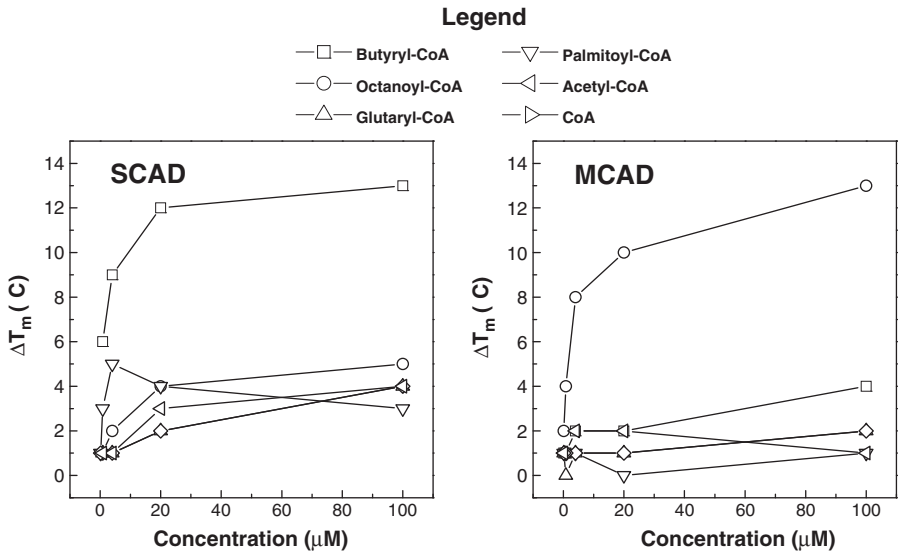

Fig. 1. Dependence of substrate concentration on ACDH thermal stability. The thermal stabilities of SCAD and MCAD were determined by DSF in the presence of various compounds and substrates at different concentrations: butyryl-CoA (□), octanoyl (○), glutaryl-CoA(△), palmitoyl-CoA (▽), acetyl-CoA (◁) and CoA (▷). Plots show relative protein stability variations (ΔT_m) compared to controls in which the midpoint of thermal unfolding has been determined in the absence of any added compound ($T_m = 50$ °C for SCAD and $T_m = 54$ °C MCAD). Protein concentration was 1.2 μM and compound concentration varied between 0.16 and 100 μM, in 10 mM hepes pH 7.8. See Materials and methods for details and supplementary materials for tables.

have a dramatic effect on the thermal stability affording a substantial stabilization ($\Delta T_m \approx +12$ °C). Interestingly, this stabilization is achieved even at very low concentrations and already at 20 μM more than 80% of the maximal stabilizing effect observed at higher substrate concentrations (100 μM) is achieved. For these two proteins, although the most significant effect is limited to the preferred catalytic substrate, some stabilization with lower magnitude ($\Delta T_m < 4$ °C) is also observed for the other molecules tested. However, substrate-induced conformational stabilization does not seem to be a property shared by acyl-CoA dehydrogenases in general, as we did not observe any effect of glutaryl-CoA on GCD (Table S1). These differences observed in respect to substrate stabilization among ACDHs suggest that these proteins, although sharing a common structural fold, can in fact have their conformational properties modulated by distinct mechanisms, a process which possibly relates to differences in protein dynamics (discussed below). The substrate-induced stabilization effects observed for SCAD and MCAD are thus suggestive of a stabilizing mechanism through which local effects resulting from substrate binding to the active site are globally propagated to the protein.

3.2. FAD but not its precursor riboflavin stabilizes ACDH

We then set to investigate if FAD, the common flavin cofactor present in all acyl-CoA dehydrogenases, has also a stabilizing action over the ACDH proteins. We had previously established that a slight excess of FAD, similar to that found in patients undergoing riboflavin supplementation therapy [26], has an important stabilizing effect over the electron transfer flavoprotein (ETF) under mild thermal stress mimicking a fever episode, even under conditions in which the protein cofactor site is fully saturated [14]. We have now extended this analysis to the three acyl-CoA dehydrogenases here studied, and for the purpose the DSF assays of the different proteins (at 1.2 μM final concentration) were performed in the presence of a slight excess of FAD at concentrations from 3 to 25 μM. Importantly, it should be noted that the as purified proteins had already fully loaded flavin binding sites (>0.9 FAD/mol). The results obtained show that FAD has a stabilizing effect on SCAD, MCAD and GCD, resulting in considerable increases of the T_m (Fig. 2, Table S2). In order to investigate if this stabilization is specific for FAD or if, on the other hand, isoalloxazine-ring mediated interactions could promote an identical effect, we have carried out experiments using riboflavin. In these cases, no stabilization was observed thus showing that at the concentrations and conditions tested, the stabilization is not afforded generically by any flavin moiety, but rather it is specific for FAD.

3.3. The combination of cofactor and substrate stabilization is nearly additive

Having established that mitochondrial cofactors and metabolites are potential stabilizers of native β-oxidation flavoprotein dehydrogenases, we have investigated for possible synergistic effects. For the purpose, we have analyzed SCAD without FAD added in parallel with assays in which a moderate excess of FAD (20 μM) was included in the assay solution; for all these conditions the additional effects of substrates were analyzed (Fig. 3, Table S3). The results obtained show that i) the tested combinations of compounds stabilize SCAD; and ii) the stabilization achieved is nearly additive in respect to that observed in experiments in which the effect of the molecules has been tested individually (Fig. 3). This observation agrees with the results described in the previous sections of this study as it confirms that substrates and the cofactor result in protein stabilization via different mechanisms: the former by binding to the active site pocket, and the latter by shifting the dissociation equilibrium of the redox cofactor toward the protein bound state. What is very interesting to note is that the combination of butyryl-CoA and FAD increases substantially the melting temperature of SCAD. This illustrates how the conformation of an ACDH can be effectively rescued by small molecule ligands, in this case natural mitochondrial metabolites at very low concentrations.

3.4. Cofactors and metabolites prevent activity loss during mild temperature stress

In order to be truly effective, protein stabilization by small molecules must not compromise the biological activity. In some cases, a substantial increase in the protein melting temperature corresponds to a rigidification of the protein structure leading to decreased dynamics and flexibility, thus compromising biological activity [27]. An adequate stability–activity balance must thus be attained so that a better protein conformation is not obtained at the expense of a

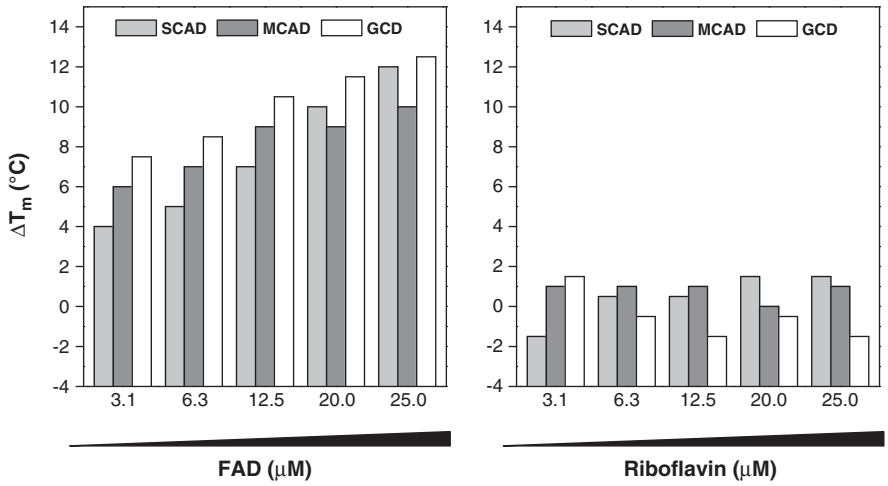

Fig. 2. Effect of FAD and riboflavin on ACDH thermal stability. The effect of FAD and riboflavin at different concentrations on the thermal stabilities of SCAD, MCAD and GCD (1.2 μM) was determined at various flavin concentrations (3.1 to 25 μM) in 10 mM hepes pH 7.8. Proteins were added to flavin solutions right before the analysis. Bars denote protein stability variations (ΔT_m) in respect to controls. Please note that the x-axis is not crossing at y = 0. See Materials and methods for details and supplementary materials for tables.

trade off with enzymatic activity. In order to investigate this possibility we have carried out experiments in which GCD, MCAD and SCAD were incubated for 1 h at 40 °C with and without FAD, in combination with butyryl-CoA, octanoyl-CoA and glutaryl-CoA. After this period, which simulates a mild temperature stress resembling a fever episode, the remaining enzymatic activity was measured. The results obtained in control experiments without added molecules show that these are rather destabilizing conditions as after this incubation the enzymes had low residual activities: 48% for GCD, 31% for MCAD and 49% for SCAD (Fig. 4, Table S4). For all cases, this activity decay was prevented if FAD was present at low concentrations (20 μM) during the thermal stress. Likewise, the preferred substrates (octanoyl-CoA for MCAD and butyryl-CoA for SCAD) also prevent activity loss and, when in combination with the flavin cofactor, an enzymatic activation is observed. These results are in agreement with the increased melting temperatures and show that the conformational stabilization does not sacrifice catalytic activity. In this respect, it is interesting to note that non-optimal substrates withheld activity during the temperature stress assay at 40 °C. This is for example the case of butyryl-CoA for MCAD: at 20 μM it yields a modest increase in the melting temperature ($\Delta T_m \approx +2$ °C), but under identical conditions it prevents activity loss during 1 h incubation at 40 °C, affording a residual activity of 70% (versus 30% in its absence). On the other hand, glutaryl-CoA, which is not a substrate for either SCAD or MCAD has no preventive effect.

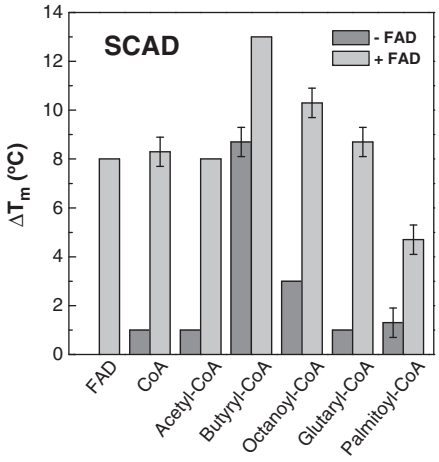

Fig. 3. Combined effect of FAD and metabolites on SCAD stability. The thermal stabilities of SCAD (1.2 μM) in the presence of CoA, acetyl-CoA, butyryl-CoA, octanoyl-CoA, glutaryl-CoA or palmitoyl-CoA (20 μM) were determined in the presence and absence of FAD (20 μM). Protein was added to flavin and metabolite solutions right before the analysis. Bars denote protein stability variations (ΔT_m) in respect to controls. See Materials and methods for details and supplementary materials for tables.

4. Discussion

In this study we have investigated the effect of mitochondrial cofactors and metabolites as potential stabilizers in three acyl-CoA dehydrogenases: GCD, SCAD and MCAD. The rationale for the study was based on the principle that ligands and substrates that increase protein stability and function could in theory be used as small molecule pharmacological chaperones, restoring levels of the affected proteins above critical disease thresholds in the associated metabolic pathologies. We have tested the hypothesis that metabolites and cofactors at micromolar physiological concentrations could play this role, and we have undertaken two complementary approaches: on the one hand testing effects on protein stabilization, and, on the other, testing effects on the protein kinetic stability upon an *in vitro* fever simulation.

Our results show that for MCAD and SCAD, the preferred substrates and the FAD cofactor have a dramatic stabilizing effect, increasing the protein thermal stability and preventing activity loss during a mild temperature stress. Interestingly, GCD substrate binding does not seem to be coupled with overall fold stabilization, whereas the FAD cofactor clearly does. This suggests that substrate-binding in GCD may result in a somehow different set of conformational changes that do not yield effective stabilization as observed in MCAD and SCAD, which reflects differences among the dynamic properties of these enzymes. In the case of SCAD, cofactor and substrate synergistically restore stability and catalytic activity. Thus, these results clearly establish the proof of principle that cofactors and metabolites at physiological levels are potential stabilizers of mitochondrial acyl-CoA dehydrogenases. The fact that these substantial effects take place at near physiological concentrations of the compounds, which have the same magnitude as the K_m for the most effective enzyme–substrate pairs, strongly suggests that the maintenance of a high active site occupancy is an effective stabilizing strategy for SCAD and MCAD. Interesting to note is the fact that in the case of SCAD patients there is accumulation of butyryl-CoA byproducts such as ethylmalonic acid (EMA) in the cells, blood and urine [28-30]. Therefore, patients could potentially benefit of a treatment that would increase the intra-mitochondrial level of butyryl-CoA or by a substrate analog which would have the ability to stabilize the enzyme. The

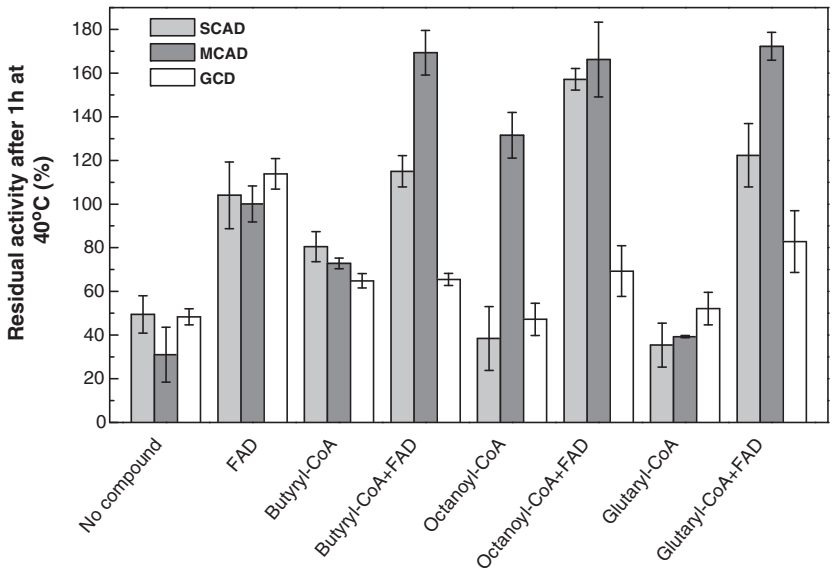

Fig. 4. Rescue of ACDH activity following thermal stress by metabolites and FAD. The residual activity after thermal stress (1 h at 40 °C) was determined for GCD, MCAD, and SCAD with no compound added or in the presence of FAD (20 μM), and butyryl-CoA, octanoyl-CoA and glutaryl-CoA (20 μM), the latter also in combination with FAD (20 μM). Protein was added to the solutions right before the incubation at 40 °C (final concentration was 1.2 μM). Values for residual activity were calculated in relation to activity at time zero, for each condition. See Materials and methods for details and supplementary materials for tables.

competition effect of a substrate analog over the native substrate would be overcome by the fact that its binding would increase protein stabilization and result in a higher level of correctly folded protein in the mitochondria with extended lifetime, thus surpassing a certain functional threshold below which there is dysfunction and disease. Thus developing such substrate analog compounds would be potentially beneficial to patients. In fact the same role could be played by inhibitors, such as in the case of those that have been developed and are used therapeutically to treat for example lysosomal disorders, such as Gaucher disease. In the latter case, inhibitor molecules bind to folding variants of the affected glucocerebrosidase enzyme, increasing their thermodynamic and kinetic stability, thus favoring their correct trafficking and an increase of enzymatic function above a critical disease threshold [31–32]. Other relevant modes of pharmacological rescue of metabolic disorders focus on increasing protein expression levels, which could also be a possibility in respect to increasing the levels of enzyme, even if partially catalytically impaired. As an example, bezafibrate was shown to induce both mRNA and protein expression levels of VLCAD and CPTII in deficient fibroblast [33], and has also proved to be effective in patients [34].

In the present case, the highly stabilizing effect of FAD is suggestive of a direct effect in the proteins, which corroborates the view that dietary administration of high doses of the riboflavin precursor results in an intracellular increase in FAD levels [26] is in fact potentially beneficial for patients suffering from inherited acyl-CoA dehydrogenase deficiencies. Although comprehensive studies on the clinical effects of high-dose riboflavin treatments are scarce, data from a subgroup of patients with SCAD deficiency is not conclusive in respect to establishing a clear relationship between high-dose riboflavin treatments and clinical improvements, therefore broader and more complete studies are needed [18]. Nevertheless, the biochemical improvements resulting from increased FAD levels, as a result of vitamin therapy are well established, and point to a pivotal role for this cofactor. Even low FAD:protein ratios substantially improve protein stability and sustain enzymatic activity, and presumably the same effect takes place in disease-associated variants. One could speculate if whether FAD could be a general regulator of β-oxidation. Under this hypothetical scenario, increased steady-state levels of β-oxidation enzymes could be induced by high FAD concentrations, both as a result of a direct stimulatory effect of FAD over translation [35] and also by significant increase in protein stability promoted by FAD, that would inhibit protease-mediated protein degradation. Future experiments will contribute to clarify this hypothesis.

The data here collected suggests that cofactor dissociation during thermal stress may be compensated by exogenous FAD, which can be provided by riboflavin treatment [26]. The relevance of our observations is that a very significant stabilization is achieved at FAD concentrations (20 μM) which are within the physiological cellular range [25]. This stabilization effect arises probably from a shift in the equilibrium between bound and unbound cofactors, in the direction that favors the bound form resulting in an improvement of the stability of the protein. Important to note is that in our experiments the initial proteins had 95% occupancy of the flavin binding site; therefore, FAD is stabilizing the holo form of the protein, in contrast with the well described stabilization of apo forms by respective cofactors. Mechanistically, the effect of FAD might be interpreted in respect to the principle of ligand-based kinetic stabilization; the availability of the FAD cofactor results in an equilibrium shift toward the bound state, further promoted by the fact that binding of a molecule to the active pocket contributes to the kinetic stability of the protein conformation, which further increases the end-point residual activity.

With this study we provide a quantitative measure for the FAD-dependent stability of mitochondrial acyl-CoA dehydrogenases, through saturation of the flavin binding site yielding conformational and structural stabilization, therefore providing a framework that explains the fact that mature acyl-CoA dehydrogenases undergo fast degradation under flavin-depletion [35]. Therefore, riboflavin may be beneficial during feverish crises in patients with the short- and the 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. Future research will contribute to clarify this possibility.

Acknowledgements

The work was supported by funds from the Fundação para a Ciência e Tecnologia (FCT/MCTES, Portugal) via research grant PTDC/SAU-GMG/70033/2006 (to C. M. G) and by CLIMB UK — Children living with metabolic disease (to C. M. G.). The Fundação para a

Ciência e Tecnologia (FCT/MCTES, Portugal) is acknowledged for fellowships BII — Bolsa de Iniciação à Investigação (to T. G. L.), SFRH/BPD/74475/2010 (to B. J. H.) and SFRH/BPD/34763/2007 (to J.V.R). The Alfred Benzon Foundation is acknowledged for an interchange grant.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbadis.2011.09.009.

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