Extensive domain motion and electron transfer in the
human ETF–MCAD complex.

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Abstract

The crystal structure of the human ETF–medium chain acyl-CoA dehydrogenase (MCAD) complex reveals a dual mode of protein—protein interaction, imparting both specificity and promiscuity in the interaction of ETF with a range of structurally distinct primary dehydrogenases. ETF partitions the functions of partner binding and electron transfer between (i) the recognition loop, which acts as a static anchor at the ETF—MCAD interface, and (ii) the highly mobile redox active FAD-domain. Together, these enable the FAD-domain of ETF to sample a range of conformations, some compatible with fast interprotein electron transfer. Disorders in amino acid or fatty acid catabolism can be attributed to mutations at the protein—protein interface. Crucially, complex formation triggers mobility of the FAD-domain, an induced-disorder that contrasts with general models of protein—protein interaction by induced-fit mechanisms. The subsequent interfacial motion in the MCAD—ETF complex is the basis for the interaction of ETF with structurally diverse protein partners. Solution studies using ETF and MCAD with mutations at the protein:protein interface support this dynamic model and indicate ionic interactions between MCAD Glu212 and ETF Argɔ249 are likely to transiently stabilise productive conformations of the FAD-domain leading to enhanced electron transfer rates between both partners.
**Introduction**

In the mammalian mitochondrial matrix, electron transferring flavoprotein (ETF) links the activity of no less than 10 different dehydrogenases to the respiratory chain by accepting and subsequently transferring electrons to the membrane-bound ETF-ubiquinone oxidoreductase (1). ETF therefore plays a role akin to the function of cytochrome c in the intermembrane space of mitochondria. Mitochondrial flavoprotein partners of ETF include the 4 fatty acyl-CoA dehydrogenases [very long chain (VLCAD), long chain (LCAD), medium chain (MCAD) and short chain (SCAD) acyl-CoA dehydrogenases] involved in mitochondrial β-oxidation of fatty acids, isovaleryl-CoA (IVD), glutaryl-CoA (GCDH) and isobutyryl-CoA (IBD) dehydrogenases involved in amino acid catabolism, and sarcosine and dimethylglycine dehydrogenase involved in 1-carbon metabolism (1).

Defects in ETF or ETF-ubiquinone oxidoreductase give rise to the human inherited metabolic disease glutaric acidemia type II (2). In this disease, electron transfer from the primary flavoprotein dehydrogenases to the main respiratory chain is impaired. Likewise, defects in the primary dehydrogenases often cause similar and lethal effects (3). MCAD deficiency is the most frequently diagnosed mitochondrial β-oxidation defect in populations of northwestern European origin. This potentially fatal disease is inherited in an autosomal recessive fashion (carrier frequency around 1 in 70), and may affect as many as 1 in 10,000 newborns.

While the primary dehydrogenases involved in fatty acid breakdown and amino acid catabolism share a common evolutionary origin and thereby retain highly similar structural features (4), the flavin dehydrogenases involved in 1-carbon
metabolism are structurally and evolutionarily distinct enzymes (5). In addition, the membrane-bound ETF-ubiquinone oxidoreductase is unrelated to either of these dehydrogenase families. The wide range of structurally different partners thus suggests a certain level of promiscuity in ETF binding, similar to that observed with cytochrome c. However, unlike the relatively small 12 kDa electron carrier cytochrome c, the 60 kDa ETF shows a remarkable specificity for its physiological partners. It is therefore intriguing that ETF manages to reconcile these contrasting properties of promiscuity and specificity.

ETFs are heterodimeric proteins that fold into three distinct domains; the FAD is bound non-covalently to domain II, which sits in a shallow bowl created by domains I and III (6). Recent models constructed using the known crystal structures of human ETF and its partner human MCAD have suggested that electron transfer from MCAD to ETF requires rotational movement of the ETF FAD-domain (7). Also, the recent structure determination of ETF from the bacterium Methylophilus methylotrophus in complex with its partner trimethylamine dehydrogenase (TMADH) reveals a highly dynamic protein-protein interface in which the FAD-domain of ETF samples the available space within the complex (8). This diffusional motion within the complex is restricted by the static component of the interaction: a small hydrophobic interface centred on the ETF conserved residue Leuβ194. While TMADH shares no significant structural similarity with any of the human ETF partners, a similar dual mode of interaction for the human ETF complexes could explain how human ETF retains both specificity (via the Leuβ195 interaction in domain III) and promiscuity (via the flexible FAD-domain).

To determine the structural basis of the interaction between human ETF and its partners, we have elucidated the crystal structure of the human ETF–MCAD
complex at 2.9 Å resolution. This structure has led us in turn to further investigate the properties of the observed contact areas by studying mutant forms of MCAD and ETF. Remarkably, the interprotein interface of the human ETF–MCAD complex shares many essential structural features with the bacterial ETF–TMADH complex, despite the very different overall structures of MCAD and TMADH. Both crystal structures, together with the obtained solution data for the mutant human ETF and MCAD forms, reveal a conserved and dual interaction mode for ETF. The static part of this interaction confers ETF specificity while the mobile section allows for the observed promiscuity. Extensive domain motion in the ETF–acyl-CoA dehydrogenase complexes is therefore needed to drive the catabolism of fatty acids.

**Material and Methods**

**Crystallogenesis and data collection**

A solution of a 1:1 mixture of recombinant human ETF and MCAD tetramer was prepared and used for subsequent crystallisation trials. Crystals of the ETF–MCAD complex were grown using the sitting drop vapour diffusion method. The reservoir solution contained 16 % PEG 5000 MME, 50 mM sodium thiocyanate and 0.1 M sodium cacodylate, pH 6.5. Crystals were flash-cooled in oil and a full 2.9 Å data set was collected on a single P2_12_1_1 crystal at ID29, ESRF (Grenoble France). Data were processed and scaled with the HKL package programs DENZO and SCALEPACK (9). Data statistics are given in Table 1.

**Structure determination and refinement**

The program AMoRe (10) was used to search for a molecular replacement solution using the available crystal structures for human MCAD and ETF (PDB codes 1EGD
and 1EFV). Only domain I and III of human ETF were used as the search model. For both search models a clear solution was found and initial phases were of sufficient quality to define unambiguously changes in the complex structure with respect to the search models. Positional and isotropic B-factor refinement was performed using REFMAC5 (11) with alternative rounds of manual rebuilding of the complex model. Tight NCS symmetry restraints on the MCAD tetramer were imposed during the initial cycles of refinement, but were removed in the final cycles. Final refinement statistics are given in Table 1.

**Protein production and site-directed mutagenesis**

The plasmids encoding MCAD (pTrc99c), hETF (pK18) and IVD (pKK223-3) were kindly supplied by S. Ghisla and J. Vockley. The plasmids were transformed into JM109 cells (Stratagene). Wild-type and mutant enzymes were grown in 2YT medium containing 100 µg/ml ampicillin (MCAD and IVD) or 34 µg/ml kanamycin (hETF) at 35 °C to an optical density at 600nm of 1 followed by a 12 h induction at 25 °C with 0.1 mM IPTG. Cells were lysed by sonication, and the extracts clarified with 0-40% ammonium sulfate. The enzymes were purified using anionic chromatography (DE52 and Q-Sepharose) and hydroxyapatite columns. The enzymes were stored in 10 mM Tris HCl buffer, pH 7.0, containing 10 % glycerol at –80 °C. Mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by automated DNA sequencing.

**Steady-state assays**
Acyl-coenzyme A dehydrogenase activity was determined at 25 °C using a modification of the DCIP-reduction method of Okamura-Ikeda et al (12). Reactions were performed in 20 mM Tris HCl buffer, pH 8.0 containing 50 µM octanoyl-CoA or isovaleryl-CoA (for MCAD and IVD assays, respectively), 60 µM DCIP with either 3.3 mM PMS (phenazine methosulfate) or 2 µM hETF as intermediate electron acceptors. Reduction of DCIP was monitored continuously at 600 nm.

Results and Discussion

Structure of the ETF–MCAD complex

The crystal structure of the human ETF–MCAD complex reveals a single ETF molecule interacting with a MCAD homotetramer in the asymmetric unit (Fig. 1a). While no solution data is available on the correct stoichiometry of the complex, a putative complex with 4 ETF molecules decorating the central MCAD tetramer can be constructed by applying the 222-fold symmetry of MCAD to the ETF partner. Any one of the resulting four ETF molecules does not impede binding of the other three, nor does it obstruct entry to the four fatty acyl-CoA binding sites. It seems likely, therefore, that in solution up to four ETF molecules can interact with a single MCAD tetramer at any given time.

The most striking observation is the total lack of any significant electron density for the FAD-domain (domain II) of ETF in the complex. However, SDS-PAGE analysis of the crystalline complex reveals that the lack of density for the FAD-domain does not arise from proteolysis, as only full-length ETF subunits are
observed (Fig. 1b), illustrating that the FAD-domain is highly disordered in the complex. To establish whether these crystals retain catalytic activity, a DCIP (dichloroindophenol) dye linked reduction assay was used. DCIP is converted from the oxidised blue form to the reduced colourless state via ETF-mediated electron transfer from MCAD; DCIP is not reduced by MCAD/substrate alone. ETF–MCAD crystals mounted in capillaries in a 10 µL crystallisation solution, containing the substrate octanoyl-CoA and the terminal electron acceptor DCIP catalyse, albeit slowly, the reduction of DCIP by octanoyl-CoA, a process that could be followed using microspectrophotometry (Fig. 1c). This observation demonstrates that the crystalline complex and the observed inherent disorder of the FAD-domain are physiologically relevant.

The ordered region of ETF interacts solely through the β-subunit with the N-terminal α-helical domain of a single MCAD monomer, consistent with published cross-linking experiments that have revealed a preferential cross-linking of the ETF β-subunit to both MCAD and membrane-bound ETF–ubiquinone oxidoreductase (13,14). The total buried interfacial surface visible for the crystallographically ordered atoms is 536 Å² and covers only 4.3% and 3.2% of the accessible surface of an MCAD monomer and ETF, respectively. That this interaction surface is rather small, albeit highly complementary [shape correlation statistic (15) of 0.704], is consistent with kinetic data that suggest a weakly interacting system (Ikeda, et al., 1985; Salazar, et al., 1997).

Central to the interaction of ETF with MCAD is the polypeptide stretch between residues β191 and β200, termed the ‘recognition loop’; this comprises the initial turn of an α-helix and the immediate preceding loop region (Fig. 2a,b). This α-helix of ETF interacts with the MCAD surface, thereby extending α-helix C of
MCAD, with near perfect alignment of the axes, and corresponding dipoles, of both helices. The close proximity of the N-terminal region of this α-helix of ETF and the C-terminal region helix C of MCAD is facilitated by the sharp turn of the polypeptide chain in both molecules. A single hydrogen bond is made between the backbone oxygen of the conserved Gly60 on MCAD and the backbone nitrogen of Leuβ195. Only three other direct hydrogen bonds are observed between ETF and MCAD; viz. (i) between the side chain of Thr26 and the oxygen backbone of Alaβ193, (ii) the Glu34 and Tyrβ192 side chains and (iii) the Glu22 and Thrβ77 side chains. The side chain of Leuβ195 is completely buried within a hydrophobic pocket lined by residues that are part of α-helices A, C and D of MCAD. This pocket is formed by residues Phe23, Gly60, Thr64, Leu61, Leu73, Leu75 and Ile83. This hydrophobic patch extends across the interface to include the ETF residues Tyrβ192, Proβ197, Ileβ198 and Metβ199. A single putative ionic interaction at the periphery of this hydrophobic patch is made between ETF Argβ76 and MCAD Glu18, although the poor electron density indicates both residues are predominantly disordered.

In addition to the contacts made with the recognition loop, MCAD interacts with a second α-helix of ETF situated immediately adjacent to the α-helix of the recognition loop (Fig 2a). This interaction also includes a weak ionic interaction between Lys21 and Gluβ73 in addition to the hydrogen bond between Glu22 and Thrβ77. However, these residues are not conserved, suggesting this interaction is specific to the ETF–MCAD complex. It is conceivable that several indirect hydrogen bonds can be made between both molecules via bridging water molecules that are not observed at this resolution.

Comparison of the complex crystal structure with the available crystal structures for the uncomplexed protein partners (6, 18) reveals no significant
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differences for the MCAD structures. The recognition loop of ETF has, however, adopted a slightly different backbone conformation with more extensive rearrangement of several side chains, including the pivotal Leuβ195, indicating an induced-fit mode of binding for the ETF partner. Furthermore, the region comprising Ileβ14 to Serβ37 of ETF forms an extended loop, is slightly displaced with respect to the other ETF structures, thereby preventing close interaction with the C-terminal residues of MCAD. This results in the concomitant rearrangement of Tyrβ16 from its normal position near the FAD isoalloxazine ring.

**Leuβ195 is essential for complex formation**

The ETF mutant Lβ195A leads to no significant rearrangement of the recognition loop, as determined by the 1.8 Å crystal structure of this mutant protein (data not shown), yet this mutant is severely impaired in complex formation with MCAD. Using MCAD as the electron donor to ETF, which in turn reduces the dye DCIP, the rate of DCIP reduction under steady-state conditions decreases by ~10-fold in the Lβ195A mutant ETF (Fig. 3a). This underpins the hypothesis that the recognition loop is the only region of ETF that interacts extensively with MCAD, with Leuβ195 a key interfacial residue. Furthermore, the rate of DCIP reduction using isovaleryl-CoA dehydrogenase (IVD), another member of the acyl-CoA dehydrogenase family, is substantially decreased with the Lβ195A ETF as electron acceptor of the enzyme (Fig. 3b). The rate of reduction of DCIP by IVD with the mutant ETF is reduced ~2-fold with respect to the rate with wild-type ETF under the defined steady-state conditions. It is anticipated that human ETF interacts with the 7 acyl-CoA primary dehydrogenases in a similar fashion to that observed with MCAD itself. It is also
interesting to note that experimental studies suggest Leuβ195, and thereby the recognition loop, are pivotal to correct docking with the structurally unrelated dimethylglycine dehydrogenase (DMGDH) and its close relative sarcosine dehydrogenase (SDH). For example, evidence for competitive binding of ETF to acyl-CoA dehydrogenases and DMGDH has been observed by microelectrospray ionisation mass spectrometry and surface plasmon resonance, thus suggesting similar or closely overlapping binding sites on ETF for both types of dehydrogenase (19). Although there is no direct evidence that ETF interacts with the membrane-bound ETF–ubiquinone oxidoreductase via the recognition loop, cross-linking experiments have suggested that the β-subunit preferentially interacts with ETF–ubiquinone oxidoreductase (14). This suggests a mode of interaction similar to that observed for the human ETF–MCAD and bacterial ETF–TMADH complexes. That the sequence of the ETF recognition loop is highly conserved throughout ETFs from a diverse range of organisms indicates that ETFs are all likely to interact with their partners in a similar manner (Fig. 2d).

**MCAD and related dehydrogenases present a similar docking site**

That ETF interacts with several different dehydrogenases via the recognition loop suggests a similar complementary hydrophobic patch is formed on the surface of these proteins; this includes the small hydrophobic pocket that can accommodate the protruding Leuβ195 side chain. Alignment of sequences of the 7 MCAD-related human dehydrogenases with MCAD reveals that very few residues involved in the ETF–MCAD interface are conserved (Fig. 2c). With the exception of residue positions equivalent to Phe30, Gly60 and Gly74 in MCAD, which are likely
conserved for folding reasons, none of the residues directly contacting the ETF recognition loop is totally conserved. Almost all positions involved in hydrophobic packing retain the hydrophobic nature of the side chain while the positions involved in direct hydrogen bonds, Thr26 and Glu34, usually retain hydrogen bonding capacity. Docking of human IVD using the available human IVD crystal structure [PDB code 1IVH (20)] on the ETF–MCAD coordinates reveals that IVD presents a very similar surface to the ETF recognition loop. The side chain of Leuβ195 is buried by IVD hydrophobic residues at similar positions in the polypeptide chain (Fig. 2e). This hydrophobic pocket is clearly malleable to some extent, and the reduction in bulk at position 83 from Ile (MCAD) to Val (IVD) is compensated by a concomitant increase at position 64 involving Thr (MCAD) to Ile (IVD) and size decrease at position 73 Leu (MCAD) to Ser (IVD) by rearrangement of the backbone.

We have generated mutant forms of MCAD designed to reduce the size of the hydrophobic pocket. Mutants L61M, L73W, L75Y and I83M are all severely impaired in electron transfer to wild-type ETF, as suggested by the decrease in DCIP reduction rates under the defined steady-state conditions (Fig. 3a). Given that these residues are remote from the MCAD active site, this effect is most likely due to decreased binding strength of the ETF–MCAD complex. It is interesting to note that even in the case of mutants with ‘filled’ hydrophobic cavities, the DCIP reduction kinetics are slower with the Leuβ195Ala mutant than with wild-type ETF, despite the fact that the latter is expected to be less complementary with these MCAD mutants than the Leuβ195Ala mutant. The inability to rescue (part of the) activity through complementary mutation on the ETF surface clearly suggests the need for a protruding element of ETF buried in the partner surface. This first of all increases surface contact area and avoids ‘slipping’ of the otherwise flat hydrophobic contact
areas, but might also ultimately be involved in inducing the observed flexibility of the FAD-domain in the complex. It is possible that upon binding to MCAD reorganisation in the recognition loop signals, by an as yet unknown mechanism, to the FAD-domain to sample the conformationally available space.

Mammalian β-oxidation of fatty acids does not occur exclusively in the mitochondria. In liver and kidney approximately 10% of the fatty acids are broken down by peroxisomal β-oxidation (21). The first step in the peroxisomal pathway is catalysed by a distant relative of MCAD, acyl-CoA oxidase (ACO) that passes electrons to oxygen generating hydrogen peroxide. Not surprisingly, the overall fold of ACO is similar to MCAD, but in comparison the MCAD regions involved in binding ETF are markedly different in ACO. Additionally, the C-terminal extension of 200 amino acids in ACO versus MCAD occupies similar space to that occupied by the FAD-domain in the ETF–MCAD complex.

**Modelling the FAD-domain in the complex**

The most striking observation is that upon complex formation the FAD-domain of ETF changes from being well ordered to a highly disordered state. The electron density is reduced to background values at residues Lysα203 and Proβ231, which function as the hinge for this movement within the complex. The ETF FAD-domain is a near perfect sphere and, perhaps not surprisingly, the ‘empty’ volume at the MCAD–ETF interface corresponds almost exactly to that expected for the ‘missing’ FAD-domain. This is clearly indicative of a ‘ball-and-socket’ type of motion, where MCAD and the ordered regions of ETF form the ‘socket’ in which the FAD-domain can apparently move freely within the conformationally allowed space. Similar molecular motions occur in the bacterial ETF–TMADH complex (8), and also
within a single protein chain [e.g. the *E. coli* 5’nucleotidase (22)]. A layer of water molecules, and the presence of relatively few direct interprotein contacts, likely facilitate these domain motions.

A superposition of the free ETF crystal structure onto the ETF–MCAD crystal structure reveals minor clashes of several side chains for the FAD-domain with the MCAD partner (Fig. 4a). With a shortest FAD-to-FAD isoalloxazine distance of 35 Å, this conformation is clearly incompatible with electron transfer, based on the 14 Å rule (23,24). Surprisingly, it is not the FAD of the MCAD monomer to which the recognition loop is bound that is closest to the ETF FAD, but that of the MCAD monomer related by 2-fold symmetry. An MCAD dimer should therefore be regarded as the partner of ETF; the N-terminal domain of one monomer binds the recognition loop, while the C-terminal FAD-binding domain of the other monomer hosts the moving FAD-domain. Molecular dynamics calculations were used to systematically sample the position occupied by the FAD-domain in the bacterial ETF–TMADH complex (8). These suggest the FAD-domain occupies a large number of different positions, some of which show increased electronic coupling between the isoalloxazine ring of the ETF cofactor and the 4Fe-4S centre of TMADH by reducing the distance to < 14 Å. Such motion of the ETF FAD-domain within the ETF–MCAD complex would lead to similar reduction in interflavin distances (Fig. 4a), and several orientations of the FAD-domain were found to reduce this distance to below 14 Å. In these cases, the C7 or C8 methyl groups of the ETF flavin are in close contact with Trp 166, a conserved aromatic residue that π-stacks against the MCAD FAD isoalloxazine ring.

Remarkably, modelled complexes that are electron transfer-competent all contain putative ionic interactions involving the conserved Argα249 in the FAD-
domain of ETF and either the negatively charged Glu212 or Glu359 on the MCAD surface (Fig. 4b). These solvent exposed glutamate residues are positioned close to Trp166. Chemical modification experiments have indicated that a single arginine in ETF (25) and carboxylates on the MCAD surface are essential for complex formation (26). Dwyer and co-workers (27) reported that mutagenesis of Rα249K leads to a decrease in electron transfer rate of ~10-fold under steady-state conditions. Mutagenesis of MCAD at Glu212 (E212A) likewise leads to similar substantial reduction in DCIP reduction rates [~10-fold under steady-state conditions (Fig. 3b)]. Mutation of MCAD at the conserved Glu359 (E359A) does not significantly affect the DCIP reduction rate. Thus, it is likely that ionic interactions between Glu212 and Argo249 (but not Glu359 and Argo249) transiently stabilise productive conformations of the FAD-domain leading to enhanced electron transfer rates between both partners. Solution data have indicated that electrostatic interactions are important in electron transfer from MCAD to ETF (28). While electrostatic interactions are essentially absent from the MCAD—ETF domain III interface, the electron transfer rate decreases with increasing ionic strength. These observations can be a consequence of the destabilisation of the protein-protein interaction between Argo249 and Glu212. Alternatively, the observations might result from enhanced hydrophobic interaction at high ionic strength involving the hydrophobic patch/ETF recognition loop. The concomitant decrease in the rate of complex dissociation following electron transfer might lead to the observed reduction in steady-state turnover rate.

The majority of the MCAD residues contacting the edges of the moving FAD-domain are located on an anti-parallel β-sheet. The sequence conservation at positions where residues are solvent exposed and therefore contacting the FAD-domain is poor.
when compared to the conservation of residues pointing inwards to the core of the protein. Furthermore, there is no apparent preference for certain types of amino acid residue in this region of MCAD or MCAD-related enzyme surfaces. These observations suggest that the overall shape of this surface, but not the chemical identity of the majority of residues on that surface, is important.

**Disease causing mutations impact on ETF–MCAD complex formation**

Many of the disease-causing mutations identified in human ETF impact on protein stability and flavin incorporation. One mutation that likely impacts on ETF–partner complex formation is the deletion of Lysβ202, which leads to a mild late-onset form of glutaric acidemia type II (29). This mutation is very close to the recognition loop and likely disrupts the local structure to the extent that complex formation is impaired, but not abolished, as suggested by the mild form of the disease.

While mutations in MCAD and related fatty acyl CoA dehydrogenases lead to defects in mitochondrial β-oxidation (30), mutations in the catabolic amino acid dehydrogenases lead to isovaleric acidemia or glutaric acidemia type I (27). In the case of glutaryl-CoA dehydrogenase (GCDH), no less than 89 different mutations leading to glutaric acidemia type I have been reported (Human Gene Mutation Database at http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). Again, many of the mutations in these related dehydrogenases impact on flavin incorporation, protein stability or folding leading to obvious metabolic problems. Throughout the 7 dehydrogenases, several mutations can be found near the ETF docking site, although few of these are at positions directly involved in complex formation. Exceptions are P18S (all residues in MCAD numbering) in VLCAD (32); F27S in GCDH that packs against residues at positions 61 and 83 (33); G70S and Gl4C in SCAD that are
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essential for local folding and therefore shape of the Leuβ195 binding pocket (34,35); mutation of the stop codon to Trp in GCDH (36) leads to an extended C-terminus. This is expected to obstruct ETF binding, since additional polypeptide would occupy the space normally populated by the FAD-domain.

Comparison with the bacterial ETF–TMADH structure

A comparison of both available ETF–partner complex crystal structures reveals remarkable similarities despite the complete lack of significant structural homology and common function for the respective partners, MCAD and TMADH (Fig 5a). In both cases, the ETF recognition loop interacts with a hydrophobic patch on the surface of the partner with Leuβ195 (β194 in M. methylotrophus ETF) buried in a small sized cavity. Few direct hydrogen bonds or ionic interactions are observed at this interface. With the exception of cavity volume and surface shape, there is no similarity between the two ETF docking sites (Fig. 5b). In both cases, a direct hydrogen bond is made between the proteins, involving the backbone of residue β192/193 and a side chain of the ETF partner (Thr23 in MCAD and Gln462 in TMADH).

Surprisingly, an overlay of both complexes aligned on the basis of the ETF coordinates reveals that the ‘empty’ space occupied by the flexible FAD-domain in both complexes is nearly identical in volume and shape. This is due to the almost identical angle formed on docking the ordered region of ETF onto the concave surfaces of the partner protein. In both complexes, the FAD-domain is presumed to sample the available space in a ‘ball-and-socket’ type of motion. With respect to the Leuβ194/195 docking site, the positions of the 4Fe-4S centre in TMADH and the FAD in MCAD are very different, suggesting a different family of conformations are
-compatible with fast electron transfer in each complex. However, in both cases it is the conserved Argα237/249 positioned close to the ETF isoalloxazine ring that is proposed to transiently interact with Tyr442 in TMADH and Glu212 in MCAD.

**Conformational sampling**

Given the available crystal structures for two very different ETF complexes, the question of how ETF achieves both specificity and promiscuity can now be addressed. Unlike other electron carriers such as cytochrome c, ferredoxins, flavodoxins or azurin, the substantially larger ETF has partitioned the functions of binding and electron transfer between different domains. Domain III of the β-subunit exclusively binds to a hydrophobic patch on ETF-partners via the recognition loop and is therefore responsible for the observed specificity. From an evolutionary viewpoint, structurally diverse dehydrogenases could easily have evolved as simple ETF-binding patch on the surface of the molecule. Upon correct docking and positioning of ETF, domain II is dislodged from the position it occupies in free ETF and this trigger causes it to sample the available space. Such conformational sampling allows the FAD-domain to search out conformations that are compatible with fast electron transfer. Short-term stabilisation of those conformations leading to fast electron transfer is achieved by appropriately positioning residues on the ETF-partner surface that transiently interact with the conserved Argα237/249. The observed promiscuity of ETF is a consequence of the relatively mild constraints it imposes on the structure of its physiological partners. The ETF docking site is relatively simple, and due to the conformational flexibility of domain II, the relative positioning of the docking site and the electron transfer site has few restraints. Removal of Leuβ194/195 either by mutagenesis or limited proteolysis (8) leads to impaired electron transfer,
supporting further the hypothesis that all ETFs interact with their partners via the recognition loop. On the other hand, mutagenesis of the conserved Argα237/249, or of residues proposed to transiently interact with Argα237/249, has led to very varied effects on reaction rates with different partners. It is clear from our present data that each ETF complex can employ a different strategy to transiently bind this residue (e.g. hydrogen bond versus ionic interaction) and thereby stabilise the electron transfer-competent conformations of the FAD-domain. Such differences could explain the different behaviours observed for these mutants.

**Conclusions**

Crystal structures of physiological electron transfer complexes involving small electron carriers have led to 'colliding billiard ball' models of biological electron transfer, viz. the electrostatically guided collision of two essentially rigid bodies for interprotein electron transfer involving small proteins such as cytochrome c (37,38). Crystal structures of ETF complexes now reveal a very different and dynamic interface, whereby extensive protein motion is required for effective interprotein electron transfer. A number of structural and kinetic studies have suggested that conformational reorganisation is an essential part of the electron transfer step in several complex multi-domain redox systems (e.g. cytochrome bc1 (39), cytochrome b6f (40); sulfite oxidase (41) and the diflavin enzymes P450 reductase (42), and sulfite reductase (43).

Domain, or loop, flexibility is a feature of several proteins that interact with multiple partners, many of which are part of cellular communication pathways. Indeed, it has been shown that yeast proteins with so-called ‘no regular secondary structure regions’ (NORS) have more protein-protein interaction partners than other
proteins (44). In the case of structurally well-characterised systems that interact with several structurally distinct partners [e.g. calmodulin (45), members of the POU family of DNA-binding proteins (46), the peptidyl-prolyl cis/trans isomerase Pin1 (47) and the translocation domain of colicin E9 protein toxin (48)] proteins have been found to contain highly flexible regions or domains in the uncomplexed state. These systems therefore sample a range of conformations prior to complex formation. This allows the binding of structurally distinct partners that require different binding conformations. When complexed, however, the mobile regions of these proteins become rigid, effectively locked into a single state by binding to the partner protein—in essence an induced-fit mechanism. The static region in the different ETF complexes is based on the same principle, the recognition loop of ETF being relatively flexible in solution, but its motion being frozen into a single, partner specific conformation by complex assembly. ETF complexes, however, are remarkable in that extensive dynamic motion occurs at the protein–protein interface where the ETF FAD-domain samples a range of conformations following complex formation. Crucially, complex formation triggers mobility of the FAD-domain, an induced-disorder mechanism that contrasts with general models of protein—protein interaction by induced-fit mechanisms. Thus, the FAD-domain of ETF in complex must move to catabolise fatty acyl CoAs and link their oxidation with the respiratory chain. To date, this behaviour has only been observed in redox systems, where productive interactions can occur in different conformations owing to the loose restrictions placed upon the relative positions of redox cofactors. Whether mechanisms of conformational sampling extend to other types of protein-protein interaction (e.g. signalling complexes) remains to be seen, but until that time the ETF
complexes provide a unique insight into extensive dynamical motion at a protein interface that can be mapped directly on to protein function.

References

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**Table 1.** Overview of data collection and refinement statistics for the ETF:MCAD crystals.

**Data collection statistics**

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**Refinement statistics**

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Figure legends

Figure 1a. Stereo ribbon diagram of the ETF–MCAD complex. The MCAD tetramer is shown in green and the crystallographically observed ETF is shown in blue, light blue for the $\alpha$ and dark blue for the $\beta$-subunit. The MCAD FAD cofactors are depicted in yellow. Three additional ETF molecules obtained by applying the four-fold MCAD symmetry to the crystallographically observed ETF molecule are shown in grey decorating the MCAD surface. Figure 1b. SDS-PAGE gel of dissolved ETF–MCAD crystals. Lane 1 contains molecular weight markers (labelled in kDa), and lane 2 a single large crystal. Figure 1c. Three-dimensional representation of the reduction of DCIP by octanyl-CoA via ETF–MCAD complex crystals monitored by a significant decrease at 610 nm using a microspectrophotometer.

Figure 2a. Stereo ribbon diagram of the ETF–MCAD hydrophobic docking site. The N-terminal region of MCAD is shown in green, surrounded by a transparent molecular surface. Residues directly involved in binding ETF are shown in atom coloured sticks. The recognition loop region of ETF and the additional $\alpha$-helix that interact with MCAD are shown in blue, with residues directly involved in the ETF–MCAD contact represented in atom coloured sticks. Figure 2b. Stereo view of the $2|Fo|-|Fc|$ electron density map contoured at 1 $\sigma$ and final atomic model at the ETF–MCAD hydrophobic docking site. Backbone and side chain carbon atoms are coloured green for MCAD and blue for ETF residues. Hydrogen bonds are shown as dotted lines. Figure 2c. Sequence alignment of the N-terminal region of the 7 human acyl-CoA dehydrogenases and MCAD, using MCAD numbering (MCAD, medium-chain acyl-CoA dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; IBD, isobutyryl-CoA dehydrogenase; LACD, long chain acyl-CoA dehydrogenase;
VLACD, very long chain acyl-CoA dehydrogenase; GCDH, glutaryl-CoA dehydrogenase; ACDS, short chain acyl-CoA dehydrogenase; SBCAD, short/branched chain acyl-CoA dehydrogenase). Residues involved in binding ETF are shown in bold red while disease related mutations are shown as bold green. Those residues involved in binding ETF and for which disease related mutations have been observed are shown as bold blue. **Figure 2d.** Sequence alignment of the ETF recognition loop from different domains of life with human ETF numbering. Residues involved in binding dehydrogenase partners are shown in bold red while a disease related mutation positions is shown in bold green. **Figure 2e.** Model of the ETF–IVD complex. Residues directly involved in the putative ETF–IVD interface are shown in sticks. Backbone and side chain carbon atoms are coloured green for IVD and blue for ETF residues. MCAD residues involved in the ETF–MCAD contact are superimposed and depicted in grey.

**Figure 3a.** Kinetic data obtained under steady-state conditions for the reduction of DCIP via ETF by acyl-CoA dehydrogenases. Light blue bars represent wild-type or mutant MCAD activity with wild-type ETF; red-brown bars represent corresponding dehydrogenase activity with the Lβ195A ETF mutant.

**Figure 3b.** Kinetic data obtained under steady-state conditions for the reduction of DCIP via ETF by IVD or mutant MCAD. A dark blue bar represents wild-type IVD activity with wild-type ETF; the red-brown bar represents corresponding IVD dehydrogenase activity with the Lβ195A ETF mutant. Both green bars represent data obtained for respectively the E212A and E359A MCAD mutants in reaction with wild-type ETF.
**Figure 4a.** Model of a 2ETF–MCAD complex. The left region depicts a ribbon representation of ETF (in blue and violet) in a possible electron transfer competent conformation. The right region depicts a ribbon representation of ETF with the FAD domain in the conformation observed in the free ETF crystal structure. Black arrows indicate the interflavin isoalloxazine ring distances for both conformations. **Figure 4b.** Detailed view of the left region of figure 4a. Redox cofactors and residues likely to be involved in electron transfer are rendered in CPK and sticks, respectively.

**Figure 5a.** Stereo view overlay of the human ETF–MCAD complex (ETF in yellow, MCAD in green) and the *M. methylotrophus* ETF–TMADH crystal structure (ETF in orange, TMADH in blue). For clarity, only a single TMADH monomer and a single MCAD dimer have been depicted. The superposition was generated by overlaying the backbone carbon atoms of the ETF recognition loop from both complexes. The pivotal recognition loop (indicated by an *), residues Leuβ194/195 and Tyrβ191/192 are shown in sticks. The redox cofactors donating electrons to the ETF FAD are shown in CPK, coloured respectively in blue for the TMADH 4Fe-4S centre and yellow for the MCAD FAD. Likewise, residues proposed to transiently interact with the conserved ETF Argβ237/249 are depicted in CPK, coloured respectively in light blue for TMADH Tyr442 and bright green for MCAD Glu212. **Figure 5b.** Stereo view of an overlay of the docking site for both ETF complexes shown in Figure 5a. Residues directly involved in contact between the ETF recognition loop and the ETF partners are depicted in sticks.
Human ETF—MCAD complex structure

Figure 1 a, b, c

![Image of Human ETF—MCAD complex structure]
Figure 2 a, b
Figure 2 c,d

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**Human ETF—MCAD complex structure**
Figure 2 e, f
Figure 3 a,b
Figure 4 a,b
Figure 5 a,b
Extensive domain motion and electron transfer in the human ETF-MCAD complex
Helen S. Toogood, Adam van Thiel, Jaswir Basran, Mike J. Sutcliffe, Nigel S. Scrutton and David Leys

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