OXIDATION OF THE FAD COFACTOR TO THE 8-FORMYL-DERIVATIVE IN HUMAN ELECTRON TRANSFERRING FLAVOPROTEIN

Peter Augustin§*, Marina Toplak§*, Katharina Fuchs§, Eva Christine Gerstmann§, Ruth Prass§, Andreas Winkler§ and Peter Macheroux§

§Institute of Biochemistry, Graz University of Technology, Petersgasse 12/II, 8010 Graz, Austria
§Institute of Biophysics, Medical University of Graz, Neue Stiftungtalstraße 6/IV, 8010 Graz, Austria

*These authors have contributed equally to this work

Running title: 8-formyl-FAD formation in hETF

*To whom correspondence should be addressed: Prof. Dr. Peter Macheroux, Graz University of Technology, Institute of Biochemistry, Petersgasse 12/II, A-8010 Graz, Telephone: +43-(0)316-873 6450; FAX: +43 (0)316-873 6952; E-mail: peter.macheroux@tugraz.at

Keywords
8-formyl-FAD, electron transfer, flavin semiquinone, mitochondrial matrix, dehydrogenases.

Abbreviations
8f-FAD, 8-formyl-FAD; (h)DMGDH, (human) dimethylglycine dehydrogenase; DPPH, α,α'-diphenyl-β-picryl hydrazyl; (h)ETF, (human) electron-transferring flavoprotein; (h)ETF-QO, (human) ETF-ubiquinone oxidoreductase; GAI, glutaric aciduria type II; (h)MCAD, (human) medium chain acyl-CoA dehydrogenase; (h)SARDH, (human) sarcosine dehydrogenase.

ABSTRACT

The heterodimeric human electron transferring flavoprotein (hETF) transfers electrons from at least thirteen different flavin dehydrogenases to the mitochondrial respiratory chain through a non-covalently bound FAD cofactor. Here, we describe the discovery of an irreversible and pH-dependent oxidation of the 8α-methyl group to 8-formyl-FAD (8f-FAD), which represents a unique chemical modification of a flavin cofactor in the human flavoproteome. Furthermore, a set of hETF variants revealed that several conserved amino acid residues in the FAD binding pocket of electron transferring flavoproteins are required for the conversion to the formyl group. Two of the variants generated in our study, namely αR249C and αT266M, cause glutaric aciduria type II (GAII), a severe inherited disease. Both of the variants showed impaired formation of 8f-FAD shedding new light on the potential molecular cause of disease development. Interestingly, the conversion of FAD to 8f-FAD yields a very stable flavin semiquinone that exhibited slightly lower rates of electron transfer in an artificial assay system than hETF containing FAD. On the other hand, the formation of 8f-FAD enhanced the affinity to human dimethylglycine dehydrogenase fivefold, indicating that formation of 8f-FAD modulates the interaction of hETF with client enzymes in the mitochondrial matrix. Thus, we hypothesize that the FAD cofactor bound to hETF is subject to oxidation in the alkaline (pH = 8) environment of the mitochondrial matrix, which may modulate electron transport between client dehydrogenases and the respiratory chain. This discovery challenges current concepts of electron transfer processes in mitochondria.

Copyright 2018 by The American Society for Biochemistry and Molecular Biology, Inc.
INTRODUCTION
In 1956, Crane and coworkers identified the electron transferring ability of an unknown flavoprotein from the pig liver, which they named electron-transferring flavoprotein (ETF) (1). Since then, numerous studies on ETF have been reported and orthologs have been described in all kingdoms of life (2). The heterodimeric human electron-transferring flavoprotein (hETF) serves as a central electron carrier in the mitochondrial matrix. hETF accepts electrons from thirteen flavin dehydrogenases and transfers them to the human ETF-ubiquinone oxidoreductase (hETF-QO), an iron-sulfur cluster containing flavoprotein bound to the inner mitochondrial membrane that feeds these electrons into the mitochondrial respiratory chain (2, 3). The flavin dehydrogenases are either part of β-oxidation, amino acid or choline degradation, as shown in Scheme 1.

Interestingly, these dehydrogenases are structurally distinct with dehydrogenases operating either in the degradation of fatty or amino acids adopting the “acyl-CoA dehydrogenase”-fold, whereas both dimethylglycine dehydrogenase (hDMGDH) and sarcosine dehydrogenase (hSARDH) are part of the amine oxidase protein family. Apparently, hETF has evolved a flexible mechanism to interact with various dehydrogenases as well as with hETF-QO (2). The protein exists in a closed, non-productive, and in an open, productive conformation with a highly flexible upper protein domain (Scheme 1). The interaction with client dehydrogenases is initiated by a recognition peptide of the β-subunit leading to the exposure of the FAD and concomitant electron transfer (4).

Recently, Toogood et al. have shown that a single amino acid replacement in the β-subunit (βE165A) favors the open conformation by removing a side chain interaction with αN259 in the α-subunit (4). As a consequence, variant βE165A displayed higher affinity to the human medium chain acyl-CoA dehydrogenase (hMCAD) (and to rat DMGDH) enabling co-crystallisation (Scheme 1). In the course of our attempts to co-crystallize hETF with hDMGDH, we have reproduced the βE165A variant as well as generated the corresponding αN259A variant. However, characterization of the variants by UV/Vis absorption spectroscopy indicated that the FAD cofactor undergoes a slow conversion affecting the isoalloxazine ring. A detailed analysis of the isolated flavin moiety showed that the observed conversion entails the oxidation of the 8α-methyl to the formyl group, i.e. 8-formyl-FAD (8F-FAD) is the product of the spontaneous conversion observed in these two variants. This prompted us to extend the study to include wild-type hETF and several variants with amino acid replacements in the binding pocket of the isoalloxazine ring. In the present study, we document that (i) conversion of FAD to 8f-FAD also occurs in wild-type hETF at alkaline pH, (ii) the 8f-FAD stabilizes an oxygen-sensitive semiquinone radical and (iii) several highly conserved amino acid residues in the FAD binding pocket are essential for the formation of 8f-FAD. In addition, we demonstrate that wild-type hETF bearing 8f-FAD as cofactor exhibits increased affinity to reduced hDMGDH under steady-state conditions suggesting a potential role in vivo. Taken together our results report entirely novel properties of hETF that may have important implications for our understanding of electron handling in the mitochondrial matrix.

RESULTS
Time-dependent absorption changes of purified recombinant hETF
Human electron transferring flavoprotein (hETF) produced in Escherichia coli host cells was purified by means of Ni-NTA affinity chromatography and yielded two mg of homogeneous protein per g of wet cell pellet (Figure 1). In order to explore the effect of the pH on protein yield and stability, purifications were performed at pH 7, 7.8 and 8.5. This gave rise to different UV/Vis absorption spectra as shown in Figure 2A. At neutral pH, the absorption spectrum featured two maxima at 375 and 436 nm, which were very similar to spectral properties reported for hETF and pig liver ETF, respectively (5, 6). However, pronounced changes were observed when hETF was purified at pH 7.8 or 8.5. These spectra were characterized by a bathochromic shift of the maximum at 436 nm to 415 nm accompanied by an increase in absorption at longer wavelength (Figure 2A). To check whether the observed spectral changes were caused by a chemical modification of the FAD cofactor, hETF purified at pH 7 and pH 8.5
was denatured and absorption spectra were recorded. As shown in Figure 2B, hETF purified at pH 7 yielded the typical absorption spectrum of free FAD with maxima at 370 and 450 nm whereas the flavin moiety released from hETF purified at pH 8.5 featured spectral shifts to 354 and 456 nm, respectively.

Isolation and identification of the modified FAD generated at alkaline pH
To identify the nature of the chemical modification in the flavin chromophore, we extracted the cofactor(s) from hETF purified and kept at pH 8.5. After extraction and purification by HPLC (Figure 3, panels A and B) the two main fractions were analyzed by mass spectrometry and NMR spectroscopy (Figure 3, panels C and D, respectively). Mass spectrometric analysis of one of the major fractions (Peak 2) clearly showed the typical fragmentation pattern (AMP: m/z = 348; FMN: m/z = 439 and 457) and mass of FAD (m/z = 786; MassBank accession number KNA00248, (7)) whereas the other fraction (Peak 1) exhibited a shift of 14 a.u. of all major peaks, except that for AMP. In agreement with the observed differences in the absorption properties, this result confirmed that the chemical modification has most likely occurred in the isalloxazine ring of the FAD cofactor. Further analysis of the two flavin containing fractions by NMR-spectroscopy revealed the presence of a resonance at ca. 10.4 ppm in the unknown flavin species that is absent in FAD. Additional differences were observed in the resonances of the methyl groups at the 7α and 8α positions (see arrows in Figure 3D). Overall, the 1H-NMR-spectrum possesses the same features as previously reported for 8-formyl-FAD isolated from formate oxidase (8). Taken together with the observed mass difference, we therefore conclude that the 8α-methyl group of FAD was oxidized to a formyl group to yield 8-formyl-FAD (8f-FAD).

Time-dependent formation of 8f-FAD in hETF
In order to obtain further insights into the formation of 8f-FAD in hETF, a sample purified at pH 7 was diluted into buffer at pH 8.5 and the spectral changes were observed over time. As shown in Figure 4A, the formation of 8f-FAD is a slow process with an approximate half time of 20 hours for hETF-WT and 4 hours for hETF-αN259A, respectively. The spectral changes are marked by a single set of isosbestic points at 340, 375, 420 and 469 nm and the appearance of a sharp absorption maximum at 415 nm as well as much less pronounced maxima at 650 and 710 nm (Figure 4). The reverse reaction, the formation of FAD from 8f-FAD at neutral or acidic pH was not observed. The formation of 8f-FAD was independent from the buffer used (HEPES, TRIS and phosphate buffer). Similarly, removal of the hexa-histidine tag using TEV protease had no effect on the oxidation of the 8α-methyl group.

Since the formation of 8f-FAD does not occur free in solution, we assumed that the oxidation is promoted by amino acid residues in the isalloxazine binding pocket. Therefore, we generated four variants of hETF featuring single amino acid replacements: αR249C, αT266M, αH286A and βY16F (Figure 5).

Interestingly, the formation of 8f-FAD was substantially affected in all variants and decreased in the order βY16F > αT266M > αR249C (Table 1). For the αH286A-variant 8f-FAD formation could not be monitored reliably since the FAD binding was apparently compromised by the amino acid replacement. Hence, we conclude that amino acid residues directly participate in the oxidation of the 8α-methyl to the formyl group. In addition, we investigated two variants, αN259A and βE165A, that presumably increase the flexibility of the FAD binding domain in order to favor the formation of a productive open conformation for electron transfer (9). Both of the variants showed a much more rapid formation of 8f-FAD, which already occurred at neutral pH (Figure 4B, data for αN259A as an example). This observation strongly suggests that the conformational dynamics of hETF affect the rate of conversion of FAD to the 8f-FAD.

Generation of 8f-FAD leads to the flavin semiquinone
Although our analysis has clearly shown the oxidation of the 8α-methyl to the formyl group, the spectral characteristics observed for the generated 8f-FAD are vastly different to those seen after release of the flavin from hETF (Figure 2B). As a matter of fact, the sharp peak at 415 nm as well as the long wavelength absorption is reminiscent of a flavin semiquinone species suggesting that the

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
8f-FAD is present as a semiquinone radical instead of the oxidized form. The presence of a flavin radical was confirmed by EPR spectroscopy of the hETF variant αN259A, which rapidly forms the 8f-FAD at pH 8.5. A g-factor of 2.0048 was calculated after calibration with DPPH (g-factor of 2.0036) and a peak-to-peak line width of 11-13 G was measured. This line width is rather narrow for a flavin radical and suggests the stabilization of the red, anionic semiquinone (10). Expectedly, hETF-variant βY16F and wild-type hETF purified at pH 7.0 (no 8f-FAD formation) did not yield an EPR-signal.

Redox behavior of wild-type hETF and the αN259A variant

The stabilization of the anionic (red) FAD semiquinone in hETF is a well-established phenomenon (5, 6). As shown in Figure 6A (main panel), photoreduction of wild-type hETF purified at neutral pH yielded the FAD semiquinone before full reduction to the hydroquinone was achieved with sodium dithionite (Figure 6A, insert). In contrast to wild-type hETF, the αN259A variant purified at pH 8.5 was already present in the semiquinone form and photoreduction yielded the fully reduced hydroquinone species featuring characteristic absorption maxima at 390 and 510 nm (11, 12). The hydroquinone form of 8f-FAD is sensitive to oxygen and is reoxidized to the semiquinone but not to the oxidized form. Thus, hETF not only stabilizes the semiquinone form of 8f-FAD but also prevents its oxidation by molecular oxygen. This differential behavior of FAD vs. 8f-FAD has important implications for the one-electron transfer processes between hETF, the serviced dehydrogenases as well as the electron acceptor hETF-QO.

Physiological relevance of 8f-FAD, effects on stability and protein-protein interaction

In order to evaluate the potential impact of FAD cofactor formylation on the electron transfer reaction, we investigated the steady-state kinetics of hETF with human dimethylglycine dehydrogenase (hDMGDH), which is one of its client dehydrogenases. For that purpose, wild-type and variant αN259A were both purified in 50 mM HEPES pH 7.0 and pH 8.5 and afterwards, the steady-state kinetic parameters were determined at pH 7.0. As shown in Figure 7 and summarized in Table 2, we observed moderate changes in the velocity of electron transfer (κcat was reduced by ca. 50%) and a 5-fold decrease in the Kcat values in the proteins harboring 8f-FAD. It should be noted in this context that hETF-αN259A purified at pH 7.0 already contains ca. 15-30% of 8f-FAD, which may contribute to the lower Kcat value compared to the wild-type hETF (Table 2). On the other hand, wild-type hETF purified at pH 8.5 contains only ~30-40% 8f-FAD and thus the obtained Kcat value reflects a mixed population of wild-type hETF, i.e. with the FAD cofactor in its methylated (native) and formylated structure, respectively. This may also contribute to the differences found between wild-type hETF and the hETF-αN259A variant (Table 2). The steady-state kinetics of protein variant βE165A purified at pH 7.0 resembled the values obtained for wild-type hETF purified at pH 7.0. In contrast to the corresponding variant αN259A, FAD formylation was absent in the freshly purified variant βE165A and was only observed during long time storage (at pH 7).

DISCUSSION

Discovery of an unusual FAD derivative in an old protein

In the present investigation, we have unambiguously demonstrated the formation of 8f-FAD in hETF by means of various spectroscopies such as HPLC/MS, UV/Vis absorption, NMR- and EPR- spectroscopy. Furthermore, it was shown that the formation of 8f-FAD strongly depended on the pH such that wild-type hETF is resistant to the formation of 8f-FAD at acidic and neutral pH while at alkaline pH the slow oxidation of the 8α-methyl of the formyl group is observable (τ1/2 = 20 h, Figure 2A and 4A). Interestingly, 8f-FAD was not present in the oxidized but in the semiquinone state, which was stable towards oxidation by dioxygen. The UV/Vis absorption spectrum featured a sharp peak at 415 nm and two minor absorption maxima at longer wavelength (650 and 720 nm, Figure 2A). The latter spectral features are clearly indicative of the (blue) neutral semiquinone although the peak-to-peak linewidth measured by EPR-spectroscopy suggested that the major fraction is the (red) anionic semiquinone. Thus, we conclude that a mixture of the blue and red semiquinone is present in hETF suggesting that our measurements were conducted near the
pertinent pKa of the flavin radical, as was also found earlier for a lysine to arginine replacement in the FMN binding pocket of lactate oxidase (13). Overall, the spectral characteristics are very similar to those reported earlier by Yorita et al. (13) and by Maeda et al. (14) for formate oxidase from *Aspergillus oryzae*. In the latter case, it was shown that oxidation of the 8a-methyl group significantly enhances enzyme activity and thus it was argued that the 8f-FAD derivative is the cognate cofactor of the enzyme.

Since detailed studies on ETF date back to the 1950s, we were very surprised that our findings were apparently not observed previously. However, closer inspection of the literature revealed that isolation of ETF from pig liver yielded two forms (designated ETFR and ETFb), which were reduced to the red (ETFb) and blue semiquinone (ETFb), respectively (11). Analysis of the isolated cofactors demonstrated the presence of a flavin species with absorption maxima at 463 and 352 nm, which are in fact very similar to the spectral features observed for the 8f-FAD spectrum after denaturation of wild-type hETF purified at pH 8.5 (Figure 2B, red solid line). Furthermore, Lehman & Thorpe reported the occurrence of a pink species upon reduction featuring an absorption maximum at 520 nm. Again, this is reminiscent of the absorption spectrum obtained when the aN259A hETF variant is photoreduced (Figure 6B, dashed black line). Similarly, Yorita et al. (13) reported the same species upon reduction of 8f-formyl FMN bound to lactate oxidase. Therefore, we assume that Lehman and Thorpe have also isolated the 8f-FAD cofactor from pig liver ETF, however, neither the chemical nature of the flavin nor the mode of its generation was further investigated (11).

Since the amino acid residues in the FAD binding pocket of mammalian and bacterial ETFs are highly conserved (Figure 8), it was tempting to assume that the generation of 8f-FAD is a common feature in this protein family. Thus, we have analyzed the crystal structures of previously published ETF structures - hETF (pdb: 1EFV), the ETF from *Methylophilus methylotrophus* (pdb: 1O96), *Paracoccus denitrificans* (pdb: 1EFP) and *Acidaminococcus fermentans* (pdb: 4KPU). Closer inspection of the electron densities obtained for the 8α-position showed obvious deviations from the expected electron density of a methyl group for some reported crystal structures (Figure 9). In fact, in those cases the additional electron density is indicative of a covalent modification of the 8α-methyl group and is in line with the presence of (partially) formylated FAD (Figure 9). With the exception of hETF, this was also found in cases where crystallization was carried out at or near pH 7, *i.e.* under conditions where oxidation of the 8α-methyl to the formyl group is very slow. The structurally very similar bacterial ETFs from *A. fermentans* and *M. methylotrophus* show clear indications of FAD modification even for wild type forms and a variant with a conserved positive charge on the re side of the flavin (Figure 9, panels C, D, F), whereas no indication of 8f-FAD formation is observed for the modification incompetent αR236A variant of *M. methylotrophus* ETF (Figure 9, panel E). With regard to ETF from *M. methylotrophus* it is also noteworthy that Byron et al. (15) clearly have observed the 8f-flavin radical species (cf. Figure 4) but did not further elucidate the cause of the drastic change in the absorption spectrum. The occurrence of the 8f-FAD in *M. methylotrophus* ETF is potentially relevant as this could (partially) explain the unusually positive redox potential of the protein (+196 mV for the ETFox/ETF redox pair) (15), which is in accordance with previous reports that the redox potential of free 8f-FAD is ca. 130-160 mV more positive than that of free FAD (8, 12).

**Proposed reaction mechanism for the formation of 8f-FAD**

We propose that the oxidation of the 8α-methyl group is initiated by proton abstraction, as previously proposed (16–18). This role is assumed by hydroxide leading to the formation of a negative charge at the N(1)-C(2) = O locus of the isovaloxazine ring. After addition of water the reduced intermediate is oxidized to yield the 8α-hydroxymethylene intermediate, which is again subject to proton abstraction at the 8α-group with the resulting enol tautomerizing to the reduced 8f-FAD. Taking the results of our mutagenesis study into account, it is conceivable that the phenolate side chain of αY16 may act as a base (instead of hydroxide). Such an involvement would be reminiscent of the formation of a covalent linkage established in 8α-tyrosyl-modified flavoenzymes (19–22). The side chains of
αR249, αT266 and αH286 support the reaction by hydrogen bonding to the N(1)-C(2)=O locus (αH286), N(5) (αT266) and by π-cation interaction (αR249), respectively. Interestingly, variants favoring the open conformation of hETF, i.e. βE165A and αN259A (see also discussion below), also promote the oxidation of the 8α-methyl group, probably due to greater solvent exposure of the relevant part of the isoalloxazine ring in line with the proposed role of hydroxide and water in the putative reaction mechanism. Since our experiments have shown that the generated 8f-FAD is preferentially present as the semiquinone, we propose that the 8f-FAD is rapidly reduced, e.g. in vitro by light and in vivo by one of the client dehydrogenases, to the very stable radical species.

**Effect of cofactor oxidation to the 8f-FAD on electron transfer**

The oxidation of FAD to 8f-FAD clearly affected the interaction with hDMGDH, which is one of the confirmed client dehydrogenases of hETF. The most striking effect was observed on the apparent $K_M$ defined here as the concentration of hETF at which the rate of electron transfer proceeds at half maximal velocity. A five-fold lower $K_M$ was observed not only in the case of wild-type hETF but also for the βE165A and αN259A variants when 8f-FAD was present as the main cofactor moiety (Table 2). A smaller effect was also seen on the maximal rate of electron transfer from reduced hDMGDH to hETF as well as the βE165A and αN259A variants, which was approximately 50-60% lower in the proteins containing 8f-FAD instead of FAD (Table 2). Thus, it is apparent that oxidation of the FAD cofactor in hETF significantly affects the interaction with hDMGDH. In fact, Toogood et al have reported that hETF variant βE165A has a higher affinity to hMCAD and rat DMGDH compared to the wild-type protein (9). Since the side chains of amino acid residues βE165 and αN259 stabilize a non-productive, closed hETF conformation (Scheme 1) the replacement by alanine populates the open conformation, which is conducive to complex formation with an electron delivering dehydrogenase and enabled the crystallization of a protein complex comprising hETF and hMCAD (9). As a consequence of this perturbation of the conformational equilibrium of the closed and open conformation, the protein complex of hETF with its client enzymes exhibits higher affinity and thus rationalizes the observed kinetic effects on $K_M$ and the rate of electron transfer under steady-state conditions (Figure 7 and Table 2).

Interestingly, formation of 8f-FAD appears to exhibit a similar effect on the interaction with client dehydrogenases as seen in the βE165A and αN259A variants. Therefore, we hypothesize that the presence of 8f-FAD in hETF leads to a significant shift of the conformational equilibrium toward the open (productive) conformation, which in turn increases the affinity to client enzymes. Currently, we are conducting hydrogen/deuterium exchange mass spectrometric (H/DX-MS) experiments to test this hypothesis.

**Potential physiological role of 8f-FAD in health and disease**

Considering the apparent impact of the formylation of the FAD cofactor, the central question that emerges here concerns the physiological relevance of the cofactor modification. As the pH of the mitochondrial matrix was found to be close to 8.0 (23, 24), formylation of wild-type hETF would clearly occur in this environment albeit, at a slow rate and thus, the overall lifetime of the protein will ultimately determine the fraction of hETF containing 8f-FAD. Although, this lifetime is currently not known for hETF, mitochondrial matrix proteins were shown to exhibit half lives in the range of 17 to more than 100 hours (25). In the case that hETF belongs to the long-lived proteins a significant fraction of the protein will in fact harbor the 8f-FAD cofactor. Moreover, it is conceivable that other proteins, such as the electron delivering dehydrogenases, promote the formation of 8f-FAD by stabilizing the open conformation of hETF as seen in the αN259A and βE165A variants.

In this context, it is very intriguing that several inborn mutations in the gene encoding human ETF are known to cause glutaric aciduria type II (GAI1), also called multiple acyl-CoA dehydrogenase deficiency (OMIM entry #231680), a disease characterized by severe non-ketotic hypoglycemia, metabolic acidosis and excretion of large amounts of fatty acid and amino acid-derived metabolites (26, 27).
Strikingly, two of the hETF variants generated in our mutagenesis study, namely αR249C and αT266M, were reported as the cause of GAI1, with the latter being the most abundant variant found in affected patients (28, 29). Because both of these variants were severely impaired in the formation of the 8f-FAD derivative in their active sites, it is tempting to speculate that the inability to catalyze the oxidation of the 8α-methyl to the formyl group contributes to the disease causing effect of the underlying genetic mutation. In a previous study, Dwyer et al. concluded that αR249 plays a crucial role in stabilizing the flavin semiquinone state, however, at that time the occurrence of 8f-FAD and its mode of generation had not been recognized (30).

The remarkable stability of the 8f-FAD radical may alter the operational basis of the electron transfer reactions of hETF with the serviced dehydrogenases and the terminal electron acceptor hETF-QO. In hETF with FAD as cofactor, it is assumed that the oxidized FAD receives one electron from a client dehydrogenase generating the FAD semiquinone, which spontaneously disproportionates and subsequently reduces hETF-QO by two single electron transfer processes (29). Only, the fully reduced hETF is then able to transfer two electrons to the hETF-QO. Thus, it will be interesting to see how formylation affects the electron transfer process between the hETF and the hETF-QO. In addition, 8f-FAD also possesses a much more positive redox potential, i.e. around -90 mV (15), and therefore formylation may also affect the rate of electron transfer between the client dehydrogenases, hETF and hETF-QO.

CONCLUSIONS

Our study has conclusively shown that hETF catalyzes the oxidation of FAD to 8f-FAD, an unusual cofactor modification that has not been reported for hETF or any other electron transferring flavoprotein. Furthermore, we have demonstrated that the generation of 8f-FAD strongly depends on the pH, is catalyzed by amino acid residues in the FAD binding pocket and is favored in variants that preferentially adopt an open conformation. Depending on the lifetime of hETF in the mitochondrial matrix formation of 8f-FAD will be physiologically relevant in particular because the 8f-FAD alters the interaction with electron delivering dehydrogenases and the electron acceptor hETF-QO in the inner mitochondrial membrane. It also remains to be seen whether additional factors, such as the interaction with client dehydrogenases or the hETF-QO, affect the formation of 8f-FAD. In any case, it is apparent that our discovery of the spontaneous cofactor oxidation in a central protein of mitochondrial electron handling raises important biochemical and physiological questions with implications for human health and disease.

EXPERIMENTAL PROCEDURES

Enzymes and reagents – Restriction enzymes and Phusion DNA polymerase were from Thermo Fisher Scientific (Waltham, MA, USA), purification columns from GE Healthcare (Chalfont St. Giles, UK). Salt free purified oligonucleotides for site directed mutagenesis were synthesized by VBC-Biotech (Vienna, Austria) or Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and media were from Carl Roth GmbH (Karlsruhe, Germany) or Sigma-Aldrich (St. Louis, MO, USA) and were of the highest grade available.

hETF-WT and hETF variants gene expression design – The hETF sequence for expression of the mature hETF consisting of α- and β-subunit was designed following a similar strategy as described by Bross et al. (31) and was optimized for expression in Escherichia coli using GeneOptimizer® (Thermo Fisher Scientific). The operon starts with a ribosomal binding site (AAGGAG), followed by a TATA box in front of the ATG start codon of the gene sequence of the β-subunit. After the stop codon of the β-subunit, a 69 base pair spacer region between the β and α gene sequence was introduced, which again comprises the same ribosomal binding site and a TATA box in front of the ATG start codon of the α-subunit. The designed gene sequence was flanked by an XbaI and an XhoI restriction site and cloned for expression into a pET-28a+ vector (Thermo Fisher Scientific). In agreement with Herrick et al. (5), the α-subunit starts with the first amino acid of the mature αETF (αGln20). For protein purification, a hexa-histidine tag was added to the N-terminus of the β-subunit. The recombinant plasmid was transformed into E. coli BL21 (DE3) cells. Positive clones were
selected by kanamycin resistance. Correct cloning and potent expression colonies were verified by automated sequencing. All investigated variants of hETF-WT (αR249C, αN259A, αT266M, αH286A, βY16F and βE165A) were constructed by two-step site directed mutagenesis with Phusion DNA polymerase and the mutation primers shown in Table 3 (the altered codons are highlighted in bold).

First, two separate PCR reactions using either forward or reverse primer with 10 ng hETF-WT template DNA, 1x Phusion HF buffer, 200 µM dNTPs, 3% DMSO, 1 unit of Phusion DNA polymerase and 0.5 µM of each primer were run in 50 µL volumes with (98 °C (2 min) – [98 °C (50 sec) – 60 °C (20 sec) – 68 °C (16.5 min)] x 5 – 4 °C ∞). Afterwards, the separated PCR reactions were combined and the same PCR program was further employed for another 20 cycles. The PCR was followed by a 2 h DpnI digestion step, the plasmid afterwards transformed into E. coli BL21 (DE3) cells and the strain selection was done as above using the pET28a+ kanamycin resistance.

**hETF production** – hETF expression was carried out in shake flasks in an HT Multitron Standard shaking system (Infors AG, Basel, Switzerland) at 150 rpm. Briefly, O/N LB medium cultures of E. coli BL21 (DE3) cells with the desired hETF variant were used to inoculate 1 L main culture in a baffled shake flask to an OD₆₀₀ of 0.1. After reaching an OD₆₀₀ of 0.6-0.8, the protein production was started by induction with 0.1 mM IPTG and expression took place O/N at 25 °C. The cell pellet was collected by centrifugation (2 704 g, 15 min, RT) and stored at -20 °C.

**hETF purification** – In general, enzyme purification was carried out according to the following protocol. Cell lysates were prepared by 4 x 3 min sonication (3 min cooling steps) using a Labsonic®-L sonication probe (B. Braun Biotech, Berlin, Germany) in a Sonopuls® rosett cell RZ (Bandelin, Berlin, Germany). The wet cell pellet was suspended in lysis buffer (3 ml 50 mM HEPES/NaOH, 15 mM imidazole, pH 7.0, 7.8 or 8.5 per g) and a spatula tip of FAD was added before sonication. The lysates were cleared by centrifugation (38,720 g, 45 min, 4 °C) and filtration through a paper filter. Nickel ion affinity chromatography was performed by applying the cell lysates onto 5 mL HisTrap HP columns (GE Healthcare). Afterwards, the columns were washed with at least 10 column volumes lysis buffer and the enzyme stripped off with elution buffer (50 mM HEPES/NaOH, 200 mM imidazole, pH 7.0, 7.8 or 8.5). The purification was monitored by SDS-PAGE and fractions containing hETF were concentrated using Amicon® ultracentrifugal filter units (10 kDa cut-off, Merck-Millipore, Darmstadt, Germany) and rebuffered to storage buffer (50 mM HEPES/NaOH, pH 7.0, 7.8 or 8.5) using Sephadex G-25 PD10 desalting columns (GE Healthcare). After rebuffering, the enzyme solution was incubated at 37 °C for 30 min and afterwards cleared by centrifugation in order to remove aggregated protein and excessive hETF β-subunits. The obtained enzyme purity was sufficient for all kinetic and spectrophotometric studies.

**hDMGDH production and purification** – hDMGDH expression and purification for use in interaction studies for steady-state kinetic analyses was performed as previously reported by Augustin et al. (32).

**SDS-PAGE** – Enzyme samples were separated by SDS-PAGE with 12.5% separation and 5% stacking gels under reducing conditions (100 mM DTT in the sample buffer) as described by Laemmli (33). Gels were stained with Coomassie Brilliant Blue R-250 for purification control and a PageRuler® Prestained protein ladder (Thermo Fisher Scientific) was employed as protein standard.

**UV/Vis absorption spectroscopy** – UV/Vis absorption spectra to assess protein concentration, activity, purity and quality as well as for steady-state kinetic measurements and photoreduction were recorded with a Spectord 210 spectrophotometer (Analytik Jena, Jena, Germany).

**Protein quantification and calculation of the extinction coefficient** – Protein concentrations of purified hETF wild-type and variants were determined using the characteristic absorption of protein bound FAD at 469 nm (isosbestic point of FAD and 8f-FAD). A molar extinction coefficient (ε) of 9,900 ± 700 M⁻¹ cm⁻¹ for hETF was determined using the method described by Macheraux (34) based on an ε of free FAD at 469 nm of 9,910 M⁻¹ cm⁻¹.
Extraction and purification of 8f-FAD – Wild-type hETF or the variant αN259A were purified in 50 mM HEPES/NaOH buffer at pH 8.5 and concentrated to a about 300-400 µM using Amicon® ultracentrifugal filter units (10 kDa cut-off). After complete denaturation, which was achieved by two times treatment at 70 °C for 10 min and subsequent centrifugation at 18,500 g for 10 min, the supernatants were transferred to HPLC-vials for purification. HPLC purification was done on a Dionex UltiMate 3000 HPLC (Thermo Fisher Scientific) equipped with an Atlantis® dC18 column (5 µm, 4.6 x 250 mm, Waters, Milford, MA, USA) equilibrated with H2O/0.1% TFA, 7% acetonitrile and a diode array detector for UV/Vis monitoring (λ = 280, 370, 450, 460 nm). An injection volume of 40 µL, a temperature of 25 °C and a flow rate of 1 mL min\(^{-1}\) were used. 8f-FAD was separated from residual free FAD and other impurities for subsequent UV-Vis absorption and NMR-spectroscopy as well as HPLC/MS analyses using the following program: 0-25 min: 7-12% acetonitrile, 25-30 min: 95% acetonitrile, 30-35 min: 7% acetonitrile. 200 µL fractions were collected between 12.0 and 18.0 min and fractions with a pure and typical 8f-FAD UV/Vis absorption spectrum were combined. The combined fractions were dried using an ISS110 Savant SpeedVac System at 45 °C, < 10 mbar vacuum and an RH64-11 rotor (Thermo Fisher Scientific), and afterwards stored at -20 °C until further use. Purity of the cofactor was controlled by HPLC measurements using the same conditions as above.

Analysis of 8f-FAD by HPLC/ESI-MS - For HPLC/MS measurements, an Agilent Technologies 1200 Series (Santa Clara, CA, USA) equipped with a G1379B degasser, G1312B binary pump SL, G1367C HiP-ALS SL autosampler, a G1314C VWD SL UV detector, G1316B TCC SL column oven and a G1956B MSD mass selective detector was used. The mass spectrometer was operated in positive electro spray ionization mode. The analytes were separated on an Atlantis® dC18 column (5 µm, 4.6 x 250 mm, Waters) at 25 °C by using aqueous eluent (0.1% formic acid) and acetonitrile at a flow rate of 1.0 mL min\(^{-1}\). The column was equilibrated with 7% acetonitrile in water (0.1% formic acid) and the following gradient was used for analysis: 0-2 min: 7% acetonitrile, 2-10 min: 7-100% acetonitrile, 10-12 min: 100% acetonitrile, 12-14 min: 7% acetonitrile. 10 µL of 300 µM HPLC purified 8f-FAD or 300 µM FAD solution for control, dissolved in water, were injected for each run.

\(^{1}\)H-NMR-spectroscopy – 4 mM solutions of purified 8f-FAD or FAD dissolved in 20% D_2O in water (v/v) were subjected to \(^{1}\)H-NMR analysis using a Varian INOVA 500 (499.82 MHz, Agilent) spectrometer. \(^{1}\)H-NMR spectra were recorded at 500 MHz at 30 °C. The signal of water protons (δ_H 4.75 ppm) was used as the reference for the observed chemical shifts.

EPR-spectroscopy – Electron paramagnetic resonance experiments were performed with 150 µM hETF-αN259A and hETF-Y16F purified in 50 mM HEPES/NaOH, pH 8.5 and with 150 µM hETF-WT purified in 50 mM HEPES/NaOH, pH 7.0 with a X-band ECS 106 spectrometer (Bruker, Billerica, MA, USA) with 9.45 GHz microwave frequency. A microwave power of 2 mW was used, with a modulation amplitude of 2.0 G at a modulation frequency of 50 kHz. Samples were run at 295 K in 100 µL capillaries and 10 scans with a conversion time of 5.12 ms, a time constant of 10.24 ms and a sweep time of 20.97 s. The magnetic field was scanned for 100 G from 3340 to 3440 G. The magnetic phase and field modulation amplitude of the signal channel of the EPR machine was calibrated with solid DPPH (α, α’-diphenyl-β-picrylhydrazyl) according to the manual to a g factor of 2.0036.

HPLC-analysis of 8f-FAD formation Protein purified at pH 7.0 was diluted to a final concentration of 40 µM to adjust the pH to 8.5 and then incubated at 25 °C. Samples were taken after 0, (1), 2, 4, 6, 8 and 24 hours and the cofactor was isolated according to the protocol described above. After HPLC analysis areas of the peaks corresponding to FAD and 8f-FAD were determined and the ratio between the two chromophores was calculated.

steady-state kinetics – Steady-state kinetic parameters were determined spectrophotometrically according to Okamura-Iked a et al. (35) using 2,6-dichlorophenolindophenol (DCPIP) as terminal electron acceptor. For the assays, 125 µM DCPIP, 100 nM hDMGDH and 0-100 µM hETF in 50 mM...
HEPES/NaOH pH 7.0 were incubated at 25 °C for 10 min before the reaction was started by addition of 25 mM dimethylglycine (DMG) and the change of absorption was monitored at 600 nm for 3 min. For each concentration, at least a triplicate measurement was performed. From these data the initial velocities were determined and $K_M$ and $k_{cat}$ were assessed using a non-linear hyperbolic fit in Origin 8.6 (OriginLab Corp., Northampton, MA, USA).

**Anaerobic photoreduction** – Photoreduction of flavoproteins was done according to the method reported by Massey and Hemmerich (36). The experimental procedure of photoreduction and reoxidation was performed as described by Augustin et al. (32). Approximately 20 µM purified enzyme in 50 mM HEPES/NaOH, pH 7.0 were reduced at 15 °C. Further reduction of hETF-WT was achieved by adding a 10-fold excess of sodium dithionite to the solution.

**Thermo FAD thermal stability** – The temperature stability of the proteins was determined by monitoring the change in the intrinsic protein fluorescence of FAD in a ThermoFluor® assay (37). Thermo FAD measurements were carried out with an FX Connect real time PCR system (Bio-Rad) in 25 µL of 50 mM HEPES/NaOH, pH 7.0 and 30 µM enzyme. The samples were pre-heated to 25 °C and then the temperature was increased in 0.5 °C/min steps to 95 °C. Fluorescence data were collected using the FRET channel. Melting temperatures (T_m) were determined using the CFX Manager 3.0 software (Bio-Rad, Hercules, CA, USA).

**Author contributions**
PA, MT, KF and ECG expressed, purified and characterized wild-type hETF and variants; RP conducted and interpreted EPR data. PA, MT, AW and PM designed biochemical experiments and interpreted the data; PA, MT, AW and PM wrote the manuscript.

**Conflict of interest**
The authors declare no conflict of interest.

**Acknowledgements**
We would like to thank Dr. Hansjörg Weber for performing NMR spectroscopy and Thorsten Bachler to set up the HPLC/MS method. We are also grateful to Prof. J.-J. Kim, Medical College of Wisconsin, for providing the electron density map of the human ETF structure (pdb:1EFV). This work was supported by a grant from the Austrian Science Foundation (FWF) to PM (Doctoral program “Molecular Enzymology” W901).

**REFERENCES**
1. Crane, F. L., Mii, S., Hauge, J. G., Green, D. E., and Beinert, H. (1956) On the mechanism of dehydrogenation of fatty acyl derivatives of coenzyme A. I. The general fatty acyl coenzyme A dehydrogenase. *J. Biol. Chem.* 218, 701–706
2. Toogood, H. S., Leys, D., and Scrutton, N. S. (2007) Dynamics driving function - New insights from electron transferring flavoproteins and partner complexes. *FEBS J.* 274, 5481–5504
3. Ghisla, S., and Thorpe, C. (2004) Acyl-CoA dehydrogenases: A mechanistic overview. *Eur. J. Biochem.* 271, 494–508
4. Toogood, H. S., Van Thiel, A., Basran, J., Sutcliffe, M. J., Scrutton, N. S., and Leys, D. (2004) Extensive domain motion and electron transfer in the human electron transferring flavoprotein medium chain acyl-CoA dehydrogenase complex. *J. Biol. Chem.* 279, 32904–32912
5. Herrick, K. R., Salazar, D., Goodman, S. I., Finocchiaro, G., Bedzyk, L. A., and Frerman, F. E. (1994) Expression and characterization of human and chimeric human-Paracoccus denitrificans electron transfer flavoproteins. *J. Biol. Chem.* 269, 32239–32245
6. Husain, M., and Steenkamp, D. J. (1983) Electron transfer flavoprotein from pig liver mitochondria. A simple purification and re-evaluation of some of the molecular properties. *Biochem. J.* 209, 541–545
7. Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K.,
Tanaka, S., Aoshima, K., Oda, Y., Kakazu, Y., Kusano, M., Tohge, T., Matsuda, F., Sawada, Y., Hirai, M. Y., Nakanishi, H., Ikeda, K., Akimoto, N., Maoka, T., Takahashi, H., Ara, T., Sakurai, N., Suzuki, H., Shibata, D., Neumann, S., Iida, T., Tanaka, K., Funatsu, K., Matsuura, F., Soga, T., Taguchi, R., Saito, K., and Nishioka, T. (2010) MassBank: A public repository for sharing mass spectral data for life sciences. *J. Mass Spectrom.* **45**, 703–714

8. Doubayashi, D., Ootake, T., Maeda, Y., Oki, M., Tokunaga, Y., Sakurai, A., Nagaosa, Y., Mikami, B., and Uchida, H. (2011) Formate oxidase, an enzyme of the glucose-methanol-choline oxidoreductase family, has a His-Arg pair and 8-formyl-FAD at the catalytic site. *Biochem. Biotechnol. Biochem.* **75**, 1662–1667

9. Toogood, H. S., Van Thiel, A., Scruton, N. S., and Leys, D. (2005) Stabilization of non-productive conformations underpins rapid electron transfer to electron-transferring flavoprotein. *J. Biol. Chem.* **280**, 30361–30366

10. Schleicher, E., Bittl, R., and Weber, S. (2009) New roles of flavoproteins in molecular cell biology: Blue-light active flavoproteins studied by electron paramagnetic resonance. *FEBS J.* **276**, 4290–4303

11. Lehman, T. C., and Thorpe, C. (1992) A new form of mammalian electron-transferring flavoprotein. *Arch. Biochem. Biophys.* **292**, 594–599

12. Edmondson, D. E. (1974) Intramolecular hemiacetal formation in 8-formylriboflavin. *Biochemistry* **13**, 2817–21

13. Yorita, K., Matsuoka, T., Misaki, H., and Massey, V. (2000) Interaction of two arginine residues in lactate oxidase with the enzyme flavin: Conversion of FMN to 8-formyl-FMN. *Proc. Natl. Acad. Sci.* **97**, 13039–13044

14. Maeda, Y., Doubayashi, D., Oki, M., Nose, H., Sakurai, A., Isa, K., Fujii, Y., and Uchida, H. (2009) Expression in *Escherichia coli* of an unnamed protein gene from *Aspergillus oryzae* RIB40 and cofactor analyses of the gene product as formate oxidase. *Biosci. Biotechnol. Biochem.* **73**, 2645–9

15. Byron, C. M., Stankovich, M. T., Husain, M., and Davidson, V. L. (1989) Unusual Redox Properties of electron-transfer flavoprotein from *Methylophilus methylophilus*. *Biochemistry*. **28**, 8582–8587

16. Jhulki, I., Chanani, P. K., Abdelwahed, S. H., and Begley, T. P. (2016) A remarkable oxidative cascade that replaces the riboflavin C8 methyl with an amino group during roseoflavin biosynthesis. *J. Am. Chem. Soc.* **138**, 8324–8327

17. Robbins, J. M., Souffrant, M. G., Hamelberg, D., Gadda, G., and Bommarius, A. S. (2017) Enzyme-mediated conversion of flavin adenine dinucleotide (FAD) to 8-formyl FAD in formate oxidase results in a modified cofactor with enhanced catalytic properties. *Biochemistry*. **56**, 3800–3807

18. Konjik, V., Brunle, S., Demmer, U., Vanselow, A., Sandhoff, R., Ermler, U., and Mack, M. (2017) The crystal structure of RosB: Insights into the reaction mechanism of the first member of a family of flavodoxin-like enzymes. *Angew. Chemie - Int. Ed.* **56**, 1146–1151

19. Mewis, M., McIntyre, W. S., and Scrutton, N. S. (1998) Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: The current state of affairs. *Protein Sci.* **7**, 7–21

20. McIntyre, W., Edmondson, D. E., Singer, T. P., and Hopper, D. J. (1981) 8α-(O-Tyrosyl)flavin adenine dinucleotide, the prosthetic group of bacterial p-cresol methylhydroxylase. *Biochemistry*. **20**, 3068–3075

21. Kim, J., Fuller, J. H., Cecchini, G., and McIntyre, W. S. (1994) Cloning, sequencing, and expression of the structural genes for the cytochrome and flavoprotein subunits of p-cresol methylhydroxylase from two strains of *Pseudomonas putida*. *J. Bacteriol.* **176**, 6349–61

22. Reeve, C. D., Carver, M. A., and Hopper, D. J. (1989) The purification and characterization of 4-ethylphenol methylenehydroxylase, a flavocytochrome from *Pseudomonas putida* JD1. *Biochem. J.* **263**, 431–437

23. Porcelli, A. M., Ghelli, A., Zanna, C., Pinton, P., Rizzuto, R., and Rugolo, M. (2005) pH difference across the outer mitochondrial membrane measured with a green fluorescent protein mutant. *Biochem. Biophys. Res. Commun.* **326**, 799–804

24. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., Tsien, R. Y., and Biology, C. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green
fluorescent proteins. *Proc. Natl. Acad. Sci.* **95**, 6803–6808

25. Hare, J. F., and Hodges, R. (1982) Turnover of mitochondrial matrix polypeptides in hepatoma monolayer cultures. *J. Biol. Chem.* **257**, 12950–12953

26. Christensen, E., Kolvraa, S., and Gregersen, N. (1984) Glutaric aciduria type II: Evidence for a defect related to the electron transfer flavoprotein or its dehydrogenase. *Pediatr. Res.* **18**, 663–667

27. Frerman, F. E., and Goodman, S. I. (2001) Defects of electron transfer flavoprotein and electron transfer flavoprotein-ubiquinone oxidoreductase: glutaric acidemia type II. in *The Metabolic and Molecular Bases of Inherited Disease. (8th ed.)*, 8th editio (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D. eds), pp. 2357–2365, McGraw-Hill, New York

28. Schiff, M., Froissart, R., Olsen, R. K. J., Acquaviva, C., and Vianey-Saban, C. (2006) Electron transfer flavoprotein deficiency: Functional and molecular aspects. *Mol. Genet. Metab.* **88**, 153–158

29. Salazar, D., Zhang, L., DeGala, G. D., and Frerman, F. E. (1997) Expression and characterization of two pathogenic mutations in human electron transfer flavoprotein. *J. Biol. Chem.* **272**, 26425–26433

30. Dwyer, T. M., Zhang, L., Muller, M., Marrugo, F., and Frerman, F. (1999) The functions of the flavin contact residues, αArg249 and βTyr16, in human electron transfer flavoprotein. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* **1433**, 139–152

31. Bross, P., Pedersen, P., Winter, V., Nyholm, M., Johansen, B. N., Olsen, R. K. J., Corydon, M. J., Andresen, B. S., Eiberg, H., Kolvraa, S., and Gregersen, N. (1999) A polymorphic variant in the human electron transfer flavoprotein α-chain (α-T171) displays decreased thermal stability and is overrepresented in very-long-chain acyl-CoA dehydrogenase-deficient patients with mild childhood presentation. *Mol. Genet. Metab.* **67**, 138–147

32. Augustin, P., Hromic, A., Pavkov-Keller, T., Gruber, K., and Macheroux, P. (2016) Structure and biochemical properties of recombinant human dimethylglycine dehydrogenase and comparison to the disease-related H109R variant. *FEBS J.* 10.1111/febs.13828

33. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**, 680–685

34. Macheroux, P. (1999) UV-visible spectroscopy as a tool to study flavoproteins. *Methods Mol. Biol.* **131**, 1–7

35. Okamura-Ikeda, K., Ikeda, Y., and Tanaka, K. (1985) An essential cysteine residue located in the vicinity of the FAD-binding site in short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases from rat liver mitochondria. *J. Biol. Chem.* **260**, 1338–1345

36. Massey, V., Hemmerich, P., Knappe, W. R., Duchstein, H. J., and Fenner, H. (1978) Photoreduction of flavoproteins and other biological compounds catalyzed by deazaflavins. Appendix: photochemical formation of deazaflavin dimers. *Biochemistry*. **17**, 9–17

37. Forneris, F., Orru, R., Bonivento, D., Chiarelli, L. R., and Mattevi, A. (2009) Thermo FAD, a Thermofluor ®-adapted flavin ad hoc detection system for protein folding and ligand binding. *FEBS J.* **276**, 2833–2840
Tables

Table 1: Formation of 8f-FAD in wild-type hETF and the variants αR249C, αT266M and βY16F: Proteins were purified at pH 7.0 and diluted to a final concentration of 40 µM using HEPES buffer pH 8.5. Aliquots were taken and analyzed with HPLC after 0, 2, 4, 6, 8 and 24 h of incubation at 25 °C. Because the FAD cofactor was bound weakly to the αH286A variant, time dependent formylation could not be measured reliably.

| Protein     | 0 h | 2 h | 4 h | 6 h | 8 h | 24 h |
|-------------|-----|-----|-----|-----|-----|------|
| WT          | < 2.5 | 15 | 17 | 21 | 23 | 47   |
| αR249C      | < 2.5 | < 2.5 | < 2.5 | < 2.5 | < 2.5 | < 2.5 |
| αT266M      | < 2.5 | < 2.5 | < 2.5 | < 2.5 | < 2.5 | 6    |
| βY16F       | < 2.5 | < 2.5 | < 2.5 | 5  | 6  | 22   |

Table 2: Steady-state kinetic parameters of hETF-WT, the variant αN259A and βE165A
To obtain protein with a high FAD content purification was carried out at pH 7, whereas pH 8.5 was used to maximize the 8f-FAD content. All kinetic parameters were determined at pH 7.

| Protein     | $K_M$ (µM) | $k_{cat}$ (µM min$^{-1}$) | $k_i$ (µM$^{-1}$ min$^{-1}$) |
|-------------|------------|--------------------------|-------------------------------|
| WT-FAD      | 71 ± 5     | 116 ± 5                  | 1.6 ± 0.2                     |
| WT-8f-FAD   | 15 ± 2     | 44 ± 1                   | 2.9 ± 0.5                     |
| αN259A-FAD  | 30 ± 2     | 114 ± 2                  | 3.8 ± 0.3                     |
| αN259A-8f-FAD | 16 ± 2   | 63 ± 3                   | 3.9 ± 0.8                     |
| βE165A-FAD  | 74 ± 9     | 122 ± 8                  | 1.6 ± 0.4                     |
| βE165A-8f-FAD | 16 ± 1  | 43 ± 2                   | 2.7 ± 0.3                     |

Table 3: Primer sequences used for site-directed mutagenesis.

| Primer     | DNA Sequence 5’ – 3’ | $T_m$ (°C) |
|------------|----------------------|------------|
| αR249C_fw  | GCAGTTGGTGCAAGCTGCAGCAGCTGGATGC | 67.3       |
| αR249C_rev | GATCAACTGCTGGCACTGGTGCACTGCAACTGC | 67.3       |
| αN259A_fw  | GCAGGTTTTTGGTGCACTGGTGCACTGCAACTGC | 64.0       |
| αN259A_rev | CCAACCTGCATATCAGCAGGGAACAAACCTGC | 64.0       |
| αT266M_fw  | GATATGCAAGTTGTCAGATTGAGTGGCGGCGGAAGATGTTTCGAC | 64.8       |
| αT266M_rev | GTGCAACAAATTTTGGTGCACTGGACAAACTCTGACATC | 64.8       |
| αH286A_fw  | CAATTCGACTTGCCAGACGATGC | 69.3       |
| αH286A_rev | CCTGCCAGTGGCCTGGAATTTGCACC | 70.5       |
| βY16F_fw   | GTTAACGTGGTTATGGATTTTGCGGCAAAATTTGGTCAC | 60.0       |
| βY16F_rev  | CACGATTCTTGACGCAGGCAAAATTTGGTTCG | 60.0       |
| βE165A_fw  | CGGAAAGATGGCAGACGATGATGATGCTG | 63.6       |
| βE165A_rev | CAGACCACATCAATCGACAGTTCAACTTTTAC | 63.6       |
Scheme

**Scheme 1 - Interaction of human flavin dehydrogenases with hETF.** So far, thirteen flavin dehydrogenases, involved in \( \beta \)-oxidation (SCAD, short chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; LCAD, long chain acyl-CoA dehydrogenase; VLCAD, very long chain acyl-CoA dehydrogenase; ACAD9-11, acyl-CoA dehydrogenase family member 9-11), amino-acid (short branched chain acyl-CoA, iso-valeryl-CoA, iso-butyryl-CoA and glutaryl-CoA dehydrogenase) and choline degradation (dimethylglycine and sarcosine dehydrogenase) were identified to interact with hETF. hETF exhibits a flexible interaction mechanism and adopts a closed, non-productive form (pdb:1EFV) and an open, productive conformation, here shown bound to hMCAD (pdb:2A1T). The \( \alpha \)- and \( \beta \)-subunits of hETF are shown in raspberry and in marine cartoon view, respectively. hETF bound hMCAD is displayed in grey. hETF bound FAD is presented in yellow sticks, hMCAD bound FAD in pink stick representation.
Figures

Figure 1

Figure 1: Purification of hETF-WT. The SDS-PAGE of hETF Ni-NTA affinity chromatography shows in lane 1, PageRuler® prestained protein ladder (Thermo Fisher Scientific); lane 2, cell lysate; lane 3, column flow through; lane 4, washing fraction and lane 5, elution fraction.

Figure 2

Figure 2: UV/Vis absorption spectra of native (A) and denatured hETF-WT (B) purified at pH 7, (7.8) and 8.5. (A) The pH conditions used in the purification of hETF-WT - pH 7 (black line), 7.8 (blue line) and 8.5 (red line) – strongly affected the absorption spectra of the isolated protein. (B) Denaturation of hETF purified at pH 7 (black) and 8.5 (red) with 20% SDS also resulted in different absorption spectra (dotted and solid lines represent spectra recorded before and after denaturation, respectively).
Figure 3: Analysis and comparison of the two main flavin-containing fractions isolated from hETF. (A) HPLC reversed phase purification of the extracted cofactor(s) gave two major fractions, which were further analyzed by MS and NMR. (B) The spectra of the two peaks of HPLC purification featured the same shifts as seen in Figure 2B. (C) Mass spectra of the two main fractions as separated by HPLC. The spectrum shown at the bottom exhibits the typical fragmentation and mass peaks of FAD. The mass spectrum at the top shows a mass shift of 14 a.u. (D) In agreement with the mass analysis, the $^1$H-NMR spectrum at the bottom can be assigned to FAD, while the additional resonance at 10.4 ppm and the shifts observed for the methyl groups in position 7α and 8α indicate chemical changes in the dimethylbenzene ring moiety of the isoalloxazine ring. Both methods indicate that a small amount of a closed, hemiacetal form of 8f-FAD is present, as previously observed (12).
Figure 4: Formation of 8f-FAD radical in wild-type hETF and the αN259A variant. (A) ~400 µM wild-type hETF purified in 50 mM HEPES, pH 7.0 was diluted 1:20 with HEPES buffer pH 8.5, and was incubated at 25 °C for 24 hours. (B) ~400 µM hETF-αN259A purified in 50 mM HEPES pH 7.0 was diluted 1:20 with HEPES buffer pH 8.5, and was incubated at 25 °C for 24 hours. The dotted line in both panels represents the spectrum measured after 24 hours. The spectra were normalized to an absorption of 1 at the isosbestic point at 469 nm to simplify comparison. The insets show the time-dependent absorption changes recorded at the indicated times at 415 nm.

Figure 5: Amino acid residues near the isoalloxazine ring system that have been targeted by site-directed mutagenesis: αT266 was replaced by a methionine, αR249 by a cysteine and αH286 and βE165 by an alanine in order to study their influence on 8f-FAD formation. Possible interactions of these residues with the isoalloxazine ring are indicated by the dashed lines.
Figure 6: Photoreduction of wild-type hETF and variant αN259A. (A) Photoreduction of wild-type hETF purified at pH 7.0 proceeded to the anionic (red) FAD semiquinone. The semiquinone resisted further photoreduction and was completely reduced to the hydroquinone upon addition of sodium dithionite (inset). (B) In contrast to wild-type hETF, photoreduction of the hETF-αN259A variant purified at pH 8.5 occurred from the radical to the hydroquinone form.
Figure 7: Steady-state kinetics of wild-type hETF and the variants αN259A and βE165A purified at pH 7 (black squares) and pH 8.5 (red dots). The measurements were performed at pH 7 with varying hETF concentrations, using hDMGDH as client dehydrogenase and DCPIP as the final electron acceptor.
Figure 8: Composition of FAD binding site of additional ETF structures. The crystal structures of bacterial ETFs from *Paracoccus denitrificans* (A, gold; pdb: 1EFP), *Methylophilus methylotrophus* (B, orange; pdb:1O96) and *Acidaminococcus fermentans* (C, blue; pdb: 4KPU) were aligned with the structure of hETF (green, D; pdb:1EFV). The alignment shows that all four structures have a very conserved active site composition.
Figure 9: Electron densities of the FAD region of selected ETF proteins. (A) Human ETF (pdb: 1EFV), (B) human ETF βE165A variant (pdb: 2A1U), (C) Acidaminococcus fermentans ETF (pdb: 4KPU), (D) Methylophilus methylotrophus ETF (pdb: 1O96), (E) M. methylotrophus ETF αR236A variant (pdb: 3CLR) and (F) M. methylotrophus ETF αR236K variant (pdb: 3CLU). In all panels, the $2F_o-F_c$ electron density map contoured at 1σ is shown as light blue mesh around the cofactor and residues of an important loop region on the re side of the flavin. The additionally shown green density map corresponds to the $F_o-F_c$ map contoured at 3σ.
Oxidation of the FAD cofactor to the 8-formyl-derivative in human electron transferring flavoprotein
Peter Augustin, Marina Toplak, Katharina Fuchs, Eva Christine Gerstmann, Ruth Prassl, Andreas Winkler and Peter Macheroux

*J. Biol. Chem.* published online January 4, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.000846

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts