Expression and Characterization of Two Pathogenic Mutations in Human Electron Transfer Flavoprotein

Denise Salazar, Lening Zhang, Gregory D. deGala, and Frank E. Frerman

From the Program in Cellular and Developmental Biology and the Department of Pediatrics, University of Colorado School of Medicine, Denver, Colorado 80262

Defects in electron transfer flavoprotein (ETF) or its electron acceptor, electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO), cause the human inherited metabolic disease glutaric acidemia type II. In this disease, electron transfer from nine primary flavoprotein dehydrogenases to the main respiratory chain is impaired. Among these dehydrogenases are the four chain length–specific flavoprotein dehydrogenases of fatty acid β-oxidation. In this investigation, two mutations in the α subunit, αT266M and αG116R, are the most frequent mutation found in patients with ETF deficiency. The crystal structure of human ETF shows that αG116 lies in a hydrophobic pocket, under a contact residue of the αβ subunit interface, and that the hydroxyl hydrogen of αT266 is hydrogen-bonded to N(5) of the FAD; the amide backbone hydrogen of αT266 is hydrogen-bonded to C(4)-O of the flavin prosthetic group (Roberts, D. L., Frerman, F. E. and Kim, J-J. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14355–14360).

Stable expression of the αG116R ETF required coexpression of the chaperonins, GroEL and GroES. αG116R ETF folds into a conformation different from the wild type, and is catalytically inactive in crude extracts. It is unstable and could not be extendively purified. The αT266M ETF was purified and characterized after stabilization to proteolysis in crude extracts. Although the global structure of this mutant protein is unchanged, its flavin environment is altered as indicated by absorption and circular dichroism spectroscopy and the kinetics of flavin release from the oxidized and reduced protein. The loss of the hydrogen bond at N(5) of the flavin and the backbone amide hydrogen of this residue of the flavin since the chemical properties of bound flavin are modulated by the protein environment (11). The αT266M allele yields a stable antigen on immunoblots, and the specific activity of this mutant ETF in the fibroblasts of a patient who is homozygous for this mutation is about 15% of control values (10). A second mutation, G116R, has also been identified in the α subunit of a patient with glutaric acidemia type II (9, 10).

Electron transfer flavoproteins (ETF) are heterodimeric, FAD-containing proteins that transfer electrons between primary dehydrogenases and respiratory chains in eukaryotic and prokaryotic cells. In mammalian systems, ETF transfers electrons from nine mitochondrial flavoprotein dehydrogenases to the main respiratory chain via the iron-sulfur flavoprotein, ETF-ubiquinone oxidoreductase (ETF-QO) (1). Porcine ETF is apparently closely related to human ETF (2, 3). Both proteins stabilize an anionic flavin semiquinone upon reduction by the flavoprotein dehydrogenases (4, 5). Reduction of ETF to the hydroquinone oxidation state by the dehydrogenases is very slow and not kinetically significant (4, 6). However, kinetic studies of the ETF-QO-catalyzed reduction of ubiquinone by reduced ETF by Ramsay et al. indicated that ETF hydroquinone, and not the semiquinone, is the reductant of ubiquinone (6). This paradox is explained by the rapid disproportionation of ETF semiquinone catalyzed by ETF-QO; ETF-QO then catalyzes the reduction of ubiquinone by ETF hydroquinone.

The x-ray crystal structure of oxidized human ETF was recently solved by Roberts et al. (7). This structure has provided insight into the side chains of amino acid residues that may be involved in stabilization of the anionic ETF semiquinone and hydroquinone and suggested a possible structure for a binary complex of ETF and medium chain acyl-CoA dehydrogenase (7). The protein is composed of three domains, two contributed by the α subunit (αI and αII), and a third contributed entirely by the β subunit, which contains the noncovalently bound AMP (8). The FAD cofactor is bound primarily in the αII domain although the dimethylbenzene ring makes van der Waals contact with Tyr-16, Pro-40, and Phe-41 in the β subunit. The structure also provides some insight into the structural bases for the effects of several missense mutations in ETF that result in the inherited metabolic disease glutaric acidemia type II (9, 10). The most frequent mutant allele thus far identified in patients with ETF deficiency is the substitution of Thr-266 by a methionine residue in the α subunit (9). The hydroxyl hydrogen of Thr-266 is hydrogen-bonded to N(5) of the flavin, and the backbone amide hydrogen of this residue is hydrogen-bonded to the C(4)-O of the flavin pyrimidine ring (7). This mutation would be expected to influence the properties of the flavin since the chemical properties of bound flavin are modulated by the protein environment (11). The αT266M allele yields a stable antigen on immunoblots, and the specific activity of this mutant ETF in the fibroblasts of a patient who is homozygous for this mutation is about 15% of control values (10). A second mutation, G116R, has also been identified in the α subunit of a patient with glutaric acidemia type II (9, 10).
Gly-116 lies just below the surface of an α/β subunit interface. This mutant allele does not yield an active protein (10). Immuno- blot analysis of a fibroblast line carrying this mutation revealed no useful information regarding the stability of the α subunit of αG116R ETF because this allele is present in a patient who is a compound heterozygote, i.e. αT266M/αG116R (9, 10).

We have constructed vectors bearing these mutations and investigated their expression in Escherichia coli and characterized the structural, redox, and kinetic properties of these proteins. The G116R α subunit is unstable in crude extracts, except when coexpressed with the chaperonins GroEL and GroES. When coexpressed with the chaperonins, this ETF apparently folds into an alternate conformation. However, attempts to extensively purify this protein, which exhibits no detectable activity in crude extracts, were unsuccessful. The three-dimensional structure suggests that the instability and alternate conformation of the protein is due to the inability to sterically accommodate the guanidinium side chain of arginine at position 116 and the thermodynamic barriers to sequestering this side chain in a hydrophobic environment. The αT266M mutant ETF is stable after inactivation of proteases in crude extracts, but spectral investigations and the kinetics of flavin dissociation from the oxidized protein indicate that the flavin environment is significantly altered in the mutant protein. The altered environment modifies the redox behavior of the protein and the turnover with ETF-QO in the oxidative half-reaction. The steady-state kinetic constants of the reductive half-reactions of the mutant ETF with several primary dehydrogenases are scarcely altered. These changes accurately reflect the alteration of ETF activity in fibroblast extracts from a patient homozygous for the αT266M mutation. To our knowledge, this is the first report of a human metabolic disease resulting from a change in the oxidation-reduction behavior of a protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild-type and mutant human ETFs were purified as described by Herrick et al. (5), and the concentration of wild-type ETF was determined using ε_{438 nm} = 13,300 M^{-1} cm^{-1}. The concentration of αG116R ETF was calculated using ε_{438 nm} = 14,600 M^{-1} cm^{-1} determined after release of FAD from the mutant protein with 5% SDS (12). Centrifications of porcine muscle chain acyl-CoA dehydrogenase, porcine sarcosine dehydrogenase, and porcine ETF-QO were determined spectrophotometrically using ε_{440 nm} = 15,400 M^{-1} cm^{-1}, ε_{440 nm} = 13,100 M^{-1} cm^{-1}, and ε_{440 nm} = 24,000 M^{-1} cm^{-1}, respectively (4, 5, 13). FAD (ε_{440 nm} = 11,300 M^{-1} cm^{-1}), riboflavin, tosyl-L-leucine-chloromethyl ketone, and pepstatin were obtained from Sigma, and leupeptin, aprotinin, and Pefabloc were obtained from Calbiochem (now Roche). Primate sera were purchased from Behring Laboratories (West Point, PA). Sodium dithionite was from George F. Smith Chemicals. SDS was purchased from Bio-Rad. Restriction endonucleases and Taq polymerase were purchased from Promega. Oligonucleotides were synthesized by McCrindle Resources, Fort Collins, CO. Pyocyanine (ε_{940 nm} = 1,003 V) (15) was prepared from phenazine methosulphate by the method of McIlwain (16), and galactocyanine was purchased from Acros (ε_{940 nm} = +0.021 V) (15). Milk xanthine oxidase was the gift of Dr. Russ Hille. Other reagents were the best grade available and were obtained from commercial sources.

**Site-directed Mutagenesis**—The αT266M and αG116R mutations were generated in the wild-type human ETF cDNA by the overlapping primer method of polynucleotide chain reaction mutagenesis (17). All reactions contained 200 ng of DNA template, 2 units of Taq polymerase, 80 μM each deoxyriboonucleotide triphosphate, 1.5 mM MgCl₂, and primers at a final concentration of 25 pmol in a 50-μl reaction. Primers to the human wild-type ETF expression vector pJR46−1 were as follows. The primer 5′-ATCAAGTTGGAGGAGCGG-3′ anneals upstream of a Stul site (nucleotide 1381) and was used with the reverse mutagenic primer 5′-ACTATTTTTTCCA′TCTGTCCCA-3′ to amplify one-half of the αT266M mutant cassette. The primer 3′-GATATCACTGACATAA-5′ anneals downstream of a KpnI site (nucleotide 2184) and, with a forward mutagenic primer 5′-GGAGCAGAT′GGGAAAAATA-3′, was used to create the second half of the mutant cassette. For the αG116R mutation, the same external primers were used with the following mutagenic primers: 5′-TCTGCTTCA′GAAGAAC-3′ and 5′-AGGGT-

CTTTT′GAAGGCCG-3′ (mutagenic nucleotides are indicated by * and nucleotide positions are as designated in Ref. 5). Small aliquots of the two individual PCR reactions were mixed, and the products were annealed before being amplified with the flanking primers for 30 cycles at 48 °C. The final full-length mutant cassette was trimmed with Stul and KpnI, and the digested fragment was isolated from a low melting agarose gel and ligated into Stul/KpnI cut pJR46−1 to yield pJR46TM3 or pJR46GR, which expressed the αT266M and αG116R ETFs, respectively. The presence of the desired C797T mutation resulting in the replacement of threonine 266 with methionine, and the G474A mutation converting glycine 116 to an arginine was verified by dideoxy sequencing (18) with Sequenase 2.0. Dideoxy sequencing also showed that no unwanted mutations were incorporated.

**Expression and Purification of Recombinant Wild-type and Mutant ETFs**—Wild-type human ETF was purified from E. coli extracts by chromatography on DEAE-Bio-Gel and carboxymethyl-Sepharose as described (5) with the modifications indicated. The protease inhibitors, aprotinin (2 μg/ml), pepstatin (1 μg/ml), Pefabloc (0.5 mM), leupeptin (2 μM), and tosyl-L-leucine-chloromethyl ketone (50 μM) were added to suspensions of cells expressing αG116R and αT266M ETFs when the cells were disrupted. Cells were disrupted with two passages through a French pressure cell at 1300 p.s.i. When the A_{260nm}/A_{650nm} of the αT266M ETF exceeded 6.5 after chromatography on carboxymethyl-Sepharose, the mutant protein was further purified by chromatography on hydroxyapatite (19).

The expression plasmid, pGroESL, which encodes the E. coli chaperonins GroEL and GroES, has been described (20) and was generously provided by Dr. Anthony Gatenby.

**Steady-state Kinetic Assays**—Medium chain acyl-CoA dehydrogenase and sarcosine dehydrogenase were assayed spectrophotometrically as described previously, with ETF as the varied substrate and 60 μM 2,6-dichlorophenolindophenol as the terminal electron acceptor (5). ETF-QO was assayed spectrophotometrically, with ETF as the varied substrate as described (5), by following the reduction of 70 μM ubiquinone-1 (Q-1) in the presence of 0.5 μM medium chain acyl-CoA dehydrogenase and 50 μM octanoyl-CoA. Catalytic activity of purified and crude ETF was also determined spectrophotometrically as described by Loehr et al. (10) in reaction mixtures containing 0.5 μM medium chain acyl-CoA dehydrogenase, 100 μM octanoyl-CoA, 0.5 μM ETF-QO, and 70 μM Q-1 in 20 mM Hepes-K⁺ buffer, pH 7.5. The rates of the reactions were determined by following Q-1 reduction at 275 nm using ∆ε = 7,500 M⁻¹ cm⁻¹.

ETF-QO-catalyzed disproportionation of ETF semiquinone was assayed under anaerobic conditions as described by Beckmann and Freman (13). Rates are expressed as mole ETF semiquinone converted per second.

**Spectroscopy**—Ultraviolet/visible absorption spectra were determined with a Shimadzu UV2401 spectrophotometer. ETF flavin and tryptophan fluorescence emission spectra were determined with a Shimadzu RF5301 fluorescence spectrophotometer at 25 °C. ETF flavin was excited at 436 nm, and emission was measured from 450 to 560 nm. Intrinsic fluorescence due to tryptophan and tyrosine was determined by excitation at 280 nm; emission was measured from 290 to 400 nm.

Infrared spectra were recorded at 25 °C with a Magna Model 550 spectrometer (Nicolet Instruments Corp.) using a deuterated tungsten-gallium-selenium detector as we have previously described (21).

Flavin circular dichroism spectra were measured with an Aviv 60DS spectropolarimeter at 4 °C in semimicro quartz cuvettes with a 1-cm light path, and molar ellipticities were calculated based on flavin concentration.

**Redox Methods**—The equilibrium constant for the disproportionation, K_{dis} of ETF_{1e} was determined by ETF-QO-catalyzed disproportionation of ETF semiquinone, which was generated by dithionite reduction, as described (13) at pH 7.5 and 15 °C as follows:

\[
2ETF_{1e} = ETF_{ox} + ETF_{hr}
\]  

(Eq. 1)

\[
K_{dis} = \frac{[ETF_{ox}][ETF_{hr}]}{[ETF_{1e}]}\]  

(Eq. 2)

This equilibrium constant is, in turn, related to the separation of redox potentials (∆E) between the oxidized/semiquinone couple, E_{ox}, and the semiquinone/hydroquinone couple, E_{hr}, as described by Clark (15),

\[
\Delta E = E_{ox} - E_{hr} = 2.3 \log 1/K_{dis}
\]  

where R is the gas constant, T is the absolute temperature, and F is Faraday's constant.
Redox potentials for the oxidized/semiquinone couple \( (E_0) \) were estimated by the method of Massey (22) using 250 \( \mu \)M xanthine, 20–70 \( \mu \)M xanthine oxidase, 1.5 \( \mu \)M benzyl viologen, 10–30 \( \mu \)M wild-type or mutant ETF and pyrocyanine or galloycyanine equimolar with the ETF. Reactions were conducted at 10 °C in 10 mM potassium phosphate, pH 7.0, containing 5% glycerol. Data were plotted according to Minneste (23). Values for the potentials of the indicator dyes are from Clark (15), and appropriate corrections were made to account for any contributions of the dye to the protein spectrum. Reduction to the semiquinone, without further reduction to hydroquinone, was judged by maintenance of isosbestic points for the oxidized/semiquinone species at 402 and 482 nm for the wild-type and 394 and 495 nm for \( \alpha \)T266M ETF. Potentials for the semiquinone/hydroquinone couples \( (E_0) \) were calculated from the value of \( E_1 \) and \( AE \).

Analytical Methods—The extinction coefficient of the oxidized \( \alpha \)T266M ETF at 438 nm was estimated after release of the flavin from the protein with 5% SDS as described previously (12). The mutant protein was titrated with sodium dithionite under anaerobic conditions, and the extinction coefficient of the flavin semiquinone was determined from the intercept of the extrapolated linear portions of a plot of the absorbance at 438 nm versus the absorbance at 377 nm, the absorption maximum of the semiquinone. The extinction coefficient of the hydroquinone at 377 nm was determined by complete reduction of the ETF with dithionite. The values for the wild-type protein were determined previously and are given in Table I with the corresponding values for \( \alpha \)T266M ETF.

FAD and AMP were released from \( \alpha \)T266M ETF by heating in the dark for 10 min at 100 °C. After centrifugation to remove precipitated protein, the extracts were analyzed by high performance liquid chromatography on a Microsorb C-18 column (5 \( \mu \)m, 4.5 × 250 mm) at a flow rate of 0.5 mL/min as described by Griffin et al. (31). Flavin Dissociation from ETF—Flavin dissociation from oxidized wild-type and \( \alpha \)T266M ETFs was continuously monitored by increase in fluorescence at 510 nm with excitation at 450 nm. Incubation mixtures contained 1 \( \mu \)M ETF in 3 \( \mu \)L urea, 50 mM potassium phosphate, 0.3 mM EDTA, pH 7.5, as described by Madden et al. (24). Fluorescence was monitored at 19 °C until about 99% of the flavin was released; the cuvette was then heated to 55 °C for 5 min to release remaining protein-bound flavin and cooled to 19 °C, and fluorescence intensity was measured to obtain a fluorescence at \( \tau_0 \). Data were fitted to first order curves, from which the rate constants were calculated. The stability of the reduced ETF flavin in the presence of buffered 3 \( \mu \)M urea was estimated at 19 °C, essentially as described by Madden et al. (24) except that ETF activity was determined in the octanoyl-CoA ubiquinone reductase assay described by Loehr et al. (10). ETF (2 \( \mu \)M) was reduced with octanoyl-CoA (60 \( \mu \)M) plus medium chain acyl-CoA dehydrogenase (0.5 \( \mu \)M) in 315 \( \mu \)L under anaerobic conditions. Deoxygenated buffer was then added such that the final concentration of ETF was 1 \( \mu \)M. Samples were removed with a gas-tight syringe at 0 time and after 2 and 6 h, corresponding to 3 and 9 half-lives of the oxidized wild-type protein in buffered 3 \( \mu \)M urea. ETF activity was determined in triplicate. Under these conditions, ETF was reduced to the hydroquinone oxidation state. In the absence of urea, wild-type and \( \alpha \)T266M ETFs were stable for at least 15 h at 19 °C in 50 mM potassium phosphate, 0.3 mM EDTA, pH 7.5, as determined in the catalytic assay.

RESULTS

Expression and Stabilization of Mutants—Initial attempts to purify the ETFs bearing the \( \alpha \)T266M and \( \alpha \)G116R mutations were unsuccessful. Based on immunoblots, the antigens were present in whole cells containing the mutant constructs at levels similar to the wild type; however, the \( \alpha \) subunits of the mutant proteins appeared unstable in the supernatant fractions of disrupted cells and were absent in eluates of DEAE-Bio-Gel columns where the wild-type ETF is normally eluted (Fig. 1, A and B) (5). The \( \alpha \)T266M subunit was stabilized easily by the addition of protease inhibitors and 100 \( \mu \)M FAD when the cells were disrupted (Fig. 1C). The addition of protease inhibitors with FAD did not yield a stable \( \alpha \)G116R subunit. However, when the chaperonins GroES and GroEL were coexpressed, the stabilized and \( \beta \) subunits were detected immunologically through the first chromatography step on an analytical DEAE-Bio-Gel column (Fig. 1D) and when purification was attempted on a preparative scale. When coexpressed with the chaperones, a single immunoreactive protein was detected on a blot of the crude extract after nondenaturing gel electrophoresis. The protein detected on the nondenaturing gel had a greater mobility than the wild-type protein, suggesting that it is folded differently than the wild-type protein (Fig. 2). In crude extracts, the specific activities of the wild-type and \( \alpha \)G116R ETFs were 34 nmol/min/mg protein and <0.02 nmol/min/mg protein, respectively. Although the \( \alpha \)G116R ETF was apparently structurally stabilized through the chromatographic step on DEAE-Bio-Gel, the protein remained very unstable and could not be purified further by the several methods that were attempted.

In contrast to the \( \alpha \)G116R ETF, the \( \alpha \)T266M ETF was purified after initial stabilization to proteolysis. The yield of highly purified \( \alpha \)T266M ETF averaged about 0.2 mg/g of wet cells. The yield of wild-type ETF was at least 0.6 mg/g of cells. Both proteins were highly purified; the \( A_{270} \)/*\( A_{360} \) ratios of wild-type and mutant ETFs were 5.9 and 5.6, respectively. Spectra of the \( \alpha \)T266M Mutant Protein—The ultraviolet-visible absorption spectrum of \( \alpha \)T266M ETF showed marked differences when compared with the wild-type spectrum (Fig. 3). The 436-nm maximum of the wild-type protein is shifted to 438 nm in the spectrum of the mutant ETF, and the 372-nm maximum is shifted 11 nm to 383 nm. The flavin transition in the
less intense in the mutant. Also, the transition at the 300-nm region, which is believed to be due to interactions with the ribityl side chain (30), appears altered in the mutant protein.

There was also a 2-fold decrease in flavin fluorescence of the mutant ETF with no significant change in $\lambda_{\text{em}}$ (Fig. 5), suggesting an increase in polarity in the flavin environment or less interaction with some collisional quencher (31). Intrinsic fluorescence due to tyrosines and tryptophans was not significantly altered in the $\alpha$T266M ETF, indicating that internal packing and the environment of aromatic residues in the immediate vicinity of the flavin are not perturbed by the mutation.

The overall structure of the wild-type and mutant proteins was also analyzed by infrared spectroscopy. The infrared spectra of the wild-type and $\alpha$T266M ETFs were essentially identical in the conformationally sensitive amide I region. Thus, structural differences between the wild-type and mutant ETFs appear to be minor and confined to the immediate flavin environment.

**Flavin Binding**—Madden et al. (24) have pointed out the correlation between the kinetic stability of flavoproteins toward flavin dissociation and the oxidation reduction potentials of the protein-bound flavin. Spectral evidence indicated that FAD is bound differently in the $\alpha$T266M mutant than in wild-type ETF. A quantitative estimate of the difference in FAD binding between the mutant and wild-type proteins was obtained by determining the rate of FAD dissociation from the two proteins in the presence of buffered 3 M urea. Flavin fluorescence of human ETF is strongly quenched in wild-type and $\alpha$T266M ETFs (5, 3), and the release of flavin in buffered 3 M urea was followed fluorometrically from the increase in flavin fluorescence. The rates of flavin release from both oxidized proteins fit well to first order plots. In the oxidized form, the rate constant for flavin release from wild-type protein was $1.02 \pm 0.05$ h$^{-1}$ (n = 3), and for the mutant, a rate constant of $0.12$ h$^{-1}$ was obtained in two experiments.

Because there is no sensitive spectral signal to follow, dissociation of reduced flavin from the ETFs was determined by assay of catalytic activity of ETF after 2 and 9 half-lives of the oxidized wild-type protein in buffered 3 M urea (Fig. 6, C and D). The data again fit well to first order curves. The rate constants for loss of reduced flavin were 0.14 and 0.41 h$^{-1}$ for the reduced wild-type and $\alpha$T266M ETFs, respectively.

**Redox Properties of Wild-type and $\alpha$T266M ETFs**—The redox behavior of the wild-type and $\alpha$T266M ETFs was first compared based on disproportionation of semiquinones stabilized by the wild-type and mutant proteins. The disproportionation equilibrium constants ($K_{\text{eq}}$) for wild-type and mutant ETF semiquinones were $0.61 \pm 0.1$ and $0.06 \pm 0.01$, respectively, demonstrating that the flavin semiquinone in the wild-type protein is about 10-fold less stable than in the mutant protein. The disproportionation equilibrium constants correspond to differences of $+0.012$ and $+0.069$ V between the two redox couples, for wild type and mutant, respectively.

Using the xanthine/xanthine oxidase method of Massey (22), the potential of the oxidized/semiquinone couple of porcine ETF was determined in preliminary experiments, and a value of $+0.006$ V was obtained. This result is in reasonable agreement with the experiments of Fox et al. ($+0.004 \pm 0.15$ V, pH 7.5) (32) and Gustafson et al. ($-0.014$ V, pH 7.0) (33). These values were obtained using pyocyanine as the indicator. The potential of the oxidized/semiquinone couple for wild-type human ETF using galloycine as the indicator was $+0.037 \pm 0.003$ V ($n = 4$), and for the $\alpha$T266M mutant, the potential of the oxidized/semiquinone couple was $-0.027 \pm 0.004$ V ($n = 6$) using pyocyanine as the indicator (Fig. 7). From these data, we calcu-
lated potentials for the semiquinone/hydroquinone couple for the wild-type and mutant ETFs of about 0.025 V and -0.093 V using the value of $\Delta E$ obtained from $K_{dis}$ (Table II).

**Kinetic Properties**—With the wild-type and $\alpha$T266M ETFs as substrates of medium chain acyl-CoA dehydrogenase or sarcosine dehydrogenase, there were no large differences in steady-state kinetic constants of the dehydrogenases (Table II). In contrast, the oxidative half-reaction of the mutant protein was substantially affected. With the mutant ETF as the substrate for ETF-QO in the acyl-CoA:ubiquinone reductase assay, the turnover number decreased 7-fold, and the $K_m$ for oxidized ETF increased 5-fold. Ubiquinone reduction in this assay requires the conversion of ETF semiquinone to the hydroquinone by disproportionation catalyzed by ETF-QO. Since the equilibrium of this reaction was perturbed with the mutant protein, we determined the kinetic constants of ETF-QO directly in the disproportionation reaction. In this reaction, there was no change in the $K_m$ for ETF semiquinone; however, the turnover number of ETF-QO with $\alpha$T266M semiquinone decreased 68%.

When ETF was present in catalytic concentrations, the specific activity of wild-type ETF in the standard assay was 88-mol Q-1 reduced/sec/mol of ETF, and the specific activity of $\alpha$T266M ETF was 6.0-mol Q-1 reduced/sec/mol of ETF. This decrease in activity of the purified, expressed mutant ETF corresponds well with the decrease in activity of the mutant ETF in crude fibroblast extracts (10). The kinetic and redox data are summarized in Table II.

**DISCUSSION**

We have determined the molecular bases of two pathogenic missense mutations on the kinetic, redox, and structural properties of human ETF. The $\alpha$G116R mutation destabilizes the $\alpha$ subunit expressed in *E. coli* and presumably in human cells. However, when the molecular chaperonins GroEL and GroES are overexpressed with the $\alpha$G116R ETF, electrophoretic evidence suggests that a mutant heterodimer is assembled in crude extracts. The two subunits are eluted from DEAE-BioGel at the same position as the wild-type protein on analytical columns (Fig. 1) and when we attempted to isolate the protein on a preparative scale. When the chaperonins are coexpressed, the G116R $\alpha$ subunit can be directed into a kinetically significant folding pathway and dimer assembly. The altered electrophoretic mobility of the mutant dimer on nondenaturing gels suggests that the protein is folded differently than the wild-type protein since mobility on these gels is a function of the charge:Stokes radius ratio of the native protein. Similar results have been reported for some mutant forms of T4 lysozyme (34). The altered folding could result from several related causes. The hydrogen atoms at C2 of Gly-116 lie in a hydrophobic pocket formed by leu-125, Ala-110, Phe-115, Leu-119, and Leu-

![Fig. 3. Visible absorption spectra of oxidized wild-type ETF and $\alpha$T266M ETF. Spectra of the wild-type (—) and mutant (---) ETFs were determined at 20 °C in 10 mM potassium phosphate, pH 7.0, containing 10% glycerol.](image)
120; therefore, the guanidinium side chain of R116 would be difficult to accommodate sterically in the native structure (7). Also, folding the mutant residue into this pocket would be energetically unfavorable, requiring desolvation and leaving Arg-116 with unsatisfied hydrogen-bonding capacity, which is also energetically unfavorable. The αG116R ETF apparently folds to an alternate form in this expression system; however, it is unlikely to fold and dimerize in the absence of the overexpressed chaperonins. Assays of ETF in fibroblast extracts from this patient exhibit no more ETF activity than that expected from the compound heterozygous condition, αG116R/αT266M (10). No activity could be demonstrated in crude extracts of E. coli expressing the αG116R allele.

Once stabilized to protease activity in crude extracts, the αT266M mutation is not intrinsically unstable. The methionine substitution eliminates hydrogen bonding at N(5) of the flavin and perhaps hydrogen bonding at C(4)-O with the peptide backbone amide hydrogen of residue 266. The alteration at N(5) is likely responsible for the spectral and redox properties of the ETF flavin; although without the three-dimensional structure of this mutant ETF and its reduced forms, compensatory changes cannot be eliminated as a factor. There are major differences between the flavin absorption, circular dichroism and fluorescence emission spectra of αT266M ETF and the wild-type ETF. The ππ* transition in the 436-nm region red shifts 2 nm in the spectrum of the mutant protein, and there is

---

**Fig. 4.** Flavin circular dichroism spectra of wild-type and αT266M ETFs. Spectra of the wild-type ETF (---) and αT266M ETF (----) were determined at 4 °C in 10 mM potassium phosphate, pH 7.0, containing 10% glycerol.

**Fig. 5.** Fluorescence emission spectra of wild-type ETF and αT266M ETF. Emission spectra of wild-type ETF (---) and αT266M ETF (----) were determined using λex = 436 nm, in 10 mM potassium phosphate buffer, pH 7.0, containing 10% glycerol at 20 °C; the concentration of the proteins was 1 μM.
a loss of resolution characteristic of the spectrum of flavin in a
protic solvent (25). The flavin transition in the 370-nm region is
ππ* with considerable nπ* character, consistent with solvent-
dependent spectral shifts of this transition with little develop-
ment of fine structure (25). This transition in αT266M ETF
undergoes an 11-nm red shift relative to the wild-type protein.
The decreased flavin fluorescence is also characteristic of in-
creased solvent exposure (31). The circular dichroism spectrum
of the mutant protein simply indicates that interactions be-
tween flavin transition dipoles and protein dipoles have
changed without any specific information regarding the nature
of that change.

The spectral and flavin-binding properties of the mutant
protein predicted that its redox properties would be altered.
Known factors influencing flavin potentials include solvent
accessibility, local electrostatic environment, flavin confor-
ma
tion, and aromatic stacking of the flavin with phenolic and
indole side chains of tyrosine and tryptophan (35, 36). In-
creased exposure of the flavin to solvent in αT266M ETF, which
is suggested by the spectral data, would be expected to stabilize
the semiquinone and hydroquinone oxidation states because
both are anions (6, 13). However, the potentials of both couples
decrease, which is opposite to the result expected for increased
exposure of the anions to solvent. Both anions have charge
localized in the region of N(1) and C(2)-O (11). The three-
dimensional structure of ETF suggests that the semiquinone
and hydroquinone anions of ETF can be stabilized by hydrogen
bonding with the 4'-ribityl hydroxyl group at N(1) and αH286
at C(2)-O. Also, residues near αT266, αQ263, αV263, and
αQ262 hydrogen bond with N(3) and C(2)-O in the pyrimidine

FIG. 6. Dissociation of FAD from ox-
idized and reduced wild-type and
αT266M ETFs. The dissociation of ox-
idized FAD from wild-type ETF (A) and
αT266M ETF (B) was determined from
the increase in flavin fluorescence at
19 °C in 3 M urea, 50 mM potassium phos-
phate, 0.3 mM EDTA, pH 7.5. The disso-
ciation of FAD from enzymatically re-
duced wild-type ETF (C) and αT266M
ETF (D) was estimated from the loss of
activity under anaerobic conditions at
19 °C in the urea buffer described. The
concentration of ETFs was 1 μM in all
experiments.

FIG. 7. Determination of the oxidation reduction potential of
the oxidized/semiquinone couple of wild-type and αT266M
ETFs. The potentials were determined by the method of Massey (22)
using 15 μM wild-type ETF (●) with equimolar gallo
cyanine and
αT266M ETF (■) with equimolar pyocyanine, 250 μM xanthine, 35 μM
xanthine oxidase, 1 μM benzyl viologen in 10 mM potassium phosphate,
pH 7.0, containing 5% glycerol. Reactions were run at 10 °C.
However, Ramsay of ETF by the reduced dehydrogenases is very slow (4, 6). The oxidative and reductive half-reactions.

Netically significant reduced product in the reaction of ETF with any acyl-CoA dehydrogenases (7). Two-electron reduction of ubiquinone in second reaction catalyzed by disproportionation reduces ubiquinone in second electron addition to the system. Finally, the loss of the hydrogen bond donor to N(5) would not alone be expected to affect formation of the anionic semiquinone because the semiquinone is unprotonated at N(5); however, the anionic hydroquinone is protonated at N(5), and this may affect the potential of the semiquinone/hydroquinone couple. Therefore, factors other than solvation among those mentioned above must be primary determinants modulating the potentials.

Kinetic experiments demonstrated that the reaction with ETF-QO is primarily affected by the mutation, and there is little effect on the reductive half-reaction of ETF with any primary flavoprotein dehydrogenase studied. ETF_{1e} is the kinetically significant reduced product in the reaction of ETF with the acyl-CoA dehydrogenases (DH),

\[ DH_{1e} P + 2ETF_{1e} = DH + P + 2ETF_{1e} \]  

where \( P \) is the 2,3-enoyl-CoA product. Two-electron reduction of ETF by the reduced dehydrogenases is very slow (4, 6). However, Ramsay et al. (6) have shown that the mechanism of ubiquinone reduction involves the ETF hydroquinone rather than the semiquinone. They proposed that the slow reduction of ETF to the hydroquinone is overcome by the rapid disproportionation of ETF_{1e} catalyzed by ETF-QO; ETF_{2e} generated by disproportionation reduces ubiquinone in second reaction catalyzed by ETF-QO (13).

\[ 2ETF_{1e} \rightarrow ETF_{1e} + ETF_{2e} \]  

The disproportionation equilibrium of the mutant ETF strongly favors the semiquinone over the hydroquinone such that ETF_{1e} available for reduction of ubiquinone catalyzed by ETF-QO may be limited. We obtained direct evidence for this hypothesis from determination of semiquinone stability and the ETF-QO catalyzed rate of semiquinone disproportionation. It is important that these results also provide supporting evidence that the disproportionation reaction is physiologically significant.

\[ \alpha T244 in Paracoccus denitrificans ETF corresponds to \alpha T266 in human ETF not only by position in the highly conserved \alpha II domain of the subunit, but also as a hydrogen bond donor to N(5) and C(4)-O of the flavin. \]

When this threonine residue in the bacterial ETF was substituted with a methionine residue, the value of \( K_{dis} \) increases from 1.3 for the wild-type protein to 3.2 for the \( \alpha T244 M \) (21). The perturbation of this equilibrium is in the opposite direction to that found with the corresponding human ETF mutant. Significant differences between human and \( P. denitrificans \) ETFs have been reported (19, 21, 37), including the inability to serve as an electron acceptor for mammalian dimethylglycine dehydrogenase and as an electron donor to a mammalian ETF-QO. These differences exist although there is about 60/72% overall sequence identity/similarity between the two proteins (37). Most of the differences exist in the \( \alpha I \) domain and on one side of the \( \beta \) domain (7). The \( \alpha T244 M \) mutation in \( P. denitrificans \) ETF appears to perturb the relatively minor region of the subunit near the flavin. This conclusion is based on the change of intrinsic tryptophan fluorescence, probably from the nearby \( \alpha W239 \) that is about 8 Å from the flavin (21)\(^2\); however, the mutation does significantly alter flavin spectra as does the corresponding mutation in the human protein. It is surprising that the same mutation in the two proteins results in such different effects on protein structure and flavin behavior. The results underscore the differences between the two proteins, which have tended to be minimized by consideration of only primary sequence identity/similarity and the capacity of the bacterial ETF to substitute for the human and porcine ETFs as an electron acceptor for some mammalian flavoprotein dehydrogenases. Since other factors, including local electrostatic effects, flavin conformation, and stacking with side chains of aromatic residues are suggested to be important in modulating oxidation reduction potentials of flavins in flavoproteins, determination of the crystal structures of these human and \( P. denitrificans \) ETF mutants, which are in progress, will contribute to understanding how the same mutation in an otherwise highly conserved region can alter the redox properties of the flavin in opposite directions.

Acknowledgments—We thank Dr. John Carpenter, Dr. Mark Manning, and Jeffrey Meyer for assistance with the circular dichroism and infrared spectroscopy.

REFERENCES

1. Thorpe, C. (1991) in Chemistry and Biochemistry of Flavoenzymes (Muller, F., ed) Vol. II, pp. 471–486, CRC Press, Boca Raton, FL.
2. Freeman, F. E., and Goodman, S. I. (1985) Procr. Natl. Acad. Sci. U. S. A. 82, 4517–4520.
3. Freeman, F. E., and Goodman, S. I. (1985) Biochem. Med. 33, 38–43.

\(^2\) D. L. Roberts, F. E. Freeman, and J. J. P. Kim, unpublished data.
4. Gorelick, R. J., Schopfer, L. M., Ballou, D. P., Massey, V., and Thorpe, C. (1985) Biochemistry 24, 6830–6839
5. Herrick, K. R., Salazar, D., Goodman, S. I., Finochiaro, G., Bedzyk, L. A., and Frerman, F. E. (1994) J. Biol. Chem. 269, 32239–32245
6. Ramsay, R. R., Steenkamp, D. J., and Husain, M. (1987) Biochemistry J. 241, 883–892
7. Roberts, D. L., Frerman, F. E., and Kim, J.-J. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 51–61
8. Sato, K., Nishina, Y., and Shiga, K. (1993) J. Biochem. 114, 215–222
9. Freneaux, E., Sheffield, V. C., Molin, L., Shires, A., and Rhead, W. J. (1992) J. Clin. Invest. 90, 1670–1686
10. Loehr, J. P., Goodman, S. I., and Frerman, F. E. (1990) Pediatr. Res. 27, 311–315
11. Massey, V., and Swoboda, B. E. P. (1963) Biochem. Z. 338, 474–484
12. Beckmann, J. D., and Frerman, F. E. (1985) Biochemistry 24, 3913–3924
13. Beckmann, J. D., and Frerman, F. E. (1983) J. Biol. Chem. 258, 1866–1870
14. Massey, V., and Swoboda, B. E. P. (1963) Biochem. Z. 338, 474–484
15. Clark, W. M. (1960) Oxidation-Reduction Potentials of Organic Systems, pp. 184–203, The Williams and Wilkins Co., Baltimore, MD
16. Melliwain, H. (1937) J. Chem. Soc. 2, 1704–1711
17. Higuchi, R., Kimmel, B., and Saiki, R. K. (1988) Nucleic Acids Res. 16, 7351–7367
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
19. Husain, M., and Steenkamp, D. J. (1983) Biochem. J. 209, 541–545
20. Gatesby, A. A. (1992) Plant Molec. Biol. 19, 677–687
21. Griffin, K. J., Dwyer, T. M., Manning, M. C., Meyer, J. D., Carpenter, J. F., and Frerman, F. E. (1997) Biochemistry 36, 4194–4202
22. Massay, V. (1990) in Flavins and Flavoproteins (Curti, B., Ronchi, S., and Zanetti, G., eds) pp. 59–66, Walter de Gruyter, Berlin
23. Minnaert, K. (1965) Biochim. Biophys. Acta 110, 42–56
24. Maren, M., Lau, S.-M., and Thorpe, C. (1984) Biochem. J. 224, 577–580
25. Eweg, J. D., Muller, F., van Dam, H., Terpstra, A., and Oskam, A. (1980) J. Am. Chem. Soc. 102, 51–61
26. Mayhew, S. G., Whitfield, C. D., Ghisla, S., and Jorns, M. (1974) Eur. J. Biochem. 44, 579–591
27. Whitfield, C. D., and Mayhew, S. G. (1974) J. Biol. Chem. 249, 2801–2810
28. Hsu, M. C., and Woody, R. W. (1971) J. Am. Chem. Soc. 93, 3515–3525
29. Blauer, G., Breitwieser, N., and Woody, R. W. (1990) Biochemistry 32, 6674–6679
30. Edmondson, D. E., and Tollin, G. (1971) Biochemistry 10, 113–123
31. Kasai, J., and Kambayashi, R. (1965) Biochim. Biophys. Acta 102, 289–300
32. Husain, M., Stankovich, M. T., and Fox, P. G. (1984) Biochem. J. 219, 1043–1047
33. Gustafson, W. G., Feinberg, B. A., and McFarland, J. T. (1986) J. Biol. Chem. 261, 7753–7761
34. Dao-pin, S., Anderson, D. E., Baase, W. A., Dahlquist, F. W., and Matthews, B. W. (1991) Biochemistry 30, 11521–11529
35. Swenson, R. F., and Krey, G. D. (1994) Biochemistry 33, 8505–8514
36. Tatwawadi, S. V., Santhanam, K. S., and Bard, A. J. (1968) J. Electroanal. Chem. 17, 411–416
37. Bedzyk, L. A., Escudero, K. W., Gill, R. E., Griffin, K. J., and Frerman, F. E. (1993) J. Biol. Chem. 268, 20211–20217