αT244M Mutation Affects the Redox, Kinetic, and *in Vitro* Folding Properties of *Paracoccus denitrificans* Electron Transfer Flavoprotein†

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ABSTRACT: Threonine 244 in the α subunit of *Paracoccus denitrificans* transfer flavoprotein (ETF) lies seven residues to the amino terminus of a proposed dinucleotide binding motif for the ADP moiety of the FAD prosthetic group. This residue is highly conserved in the α subunits of all known ETFs, and the most frequent pathogenic mutation in human ETF encodes a methionine substitution at the corresponding position, αT266. The X-ray crystal structures of human and *P. denitrificans* ETFs are very similar. The hydroxyl hydrogen and a backbone amide hydrogen of αT266 are hydrogen bonded to N(5) and C(4)O of the flavin, respectively, and the corresponding αT244 has the same structural role in *P. denitrificans* ETF. We substituted a methionine for T244 in the α subunit of *P. denitrificans* ETF and expressed the mutant ETF in *Escherichia coli*. The mutant protein was purified, characterized, and compared with wild type *P. denitrificans* ETF. The mutation has no significant effect on the global structure of the protein as inferred from visible and near-ultraviolet absorption and circular dichroism spectra, far-ultraviolet circular dichroism spectra, and infrared spectra in ^1H_2O and ^2H_2O. Intrinsic fluorescence due to tryptophan of the mutant protein is 60% greater than that of the wild type ETF. This increased tryptophan fluorescence is probably due to a change in the environment of the nearby W239. Tyrosine fluorescence is unchanged in the mutant protein, although two tyrosine residues are close to the site of the mutation. These results indicate that a change in structure is minor and localized. Kinetic constants of the reductive half-reaction of ETF with porcine medium chain acyl-CoA dehydrogenase are unaltered when αT244M ETF serves as the substrate; however, the mutant ETF fails to exhibit saturation kinetics when the semiquinone form of the protein is used as the substrate in the disproportionation reaction catalyzed by *P. denitrificans* electron transfer flavoprotein−ubiquinone oxidoreductase (ETF−QO). The redox behavior of the mutant ETF was also altered as determined from the equilibrium constant of the disproportionation reaction. The separation of flavin redox potentials between the oxidized/semiquinone couple and semiquinone/hydroquinone couple are −6 mV in the wild type ETF and −27 mV in the mutant ETF. The mutation does not alter the AMP content of the protein, although the extent and fidelity of AMP-dependent, *in vitro* renaturation of the mutant AMP-free apoETF is reduced by 57% compared to renaturation of wild type apoETF, likely due to the absence of the potential hydrogen bond donor T244.

Electron transfer flavoproteins (ETF)[1] are FAD-containing heterodimers that commonly act as electron carriers between flavoprotein dehydrogenases and the respiratory chains of bacteria and mitochondria (Thorpe, 1991). The most extensively investigated electron transfer flavoproteins are those isolated from pig liver (Gorelick et al., 1982; McKean et al., 1983), the methylotrophic bacterium W3A1 (Davidson et al., 1986), and the soil bacterium *Paracoccus denitrificans* (Husain & Steenkamp, 1985; Watmough et al., 1992). The genes encoding the subunits of the two bacterial ETFs have been cloned and expressed in *Escherichia coli* (Bedzyk et al., 1993; Chen & Swenson, 1994), as have the cDNAs encoding the α and β subunits of human ETF (Finocchiaro et al., 1988, 1993; Herrick et al., 1994). In addition to the single flavin nucleotide, porcine liver ETF and bacterium W3A1 ETF have been recently shown to contain noncovalently bound AMP, equimolar with FAD (Sato et al., 1993; DuPlessis et al., 1994). The role of AMP in ETFs is unclear, but it enhances the rate of reconstitution of porcine AMP-free apoETF when incubated with FAD (Sato et al., 1993).

The crystallization and preliminary X-ray analysis of human and *P. denitrificans* ETFs have been reported (Roberts et al., 1995), and more recently, the structure of human ETF was solved to 2.1 Å resolution (Roberts et al., 1996). These proteins have about 60% sequence identity and 72% sequence similarity. The structure of the human protein shows that the hydroxyl function of T266 in the α subunit is within hydrogen-bonding distance (2.77 Å) of N(5) of the flavin ring; an amide hydrogen of T266 is hydrogen bonded to

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[1] Abbreviations: ETF, electron transfer flavoprotein; ETF_{ox}, ETF_{1e-}, and ETF_{2e-}, the oxidized, semiquinone, and hydroquinone oxidation states of electron transfer flavoprotein; ETF−QO, electron transfer flavoprotein−ubiquinone oxidoreductase.

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C(4)O. This is also the case for the corresponding T244 in *P. denitrificans* ETF[2] which has a three-dimensional structure extremely similar to that of the human protein. This threonine residue lies in a highly conserved region in the carboxyl terminal region of all known α subunits, including those from *P. denitrificans*, rat, man, and nitrogen-fixing bacteria (Chen & Swenson, 1994). There is a conservative replacement of the threonine residue by serine in the high-potential ETF from the methylotroph W3A1 (Chen & Swenson, 1994). Further, substitution of this residue by methionine in human ETF is a frequent pathogenic mutation (Freneaux et al., 1992).

Because of the location of T244 relative to the flavin, and its potential influence on the redox properties of the flavin and activity of the ETF, we introduced the T244M mutation into the α subunit of *P. denitrificans* ETF by site-directed mutagenesis to investigate the effects of this residue on the kinetic and redox properties of ETF. Significant changes in the properties of the ETF are to be expected since the properties of the flavin are modulated by its protein environment (Ghisla & Massey, 1986). Our experiments show that the structural effects of the mutation on the ETF are very localized and affect the reaction of ETF with *P. denitrificans* electron transfer flavoprotein−ubiquinone oxidoreductase but not the reaction with a primary flavoprotein dehydrogenase, porcine medium chain acyl-CoA dehydrogenase. These changes in reactivity are accompanied by a change in the redox properties of the ETF.

MATERIALS AND METHODS

Materials. *P. denitrificans* ETF was expressed in *E. coli* and purified and quantitated using $\epsilon_{436nm} = 13.6 \times 10^3\ M^{-1}$ (Bedzyk et al., 1993). Pig liver medium chain acyl-CoA dehydrogenase was purified and quantitated using $\epsilon_{448nm} = 15.2 \times 10^3\ M^{-1}$ (Gorelick et al., 1985). *P. denitrificans* electron transfer flavoprotein−ubiquinone oxidoreductase (ETF−QO) was purified (Husain & Steenkamp, 1985; Watmough et al., 1992) and quantitated assuming $\epsilon_{424nm} = 24 \times 10^3\ M^{-1}$ (Paulsen et al., 1992).

FAD ($\epsilon_{448nm} = 11.3 \times 10^3\ M^{-1}$) was obtained from Sigma and purified by chromatography on DEAE-cellulose (Massey & Swoboda, 1963). AMP was purchased from Boehringer-Mannheim and quantitated using $\epsilon_{259nm} = 15.4 \times 10^3\ M^{-1}$. GMP, IMP, UMP, and adenosine were purchased from Sigma and quantitated using $\epsilon_{252nm} = 13.7 \times 10^3\ M^{-1}$, $\epsilon_{249nm} = 12.2 \times 10^3\ M^{-1}$, $\epsilon_{262nm} = 10.0 \times 10^3\ M^{-1}$, and $\epsilon_{258nm} = 14.6 \times 10^3\ M^{-1}$, respectively.

Site-Directed Mutagenesis. The *P. denitrificans* αT244M mutant was generated by site-directed mutagenesis using the overlap extension polymerase chain reaction method of Higuchi et al. (1988). Briefly, we generated a mutant cassette using one mutagenic primer containing the Xba*I* site (nucleotide 1719) and a second mutagenic primer containing the Hind*III* site (nucleotide 2142). Nucleotide positions correspond to those in Bedzyk et al. (1993). The template was pLE 40, a derivative of pLE 20, which lacks approximately 2 kb of *P. denitrificans* DNA 3′ to the coding sequences of the β and α subunit genes (Herrick et al., 1994). The amplified region was trimmed with XbaI and Hind*III*, and the gel-purified product was ligated into pUC18 to yield pKG1, which was transformed into *E. coli* DH5α. pKG1 yielded the expected restriction fragments when cut with Xba*I*/Hind*III* and Bsu*36I*/Eco*NI*. The Bsu*36I*/Eco*NI* fragment was ligated into pLE 40, which had been cut with Bsu*36I* and Eco*NI* and gel-purified, to yield the expression vector for the mutant ETF, pKG3. The sequence of the inserted fragment was determined by dideoxy sequencing (Sanger et al., 1977) and the desired mutations, $C^{1900}G$ and $C^{1899}T$, were detected along with one additional base change, $G^{1858}A$, which is a silent mutation. The mutant protein was expressed and purified as described above.

Analytical Methods. The purified ETFs expressed in *E. coli* were extracted with 60% methanol or heated for 5 min at 95 °C in the dark to determine the contents of AMP and FAD in the proteins. The two methods yielded identical results. Extracts were analyzed by HPLC on a Microsorb C18 column (5 μm, 4.5 × 250 mm) at a flow rate of 0.5 mL/min. Nucleotides were eluted with a methanol (solvent B) gradient in 20 mM potassium phosphate, pH 7.0 (solvent A). Elution was carried out with solvent A for 10 min and then a 0−55% gradient of solvent B in solvent A from 10 to 34 min. We also used a gradient elution system with methanol−triethylammonium phosphate buffer (83.3 mM, pH 6.0) to confirm the identity of AMP (Perrett et al., 1981). FAD and AMP were quantitated from the areas under peaks, using standard curves derived with authentic standards of known concentration which related integrated areas to nucleotide concentrations.

The molar absorptivity of the AMP-containing apoETF generated by treatment with buffered KBr (see below) was determined as described by Beckmann and Frerman (1985a). The protein, denatured with sodium dodecyl sulfate, was exhaustively trinitrophenylated with trinitrobenzenesulfonate, and the concentration of lysine was determined, taking into account the 2 equiv of α amino groups at the amino termini. The absorbance of the apoprotein at 272 nm could then be related directly to the concentration of protein based on lysine concentration. Using this method, we estimated an $\epsilon_{272nm} = 38\,870 \pm 2110\ M^{-1}$; this value agreed with the molar absorptivity, $\epsilon_{272nm} = 37\,600\ M^{-1}$, when the protein concentration was estimated according to Miller (1956). The same molar absorptivity was assumed for the αT244M mutant since the mutation does not affect a chromophore absorbing in this region. The reliability of the ϵ_{272nm} is indicated by fluorometric titration of the wild type apoETF with FAD which showed an equivalence point of about 1.0. The apoETF in 50 mM potassium phosphate buffer, 0.3 mM EDTA, and 5% glycerol, pH 6.8, was titrated with purified FAD at 4 °C; a blank containing buffer was titrated identically. The samples were excited at 436 nm, and because fluorescence of bound FAD is blue-shifted relative to free FAD, emission was determined at 485 nm where the fluorescence of free FAD is considerably less. The values of the buffer blank were subtracted from the values obtained by titrating the apoprotein. FAD binding was essentially complete in 4 min, but samples were allowed to equilibrate for 15 min before making the fluorescence measurements. Essentially identical results were obtained with the mutant protein except that the equivalence point was reached at 0.82 equiv of flavin/mol of apoprotein, likely due to the mutation at T244.

[2] D. L. Roberts, F. E. Frerman, and J. J. P. Kim, manuscript in preparation.

Generation and Reconstitution of ApoETFs. An AMP-containing apoETF was generated by treatment of the holoETF with KBr as described by Sato et al. (1992). KBr and the released flavin were separated from the apoprotein by gel filtration on a column (1 × 15 cm) of Bio-Gel P-6 DG. The AMP-containing apoproteins were extremely stable and could be stored for at least 5 days at 4 °C without affecting the capacity to quantitatively reincorporate FAD.

P. denitrificans and human AMP-free apoETFs were prepared for reconstitution by extraction of the holoETFs four times with 5.4 M guanidine-HCl in 50 mM potassium phosphate, pH 7.6, and 1 mM dithiothreitol, as described by Sato et al. (1993). Analysis of the combined guanidine-HCl extracts for AMP and FAD by high-performance liquid chromatography indicated that this procedure removed at least 97% of the two ligands from *P. denitrificans* ETF. The AMP-free apoETF was reconstituted exactly as described by Sato et al. (1993) except when renaturation was attempted with IMP, UMP, GMP, or adenosine, which were present at 4 μM.

Enzyme Assays. The reductive half-reaction of *P. denitrificans* ETF with catalytic porcine liver medium chain acyl-CoA dehydrogenase was assayed fluorimetrically, following conversion of the ETF_{ox} to ETF_{1e-} as previously described (Beckmann & Frerman, 1983) except that the buffer was 75 mM potassium phosphate, pH 8.0. Rates are expressed as moles of ETF_{1e-} formed per second. The reaction of *P. denitrificans* ETF with ETF−QO was assayed by disproportionation of the ETF semiquinone as described by Beckmann and Frerman (1985a), except that the reaction mixtures also contained 30 mM KCl. Rates are expressed as moles of ETF_{1e-} consumed per second. Steady state kinetic constants and their standard errors were calculated by weighted least-squares regression analysis (Beckmann & Frerman, 1983).

The catalytic activity of *Paracoccus* ETF was assayed in 0.75 mL reactions containing 75 mM potassium phosphate, pH 8.0, 1 μM medium chain acyl-CoA dehydrogenase, 60 μM 2,6-dichloroindophenol, and 20−150 nM ETF. The reactions were started by addition of octanoyl-CoA, 50 μM, and dichloroindophenol reduction was followed at 600 nm ($\epsilon = 2.1 \times 10^4$ M^{-1}).

Semiquinone Stability. The thermodynamic stability of ETF semiquinone was evaluated from the equilibrium constant, κ, for the disproportionation of ETF semiquinone catalyzed by ETF−QO (Beckmann & Frerman, 1985a; Watmough et al., 1992).

$$2ETF_{1e-} \rightleftharpoons ETF_{ox} + ETF_{2e-}$$

The reciprocal of κ is the formation constant, K, for the semiquinone (Clark, 1960):

$$K = \frac{[ETF1_{e-}]^2}{[ETF_{ox}][ETF2_{e-}]} = 1/\kappa$$

The semiquinone formation constant is related to the difference (ΔE) between the oxidation−reduction potentials of the oxidized/semiquinone (E_2) and semiquinone/hydroquinone (E_1) couples

$$\Delta E = E_2 - E_1 = \frac{2.303RT}{F} \log K$$

where R is the gas constant, T is absolute temperature, and F is Faraday's constant. κ was determined at 25 °C as described by Beckmann and Frerman (1985a) under conditions of the kinetic disproportionation assay described above.

Spectroscopy. Ultraviolet and visible absorption spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer or a Shimadzu UV3000 spectrophotometer. Circular dichroism spectra were determined with an AVIV 60DS spectropolarimeter at 4 °C. Measurements in the 300−600 nm range were determined in semimicro quartz cuvettes with a 1 cm light path; molar elipticities were calculated based on flavin concentration. Spectra in the 190−300 nm range were determined in 0.01 mm path length cells, and molar elipticities were calculated based on peptide bond concentration. All circular dichroism spectra were determined in 50 mM potassium phosphate, 0.3 mM EDTA, 5% glycerol, pH 7.6.

Fluorescence emission spectra were determined at 4 °C with a Perkin-Elmer MPF-66 fluorescence spectrophotometer. Quenching of tryptophan fluorescence by Cs^+ ion in native and AMP-containing apoETFs was determined in 50 mM potassium phosphate, 0.3 mM EDTA, 5% glycerol, pH 6.8. Tryptophan fluorescence ($\lambda_{ex} = 295$ nm and $\lambda_{em} = 329$ nm) was determined by titration with CsCl. An identical sample was titrated with NaCl to control for the change in ionic strength. Data were corrected for inner filter effect (Watmough et al., 1991) and plotted according to the Stern−Volmer equation:

$$F_o/F_q = 1 + K_{SV}[Q]$$

where F_o is the intensity of fluorescence in the absence of the quenching agent, Q, F_q is the fluorescence in the presence of the quenching agent, Q, [Q] is the concentration of quenching agent, and K_{SV} is the Stern−Volmer quenching constant. The fractional exposure of tryptophan residues, f_a, was calculated from the relationship:

$$\frac{F_o}{\Delta F} = \frac{1}{f_a K_d [Q]} + \frac{1}{f_a}$$

where F_o is the fluorescence in the absence of quencher, F_q is the fluorescence in the presence of quencher, and $\Delta F = F_o - F_q$; [Q] is the concentration of the quenching agent, and K_d is the association constant of the quenching agent (Lehrer, 1971). Tyrosine fluorescence was estimated by excitation at 280 nm and subtraction of the fluorescence due to tryptophan (Watmough et al., 1991).

Protein solutions were prepared for infrared measurement in a CaF_2 cell (Beckman FH-01) with a 6 μm spacer. Infrared spectra were recorded at 25 °C with a Magna Model 550 spectrometer (Nicolet) using a dTGS detector. For each spectrum, a 512 scan interferogram was collected in single-beam mode with a 4 cm^{-1} resolution. Reference spectra were recorded under identical scan conditions with only the corresponding buffer in the cell. The spectra of liquid and gaseous water were subtracted from the observed protein spectra according to previously established criteria (Dong et al., 1992) and double-subtraction procedure (Dong et al., 1995). The final protein spectrum was smoothed with a 7-point Savitsky−Golay smooth function to remove the white noise. Second-derivative spectra were obtained with the derivative function of Omnic software (Nicolet). The

Table 1: Kinetic and Redox Properties of Wild Type and αT244M Electron Transfer Flavoproteins

	wild type	αT244M mutant
medium chain acyl-CoA dehydrogenase $K_{K_m}^{ETF}$ (μM)	13.5 ± 3.5	17.3 ± 5.0
medium chain acyl-CoA dehydrogenase TN[a] (s^{-1})	30.5 ± 5.5	28.9 ± 6.4
ETF−ubiquinone oxidoreductase $K_{K_m}^{ETF}$ (μM)	4.7 ± 1.8	NS[b]
ETF−ubiquinone oxidoreductase TN[a] (s^{-1})	181 ± 30	NS[b]
equilibrium constant, disproportionation of ETF_{1e-}	1.3 ± 0.5[c]	3.2 ± 0.2[d]
separation of potentials, $E_2 - E_1$ (mV)	−6.0 ± 1.0	−26.8 ± 2.0

[a] Turnover number. [b] Not saturable; the second-order rate constant is 1.49×10^7 M^{-1} s^{-1}. [c] $n = 6$. [d] $n = 7$ (n is the number of determinations). Values for footnotes c and d were determined under conditions of the kinetic disproportionation assay.

inverted second-derivative spectra were obtained by factoring by −1 and then curve-fitted (SpectraCalc, Galactic Industries) with Gaussian band profiles (Dong et al., 1992, 1994, 1996). When spectra of the deuterated protein were measured, the buffer was prepared in ^2H_2O and the relation between pH and pD was calculated from pH − 0.4 = pD. Deuterium was exchanged into the protein by concentrating from the ^2H_2O-containing buffer four times with 10-fold dilutions; the protein was incubated overnight at 4 °C, and the buffer was exchanged twice more before the spectra were determined.

RESULTS

Kinetic and Redox Properties of the αT244M Mutant ETF. When the wild type and mutant ETFs were investigated as substrates of porcine medium chain acyl-CoA dehydrogenase, there was no significant difference in the steady state kinetic constants of the dehydrogenase in the reductive half-reaction with the two ETFs (Table 1). When ETF was catalytic and reduced with octanoyl-CoA/medium chain acyl-CoA dehydrogenase with 2,6-dichloroindophenol as the terminal electron acceptor, the specific activities of the wild type and mutant ETFs were 0.42 ± 0.02 and 0.44 ± 0.03 μmol of dye reduced/s/μmol of ETF flavin, respectively. These values are comparable to that reported for pig liver ETF (Sato et al., 1993). However, significant differences were found when the two ETFs were substrates of *P. denitrificans* ETF−QO. In the ETF−QO-catalyzed disproportionation reaction, the K_m for wild type ETF_{1e-} was 4.7 ± 1.8 μM and the turnover number was 181 ± 30 s^{-1} (Figure 1). These values are very similar to those determined for the kinetics of disproportionation of porcine ETF_{1e-} catalyzed by porcine ETF−QO (turnover number = 200 s^{-1}; K_m = 6.8 μM; Beckmann & Frerman, 1985a). The αT244M ETF_{1e-} did not exhibit saturation kinetics with ETF−QO (Figure 1). From a direct plot of the data with the mutant ETF, a second-order rate constant of 1.49×10^7 M^{-1} s^{-1} was calculated. This result was obtained in four experiments.

At pH 7.4, the mutant and wild type ETFs quantitatively stabilized the anionic flavin semiquinone generated by photochemical reduction and reduction with $Na_2S_2O_4$, as we previously reported (Watmough et al., 1992; Bedzyk et al., 1993). The stability of the mutant ETF was evaluated from the disproportionation equilibrium of ETF_{1e-} catalyzed by ETF−QO. In 20 mM Tris-HCl, pH 7.4, 15 °C, the equilibrium constant for disproportionation (κ) of the wild

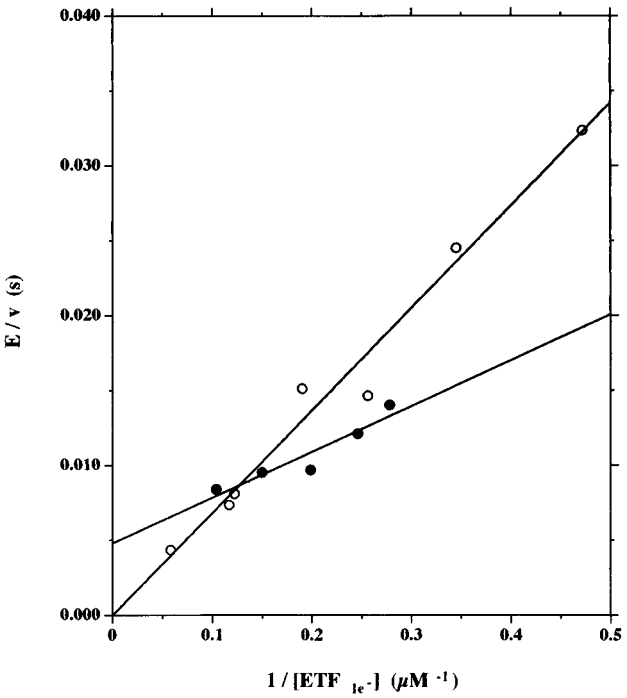

FIGURE 1: Double-reciprocal plot of the dependence of the rate of ETF_{1e-} disproportionation on the concentration of wild type ETF and αT244M ETF. The rate of wild type (●) and mutant (○) ETF_{1e-} disproportionation catalyzed by 0.96 nM *P. denitrificans* ETF−QO was determined spectrophotometrically.

type semiquinone was 1.26 ± 0.17, in good agreement with a previous determination (Watmough et al., 1992) and about 2-fold greater than values reported for porcine and human ETFs (Beckmann & Frerman, 1985a; Ramsay et al., 1985; Herrick et al., 1994). From this value, the calculated separation of oxidation−reduction potentials of the oxidized/semiquinone couple (E_2) and semiquinone/hydroquinone couple (E_1) was calculated to be −2.2 ± 2.8 mV (n = 5). κ for the αT244M mutant was 5.52 ± 0.99, corresponding to ΔE of −37.6 ± 4.3 mV. Under conditions of the kinetic assay, the values of κ for the wild type and mutant ETF semiquinones were 1.30 ± 0.05 and 3.19 ± 0.23, respectively. From these data, the values of ΔE are −6.04 ± 1.00 mV (n = 6) for the wild type ETF and −26.8 ± 2.0 mV (n = 7) for the αT244M mutant, where n is the number of determinations. Therefore, the mutation does not affect the steady state kinetic properties of the half-reaction with medium chain acyl-CoA dehydrogenase but significantly affects the reaction with ETF−QO and the redox properties of the mutant protein. These data are summarized in Table 1.

Structural Properties of the αT244M Mutant. Higher order structure of the ETFs and flavin environments were investigated using ultraviolet/visible, fluorescence, circular dichroism, and infrared spectroscopy. In some instances, the spectra of the holoproteins and AMP-containing apoproteins were compared. There were only minor differences between the absorption and circular dichroism spectra of the two proteins, and the fluorescence emission spectra of the flavin in the two proteins were indistinguishable (data not shown).

Tryptophan fluorescence emission spectra showed clear differences between the wild type and mutant proteins. In these experiments we compared the fluorescence emission spectra of tryptophan (λ_{ex} = 295 nm) of the native holopro-

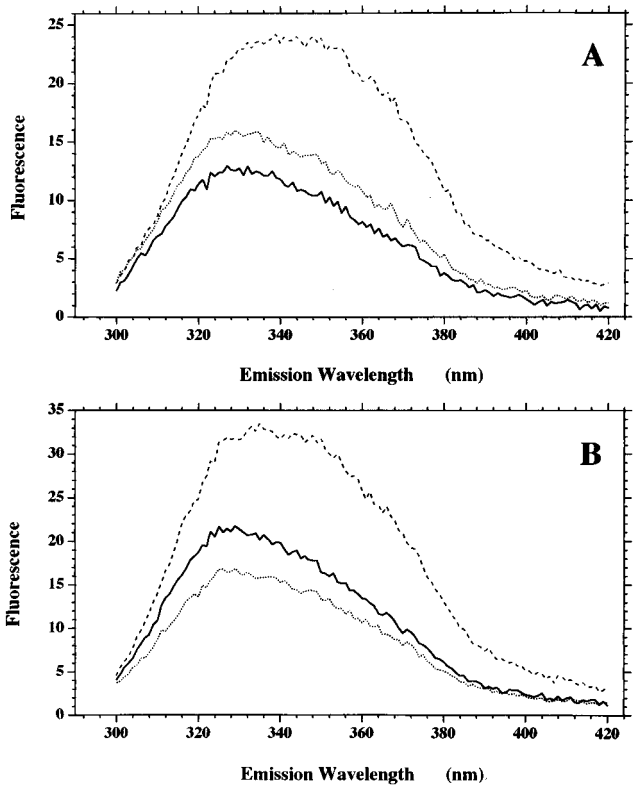

FIGURE 2: Tryptophan fluorescence emission spectra of the wild type and αT244M mutant ETFs. Fluorescence emission spectra of the wild type ETF (A) and the mutant ETF (B) were determined at 4 °C in 50 mM potassium phosphate, 0.3 mM EDTA, 5% glycerol, pH 6.8; the the excitation wavelength was 295 nm. The concentration of the proteins was 1 μM, or the spectra were normalized to this concentration. The spectra of native ETF (—), apoETF (− −), and reconstituted ETF (· · ·) are shown.

teins, apoproteins, and reconstituted holoproteins. FAD was resolved from the native holoETFs by treatment with KBr. Although we did not further characterize the proteins, the KBr-generated FAD-free apoproteins presumably contain AMP (Sato et al., 1992, 1993; see below). Fluorescence spectra of both native holoproteins suggested that tryptophan residues lie in hydrophobic environments with λ_{max} of 327 nm for the wild type and 329 nm for the αT244M mutant (Figure 2). The fluorescence emission intensity (F_{max}) of the mutant protein at λ_{max} was 60% greater than that of the wild type ETF. After removal of the flavin, tryptophan emission of both proteins increases drastically, presumably due to absence of quenching by the flavin or a change in the immediate environment. The emission envelopes of the apoETFs broaden, and λ_{max} of both proteins shifts to about 339 nm. F_{max} of the mutant apoETF is about 50% greater than the value for the wild type apoprotein. When the apoproteins were reconstituted with FAD, the fluorescent spectra of the native proteins were essentially restored to those more characteristic of the native proteins (Figure 2), and 93−97% of ETF catalytic activity with medium chain acyl-CoA dehydrogenase was recovered, based on ETF flavin. The absorption spectra and flavin circular dichroism spectra of both reconstituted proteins were almost identical to the spectra of the native proteins (data not shown). The small red shift of λ_{max} of the native αT244M mutant suggested partial exposure to solvent. This idea is supported by fluorescence quenching experiments with Cs^+. Tryptophan fluorescence of the native wild type ETF is not

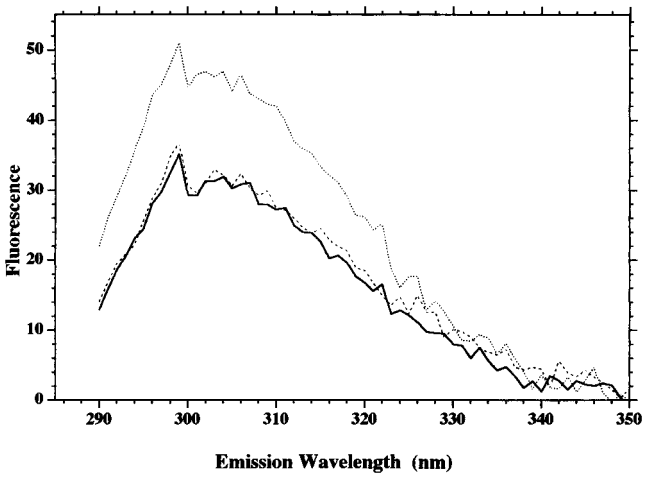

FIGURE 3: Tyrosine fluorescence of wild type ETF. Tyrosine fluorescence of ETF was determined for the native protein (—), the apoprotein (· ·), and the holoprotein after reconstitution with FAD (− −). Tyrosine fluorescence was estimated by excitation at 280 nm and subtraction of the contribution tryptophan as described in Watmough et al. (1991).

quenched by Cs^+; in contrast, fluorescence of native αT244M is quenched by Cs^+ ($K_{SV} = 0.12\ M^{-1}$). In both apoproteins, tryptophan fluorescence is quenched by Cs^+, indicating increased exposure to solvent; the fractional exposures calculated for the wild type and mutant proteins are 0.46 and 0.2, respectively. Assuming that the three tryptophan residues in the molecule have the same quantum yield, the experimentally determined fractional exposures suggest that approximately one tryptophan is accessible to Cs^+ after removal of the FAD. These data indicate that tryptophan residues in the native protein lie in hydrophobic environments, although tryptophan appears partially exposed in the αT244M mutant ETF. Tyrosine was excited at 280 nm, and tyrosine emission was calculated by subtracting the contribution of tryptophan (Watmough et al., 1991). Tyrosine fluorescence emission spectra of the two proteins were identical in native ETF, apoETF, and reconstituted ETF, but F_{max} due to tyrosine of both proteins increased by about 50% upon generation of the apoprotein with KBr. The calculated tyrosine emission spectra of the wild type protein is shown in Figure 3.

The near- and far-ultraviolet circular dichroism spectra of wild type and αT244M ETF show no significant differences between the two proteins. From the far-ultraviolet circular dichroism spectrum, the content of α helix in the wild type protein is 31−35%. However, we did observe some concentration dependence of molar elipticities in the far-ultraviolet. Between 1 and 20 mg/mL, the spectra of the wild type protein were always slightly more intense than those of the mutant at the same concentration. The concentration dependence may be due to microaggregation which was otherwise undetectable.

To compare the secondary structures of the proteins further, we determined the infrared spectra of the two proteins in buffers prepared in ^1H_2O and ^2H_2O. The second-derivative infrared spectra in the conformationally sensitive amide I region (Dong et al., 1990, 1996; Susi & Byler, 1986; Surewicz & Mantsch, 1988) for the two proteins were essentially identical, when the proteins were prepared in ^1H_2O buffer (Figure 4A). Thus, the secondary structure of the protein is not altered by the mutation. Furthermore, after

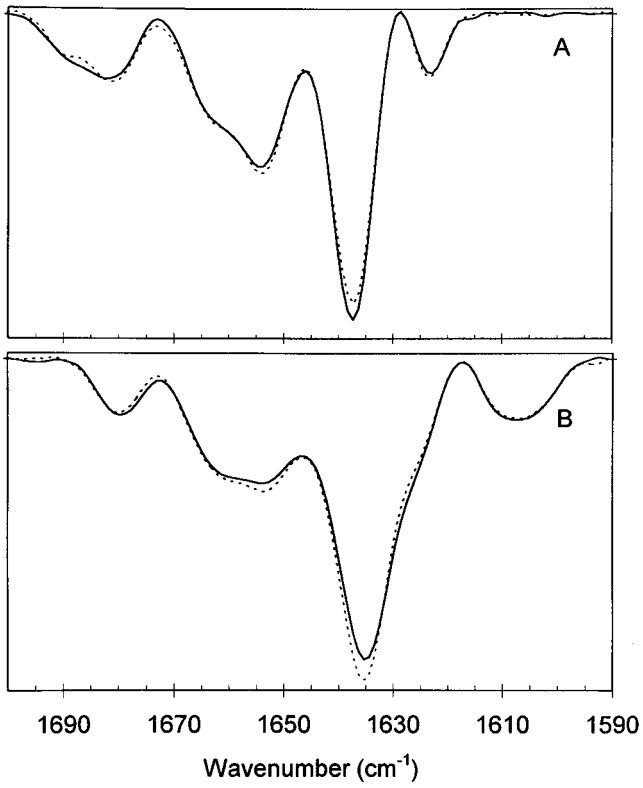

FIGURE 4: Second-derivative infrared spectra of wild type ETF and αT244M mutant ETF in ^1H_2O and ^2H_2O. In both panels spectra of the wild type protein are indicated by (—) and spectra of the mutant protein are indicated by (– –): (A) spectra of proteins determined in buffer prepared in ^1H_2O and (B) spectra of proteins determined in buffer prepared in ^2H_2O and after hydrogen–deuterium exchange. Spectra of the proteins (20 mg/mL) were determined at 25 °C in 50 mM potassium phosphate, 0.3 mM EDTA, 5% glycerol, pH 7.6 or pD 8.0.

hydrogen–deuterium exchange, the spectra of both proteins shift and broaden, as expected, and are virtually indistinguishable (Figure 4B). This result documents that the extent of hydrogen–deuterium exchange is the same for both proteins, which suggests that the mutation does not significantly alter the protein's conformational mobility (Dong et al., 1995). To quantitate the secondary structural content, curve-fitting analysis of the amide I second-derivative spectrum was carried out. The sum of the areas of the fitted curves overlap the experimental spectrum by 98.7% (Kendrick et al., 1996). Based on the relative band areas and assignment of component bands to secondary structural types (Dong et al., 1992, 1995, 1994; Kendrick et al., 1996), the following results were obtained. The 1623 and 1637 cm^{-1} bands are due to β sheet and represent 5% and 41% of the structure, respectively. The 1655 cm^{-1} band, which is due to α helix, represents 30% of the structure. The bands at 1665, 1680, and 1687 cm^{-1} are due to β turns and represent 9%, 6%, and 9% of the structure, respectively. Overall the protein consists of 46% β sheet, 30% α helix, and 24% β turn. These results are in good agreement with the X-ray crystal structures (Roberts et al., 1996).[2]

AMP Content of Wild Type and αT244M Mutant ETFs. The mutation has no effect on the content of noncovalently bound AMP in the protein. The AMP is located entirely in the β subunit (Roberts et al., 1996).[2] Extracts of recombinant *P. denitrificans* ETF expressed in *E. coli* contained FAD and AMP in ratio of 1:0.97. There was no difference in the FAD:AMP ratio (1:1.03) in the αT244M mutant. Similarly, the

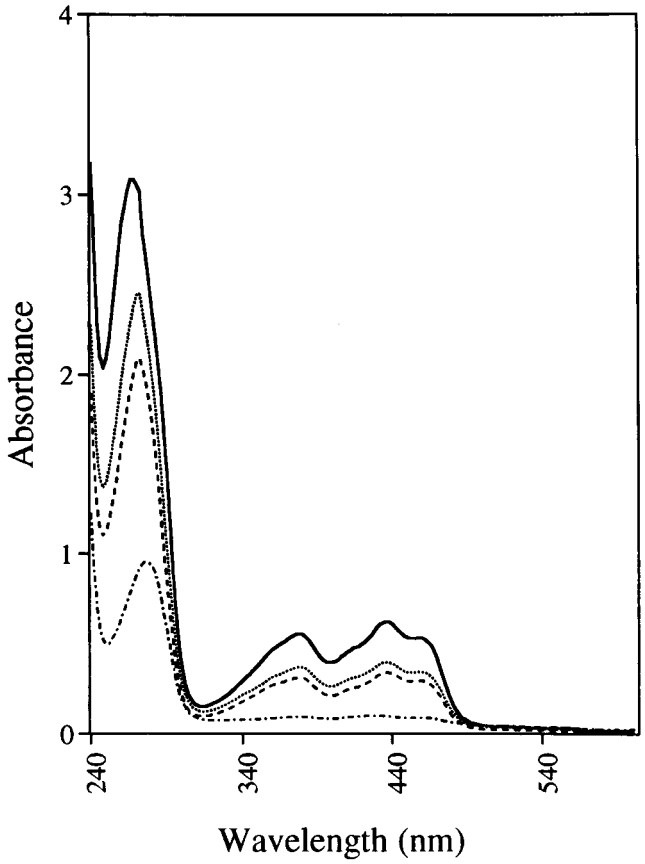

FIGURE 5: Absorption spectra of native and reconstituted wild type ETF. FAD and AMP were extracted from ETF with buffered 5.4 M guanidine-HCl, pH 7.6. The protein was then renatured at 25 °C by 400-fold dilution in 50 mM potassium phosphate, 1 mM dithiothreitol, pH 7.6. The spectra are those of the native protein prior to denaturation (—), the apoprotein renatured in the presence of 2 μM FAD and 2 μM AMP (· ·), the apoprotein renatured in the presence of both nucleotides but without dithiothreitol (– –), and the apoprotein renatured in the presence of 2 μM FAD but without AMP (– · –). Excess nucleotides were removed by four 20-fold dilutions and concentration using a Centricon 10 instrument. The recovered proteins were suspended in the same volume as the original native ETF sample to determine the spectra of the products and quantitate the recoveries.

expressed human ETF contains FAD and AMP in a ratio of 1:0.90.[3] Extraction of wild type and αT244M *P. denitrificans* ETFs with buffered 5.4 M guanidine-HCl releases FAD and AMP in an approximate 1:1 ratio. After four extractions, we could account for at least 97% of each nucleotide in the extracts of wild type and mutant *P. denitrificans* ETFs and human ETF. Figure 5 shows the absorption spectra of wild type *P. denitrificans* ETF before and after renaturation of the AMP-free apoprotein in the presence of FAD and AMP. The recovery of ETF based on flavin was about 60%, and the protein appears to renature with a high degree of fidelity because the $A_{272\ nm}$:$A_{436\ nm}$ ratio in several experiments was always identical to that of the native protein and the recovery of activity was quantitative. The αT244M mutant does not renature to the same extent or with the same fidelity. The results of experiments comparing renaturation of the wild type and mutant ETFs are summarized in Table 2 and represent the means and standard errors of several experiments with each AMP-free apoETF. Most apparent is the finding that the recovery of the mutant protein after guanidine

[3] T. M. Dwyer and F. E. Frerman, unpublished data.

Table 2: Properties of Wild Type and αT244M Native and FAD/AMP Renatured ETFs

	renatured	native
Wild Type[a]		
recovery, flavoprotein (%)	62.3 ± 1.7	
recovery, activity (%)	99.5 ± 2.5	100
A_{272nm}:A_{436nm}	6.3	6.3
FAD:AMP	1:(0.94 ± 0.03)	1:0.97
αT244M Mutant[b]		
recovery, flavoprotein (%)	27.6 ± 1.7	
recovery, activity (%)	83.6 ± 8.6	100
A_{272nm}:A_{436nm}	6.9−8.7	6.3
FAD:AMP	1:(1.03 ± 0.02)	1:1.01

[a] $n = 5$. [b] $n = 4$ (n is the number of determinations). Data are given as ± standard error.

denaturation was only 43% that obtained with the wild type protein. The recovery of ETF protein is based on the recovery of the characteristic absorption spectrum and was calculated assuming that the molar absorptivity of the protein-bound flavin in the mutant protein is identical to the value for the wild type protein. The recovery of activity of the mutant ETF, based on flavin, was slightly less than that of the wild type protein, and the A_{272nm}:A_{436nm} ratio was always significantly greater than the ratio for the native mutant protein.

In contrast with previous results with the pig liver ETF reported by Sato et al. (1993), recovery of active *P. denitrificans* and human[3] ETFs was absolutely dependent on both AMP and FAD in the reconstitution buffer, and neither IMP, GMP, UMP, nor adenosine could replace AMP in the reconstitutions. Unlike human apoETF, *P. denitrificans* apoETF did not require dithiothreitol in the denaturation and reconstitution buffers to recover active protein (Figure 5). In the absence of dithiothreitol, the yield of reconstituted bacterial ETF was 55%, compared to 62 ± 2% in the presence of the thiol.

DISCUSSION

The side chain hydoxyl hydrogen of threonine 244 in the α subunit of *P. denitrificans* is hydrogen bonded to N(5) of the flavin, and the amide hydrogen of this residue in the protein backbone is hydrogen bonded to C(4)O[2] as in the human protein (Roberts et al., 1996). This mutation would be expected to alter the properties of FAD in the αT44M ETF; however, the spectral properties of the flavin are scarcely affected. The near-ultraviolet circular dichroism spectra indicate that there is no difference in the packing of the proteins, and the far-ultraviolet circular dichroism spectra indicate that there is no global change of secondary structure due to the mutation. This latter point is reinforced by the infrared spectra. However, the intrinsic fluorescence due to tryptophan residues in αT244M ETF is 60% greater than in the wild type protein, and tryptophan fluorescence of the mutant protein is slightly quenched by Cs^+, suggesting that at least one of the three tryptophan residues in the native mutant protein is slightly exposed to solvent. W239 is most likely the tryptophan residue that responds to the mutation. W239 is 12.33 Å away from N(3) of the flavin[2] and five residues amino terminal of the mutation site. The increase in fluorescence may be due to a slight turning of the loop behind the flavin on which the W239 resides, since the quantum yield of tryptophan increases in an aqueous environment (Burstein et al., 1973). Further, when FAD is resolved from the wild type and mutant proteins, tryptophan fluorescence increases by 1.6−2-fold, respectively, suggesting that flavin quenches tryptophan fluorescence, or, alternatively, the quantum yield changes even more drastically in the apoproteins when W239 becomes further exposed to an aqueous environment. In the apoproteins, tryptophan emission red shifts and approximately one tryptophan residue becomes more accessible to quenching by Cs^+, assuming that all tryptophan residues have the same quantum yield. These changes are almost completely reversed by reconstitution of the apoproteins with FAD. The other two tryptophan residues, αW177 and βW141, are considerably more distant from the flavin and the site of the mutation. Tyrosine fluorescence of the mutant and wild type native proteins is identical and increases equally upon generation of the apoproteins. αY253 and αY234 lie 7.28 and 13.38 Å from C(2)O of the flavin, respectively, and βY13 lies 4.85 Å from the C(8) methyl group of the flavin.[2] These tyrosine residues do not report the mutation but are the most likely reporter(s) for the removal of the flavin. Taken together, the fluorescence spectra indicate that the αT244M mutation affects the structure of a very localized region of the polypeptide.

The localized structural alterations modify the redox and kinetic properties of the mutant protein. The formation constant for the semiquinone is reduced 2.5-fold. The oxidation−reduction potentials were not explicitly determined, but a 20 mV decrease in difference in potentials was calculated from the semiquinone formation constant. This is a relatively small change; however, neither the semiquinone nor the hydroquinone forms of ETF flavin are protonated at N(5), and this may minimize the effects of the loss of T244, a proton donor in the oxidized state. This change does not affect the reaction with medium chain acyl-CoA dehydrogenase but selectively affects the reaction with ETF-QO. In the disproportionation kinetic assay ETF_{1e-} is the electron donor and electron acceptor. The k_{cat}/K_m with wild type ETF_{1e-} as substrate is 3.9×10^7 M^{-1} s^{-1}. With the mutant ETF_{1e-} as substrate, saturation was not observed and a second-order rate constant, 1.49×10^7 M^{-1} s^{-1}, was calculated. The mutation, and compensating structural changes which may include disruption of the hydrogen bond at C(4)O, may decrease the on-rate of ETF_{1e-} or increase the off-rate of ETF_{ox} and ETF_{2e-} such that a Michaelis complex cannot be demonstrated. It is significant that the mutation had no effect on the reaction of ETF with the artificial electron acceptor, dichloroindophenol. The structural change need not affect the reaction with medium chain acyl-CoA dehydrogenase. Previous work with human ETF and a *Paracoccus*−human ETF chimera suggested nonidentical docking sites for medium chain acyl-CoA dehydrogenase and ETF−QO on ETF (Herrick et al., 1994). In the mutant, the altered W239- and T244-containing loop lies on the opposite side of the flavin from the predicted binding site for medium chain acyl-CoA dehydrogenase (Roberts et al., 1996). The predicted changes in the *P. denitrificans* mutant should become clearer when the crystal structure of this protein is refined.[2]

Studies on the effects of the mutation on the AMP content of *P. denitrificans* ETF were undertaken before crystallographic studies showed the mononucleotide was located entirely in the β subunit. Understandably, the αT244M

mutation does not affect the AMP content of the mutant protein. However, our experiments do make several additional points related to the AMP ligand of ETF. First, AMP, equimolar with FAD, was detected in both heterologously expressed human[3] and *P. denitrificans* ETFs. Previously, AMP was detected in ETFs isolated from porcine liver and the methylotrophic bacterium W3A1 (Sato et al., 1993; DuPlessis et al., 1994). The presence of AMP in heterologously expressed ETFs indicates that AMP is an integral component of ETFs and is independent of the cell in which the protein is expressed. Second, and in contrast with the results of Sato et al. (1993), we found that AMP is absolutely required for the renaturation of guanidine-denatured, AMP-free ETFs. Sato et al. showed that AMP enhanced the rate of FAD incorporation into porcine ETF but was not absolutely required for renaturation of the apoprotein. In our experiments, AMP was absolutely required for renaturation and the mononucleotide requirement was highly specific. We have no explanation for the difference between our results and those of Sato et al. (1993). The renaturation of *P. denitrificans* AMP-free ETF did provide additional insight into the effects of the αT244M mutation. Renaturation of the guanidine-denatured mutant apoETF was consistently less efficient than renaturation of the wild type apoETF. Also, the fidelity of renaturation of the mutant apoETF was less since the recovery of protein, judged by recovery of the resolved absorption spectrum of native ETF, and the recovery of activity on a flavin basis were significantly less than the recovery of active wild type active protein. Further, the A_{272nm}:A_{436nm} ratio of the renatured mutant protein was always greater than the ratio of the renatured wild type or native proteins. The differences between the renatured wild type and mutant ETF are not likely to be due to differences in the kinetics of renaturation because identical results were obtained when the renaturation period was varied from 12 to 18 h. Renaturation of both proteins was also absolutely dependent on FAD. These results point to the participation of an appropriate hydrogen bond donor at N(5) for flavin binding in ETFs and related proteins. The effects of ligands on protein refolding have been most extensively investigated with pyridoxal- and heme-containing proteins. Although these ligands can promote tightening of subunit structure and subunit dimerization of aspartate aminotransferase, in no case does the ligand direct *in vitro* refolding (Cai et al., 1995; Blond & Goldberg, 1985; Banik et al., 1995; Schechter & Epstein, 1968; Artigues et al., 1994). In contrast, L-valine directs the renaturation of the monomer of guanidine-denatured yeast pyruvate kinase and remains noncovalently bound in the holoprotein (Hess & Bornman, 1975).

In summary, the αT244M mutation has only modest effects on the kinetic and redox properties of *P. denitrificans* ETF but substantially affects the binding of the flavin prosthetic group. Thus the decreased activity in the corresponding human mutant ETF may be due to lability of the flavin.

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