αArg-237 in *Methylophilus methylotrophus* (sp. W3A1) Electron-transferring Flavoprotein Affords ~200-Millivolt Stabilization of the FAD Anionic Semiquinone and a Kinetic Block on Full Reduction to the Dihydroquinone*

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The midpoint reduction potentials of the FAD cofactor in wild-type *Methylophilus methylotrophus* (sp. W3A1) electron-transferring flavoprotein (ETF) and the αR237A mutant were determined by anaerobic redox titration. The FAD reduction potential of the oxidized-semiquinone couple in wild-type ETF (E'_1) is +153 ± 2 mV, indicating exceptional stabilization of the flavin anionic semiquinone species. Conversion to the dihydroquinone is incomplete (E'_2 < ~250 mV), because of the presence of both kinetic and thermodynamic blocks on full reduction of the FAD. A structural model of ETF (Chohan, K. K., Scrutton, N. S., and Sutcliffe, M. J. (1998) *Protein Pept. Lett.* 5, 231–236) suggests that the guanidinium group of Arg-237, which is located over the si face of the flavin isoalloxazine ring, plays a key role in the exceptional stabilization of the anionic semiquinone in wild-type ETF. The major effect of changing αArg-237 for Ala in *M. methylotrophus* ETF is to engineer a remarkable ~200-mV destabilization of the flavin anionic semiquinone (E'_2 = ~31 ± 2 mV, and E'_1 = ~43 ± 2 mV). In addition, reduction to the ETF dihydroquinone in αR237A ETF is relatively facile, indicating that the kinetic block seen in wild-type ETF is substantially removed in the αR237A ETF. Thus, kinetic (as well as thermodynamic) considerations are important in populating the redox forms of the protein-bound flavin. Additionally, we show that electron transfer from trimethylamine dehydrogenase to αR237A ETF is severely compromised, because of impaired assembly of the electron transfer complex.

Electron-transferring flavoproteins (ETFs)* act as carriers of electrons in bacteria and mitochondria. They mediate electron transfer between degradative enzymes and membrane-bound electron acceptors (1). ETFs have been classified into two functional groups (2). Housekeeping ETFs function in the oxidation of fatty acids and some amino acids and have been isolated from mammalian and bacterial sources (1, 3). Specialized ETFs are restricted to prokaryotes and are synthesized under defined nutritional conditions. Specialized ETFs are involved in the oxidation of trimethylamine (4) and carnitine (5, 6) and are also important in nitrogen fixation (7). All ETFs possess one equivalent of non-covalently bound FAD per ETF heterodimer, except the ETF from *Megasphaera elsdenii*, which contains 2 equivalents of FAD per dimer (8). It has been shown that AMP (1 equivalent) is associated with the housekeeping ETFs from pigs (9), humans (10), and *Paracoccus denitrificans* (11) and with the specialized ETF from *Methylophilus methylotrophus* (12).

Mammalian and bacterial ETF proteins act as one-electron carriers, cycling through the oxidized and anionic flavin semiquinone forms. The ETF from *M. elsdenii* is unusual in acting physiologically as a two-electron carrier. ETF from mammalian sources and *P. denitrificans* can be reduced to the dihydroquinone form, by reduction with dithionite or by photoreduction (13–15), although reduction to the two-electron level is relatively slow. *M. methylotrophus* ETF is readily converted to the semiquinone form in reactions with its physiological electron donor, trimethylamine dehydrogenase (TMADH) (4), or during artificial reduction with dithionite (16). However, further reduction to the dihydroquinone is not observed with dithionite (16) or with catalytic amounts of TMADH (4). Reduction of *M. methylotrophus* ETF to the dihydroquinone form can be achieved (albeit sluggishly) by electrochemical methods (17). In addition, when ETF is in complex with TMADH, the FAD is more readily reduced to the two-electron level (18). In this latter case, further reduction to the dihydroquinone is likely to be a consequence of a large scale structural reorganization in ETF that accompanies complex assembly with TMADH (18–20).

The midpoint reduction potentials of the E'_1 (quinone-semiquinone) and E'_2 (semiquinone-dihydroquinone) couples of the FAD in *M. methylotrophus* ETF have been determined. The potential of the quinone-semiquinone couple is exceptionally high (~196 mV (17) and ~141 mV (21)) as determined by electrochemical and spectrophotometric methods, respectively, consistent with a need to accept electrons from the 4Fe-4S center of TMADH (midpoint potential, +102 mV (22)). The potential of the semiquinone-dihydroquinone couple is more conventional (~197 mV (17)), indicating that there is a substan-

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1 The abbreviations used are: ETF, electron-transferring flavoprotein; TMADH, trimethylamine dehydrogenase.

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Note (i) the potential intersubunit salt bridge (a subunits (subunit (the C terminus of the methylotrophus kinetic) of protein-bound flavin.

of a single residue close to the flavin isoalloxazine ring can have FAD. These findings demonstrate that the chemical properties anionic semiquinone in native ETF and that mutation of Arg- tioned over the ETF suggests that residue Arg-237 is located close to the FAD isoalloxazine ring, with its guanidinium group posi-

demonstrated that domain II is mobile with respect to domains I and III (20). The isoalloxazine ring of FAD interacts almost exclusively with domain II (Fig. 1). The model of si ETF have been determined at 2.1 Å (10, 11) and 2.6 Å (11) resolution, respectively. Using the x-ray crystallographic studies of ETF have been reported; for pig pig ETF requires about 1 h for equilibration (13, 14). The potentials measured for free M. methylotrophus ETF (as with any ETF) may not of course reflect the situation in the electron transfer complex with its physiological electron donor (TMADH) but nevertheless are likely to serve as a reasonable guide.

M. methylotrophus ETF shares considerable sequence identity with bacterial and mammalian ETFs (10, 11). Preliminary crystallographic studies of M. methylotrophus ETF have been reported (25), but to date no crystallographic structure for the protein is available. However, crystallographic structures of human and P. denitrificans ETF have been determined at 2.1 Å (10) and 2.6 Å (11) resolution, respectively. Using the x-ray structure of human ETF as a template (10), we built a model of the structure of M. methylotrophus ETF, in free solution and in complex with TMADH (19). The model predicts that the two subunits (subunit α (residues 1–321) and subunit β (residues 322–585)) of M. methylotrophus ETF comprise three domains. Domain I (the N-terminal region of the α subunit), domain II (the C terminus of the α subunit and a small C-terminal region of the β subunit), and domain III (the majority of the β subunit) form a Y-shaped structure, with domains I and III forming a shallow “bowl” in which domain II rests. Domain II is connected to domains I and III by two flexible regions of polypeptide chain (19). Small angle x-ray scattering studies have demonstrated that domain II is mobile with respect to domains I and III (20). The isoalloxazine ring of FAD interacts almost exclusively with domain II (Fig. 1). The model of M. methylotrophus ETF suggests that residue Arg-237 is located close to the FAD isoalloxazine ring, with its guanidinium group positioned over the si face of the dimethylbenzene subnucleus. The guanidinium group is thus located to help stabilize the increased electron density (which resides predominantly in the pyrimidine subnucleus) on reduction of the flavin to the anionic semiquinone. In this paper, we report the redox properties of a mutant ETF in which Arg-237 is replaced by Ala. We show that Arg-237 plays a key role in the exceptional stabilization of the anionic semiquinone in native ETF and that mutation of Arg-237 to Ala removes the kinetic block to full reduction of the FAD. These findings demonstrate that the chemical properties of a single residue close to the flavin isoalloxazine ring can have profound effects on the redox properties (thermodynamic and kinetic) of protein-bound flavin.

### EXPERIMENTAL PROCEDURES

**Isolation of the αR237A Mutant and Expression of M. methylotrophus ETFs in Escherichia coli**—Isolation of the αR237A mutant form of ETF was performed using the QuikChange site-directed mutagenesis kit supplied by Stratagene and oligonucleotides 5′-CTT TGC TGC TCA GCT CCG ATT GCG GAT-3′ and 5′-ATC CCG AAT CCG GAT GCA GCA AAG-3′. The ETF expression plasmid pED1 (20) was used as template DNA for the mutagenesis reaction. Recombinant wild-type ETF was expressed from plasmid pED1 in E. coli strain TG1 as desuged (20). To ensure that no spurious changes had arisen as a result of the mutagenesis reaction, the entire ETF gene was resequenced using the Amersham Pharmacia Biotech T7 sequencing kit and protocols. Recombinant wild-type and mutant ETF proteins were expressed from plasmids pED1 and pED1R237A, respectively, in the E. coli strain TG1. Recombinant strains were grown at 20 °C in 2xYT medium supplemented with 100 µg/ml ampicillin. ETF was purified in large quantities (30 mg/liter of late exponential phase culture) from recombinant strains of E. coli. Harvested cells were resuspended in buffer A (50 mM potassium phosphate buffer, pH 7.2, 0.2 mM EDTA) and broken in a French press (140 megapascals, 4 °C). The extract was clarified by centrifugation at 15,000 × g for 90 min, and solid ammonium sulfate was added to 50% saturation. The precipitate was removed by centrifugation, and the supernatant was applied to a Q-Sepharose column equilibrated with buffer A. After washing with buffer A, protein was eluted using a descending gradient (1.5 to 0 M) of ammonium sulfate. Fractions containing ETF were dialyzed exhaustively against buffer A and applied to a Q-Sepharose column equilibrated with buffer A. After washing with buffer A, protein was eluted using a gradient (0 to 2 M KCl); ETF was eluted at ~0.5 M KCl. Samples were dialyzed exhaustively against potassium phosphate buffer, pH 7.2 and stored (~70 °C) in the presence of 20% ethylene glycol. Anaerobic titration buffer was prepared by flushing freshly prepared buffer with oxygen-free nitrogen. Protein samples admitted to the glove box were deoxygenated by passing through a Bio-Rad 10DG column, with final dilution of the eluted protein to give an ETF concentration of 70–80 µM. Solutions of benzyl viologen, methyl viologen, 2-hydroxy-1.4-naphthaquinone, and phenazine methosulfate were added to a final concentration of 0.5 µM as redox mediators for the titrations. Absorption spectra (300–750 nm) were recorded on a Shimadzu 2101 UV-visible spectrophotometer, and the electrochemical potential was monitored using a CD 740 m combination pH/voltmeter coupled to a Russell platinum/ calomel electrode. The electrode used for the Fe(II)/Fe(III)–EDTA couple (108 mV) as a standard. The flavoprotein solutions were titrated electrochemically using sodium dithionate as reductant and potassium ferricyanide as oxidant, as described by Dutton (24). After the addition of each aliquot of reductant, and allowing equilibration to occur (stabilization of the observed potential), the spectrum was recorded, and the potential was noted. The process was repeated at several (typically ~40) different potentials. In this way, a set of spectra representing reductive and oxidative titrations was obtained. Small corrections were made for any drift in the base line by correcting the absorbance at 750 nm to zero, and spectrophotometric contributions from the mediators were removed prior to data analysis. The observed potentials were corrected to those for the standard hydrogen electrode (platinum/calomel + 244 mV).

**Treatment of Data**—Data manipulation and analysis were performed using Origin software (Microcal). Absorbance values at wavelengths of 470 nm (near the oxidized flavin maximum) and 370 nm (close to the maximum for the anionic semiquinone) were plotted against potential. Maximum for the anionic semiquinone) were plotted against potential.

The Beer–Lambert law, as described previously (24, 25),

\[
A = \frac{a_{0}E_{0} \cdot E_{0}^{b} + b + c \cdot 10^{d \cdot E_{0}}}{1 + 10^{10^{-3} \cdot E_{0}}} + 10^{4 \cdot E_{0}}
\]

(Eq. 1)

In Equation 1, A is the total absorbance; \(a, b, c\) and \(E\) are component absorbance values contributed by one flavin in the oxidized, semiqui-

none, and reduced states, respectively. \(E\) is the observed potential; \(E'\),
and \( E^{-}_{2} \) are the midpoint potentials for oxidized-semiquinone and semiquinone-reduced couples, respectively. In using Equation 1 to fit the absorbance-potential data, the variables were unconstrained, and regression analysis provided values in close agreement with those of the initial estimates.

RESULTS

Purification of the \( aR237A \) Mutant ETF and Reduction of Wild-type and \( aR237A \) ETF with Dithionite—The \( aR237A \) mutant ETF was purified essentially as described for the wild-type protein. A notable difference between the mutant and wild-type proteins is the redox state of the flavin. \( aR237A \) ETF is purified in the oxidized form, whereas wild-type ETF is isolated as a mixture of oxidized and anionic flavin semiquinone forms (Fig. 2). Oxidation of the wild-type ETF with potassium ferricyanide, followed by immediate rapid gel filtration to remove the oxidant, generates the oxidized form of wild-type ETF. A comparison of the spectral properties of the oxidized wild-type and \( aR237A \) mutant ETF proteins indicates that the peak of flavin absorption (446 nm) in the \( aR237A \) mutant is shifted compared with the corresponding peak (438 nm) in wild-type ETF. In addition, the \( A_{388}/A_{446} \) ratio (1.01) for \( aR237A \) ETF is greater than the \( A_{380}/A_{438} \) ratio (0.89) for wild-type ETF. These observations suggest that the isoalloxazine ring of FAD in \( aR237A \) ETF is more exposed to solvent than in wild-type ETF (26), an observation that is consistent with the structural model for \( M. \) methylotrophus ETF (19).

Consistent with previous reports, anaerobic titration of wild-type ETF with sodium dithionite (16) or enzymatic reduction with TMADH (4) reduces the protein only to the level of the flavin anionic semiquinone (Fig. 3A). The addition of excess dithionite (6× molar excess) does not reduce the protein further to the dihydroquinone form, even following prolonged incubation (30 min). The potential of the \( E^{-}_{2} \) couple for wild-type ETF is almost certainly more negative than \(-250 \) mV (see below) but is more positive than the reduction potential of dithionite (-530 mV). This indicates that there is a substantial kinetic block on full reduction of the flavin. By contrast, reduction of the \( aR237A \) mutant ETF with dithionite proceeds to full reduction (Fig. 3, B and C). The two reductive phases (oxidized-semiquinone and semiquinone-dihydroquinone couples) are clearly resolved. The spectral changes accompanying reduction of the oxidized FAD to the anionic semiquinone have isosbestic points at 491 and 391 nm, and a single isosbestic point at 342 nm is seen for reduction of the anionic semiquinone to the dihydroquinone.

Potentiometric Titrations of Wild-type ETF—In all cases, titrations were initiated from fully oxidized ETF and proceeded gradually to the end point of the titration by the addition of small aliquots of sodium dithionite (from 1 and 10 mM stocks) and then back again to oxidized ETF by addition of aliquots of potassium ferricyanide stocks of the same concentration. The protein samples remained completely soluble and stable throughout the course of the titration, enabling collection of good quality sets of spectra. No hysteretical effects were observed in any of the redox titrations. Spectra recorded at similar potentials during oxidative and reductive titrations were essentially identical. Representative spectra for the reductive titration of wild-type ETF and plots of the absorbance versus potential are shown in Fig. 4, A and B.

In the reductive titration with wild-type ETF in the presence
of mediators, reduction occurs first to the flavin anionic semiquinone, and a small proportion of the flavin is subsequently reduced to the dihydroquinone (Fig. 4A). Partial reduction to the dihydroquinone does not occur in the absence of mediators (Fig. 3), with reduction taking place only to the level of the flavin anionic semiquinone; this requires further comment. The time taken to complete reductive titrations in the potentiometry experiments was typically around 3–4 h. This is clearly much longer than the time taken to complete the simple spectral analysis shown in Fig. 3. Redox mediators were also included in the potentiometric analysis but were absent in the spectroscopic characterization shown in Fig. 3. The presence of redox mediators and also the long time period for protein reduction ensured more complete equilibration of the system in the potentiometric analyses, but this is clearly not the case with the spectral changes displayed in Fig. 3. Notwithstanding the inclusion of redox mediators and the prolonged time periods of the potentiometric measurements, the spectral changes accompanying reduction of wild-type ETF in the presence of redox mediators indicates that complete reduction to the dihydroflavin is not obtained. This is indicated by the presence of considerable semiquinone signature at 370 nm at the end of the titration. Further addition of dithionite did not reduce the absorption at 370 nm to the level seen for the aR237A mutant. That a substantial proportion of the wild-type ETF is reduced beyond the anionic semiquinone level, however, is indicated by the extent of bleaching in the absorption range of 440–470 nm (compared with titrations in the absence of mediators) and the partial bleaching of the absorption at 370 nm (Fig. 4A and B). The inability to completely reduce wild-type ETF probably reflects the presence of a substantial kinetic block on full reduction for a proportion of the protein sample.

Titrations performed in the presence and absence of mediators serve to illustrate the kinetic limitation on reduction to the dihydroquinone form in wild-type ETF, a kinetic bottleneck that can be overcome (at least in part) by the inclusion of redox mediators during the course of reductive titration. That equilibration was achieved with wild-type ETF in the majority of the potentiometric analyses is evident from the stability of the potential readings throughout the reductive titration and from the well defined transitions (Fig. 4B). The lack of hysteresis on performing the oxidative titration likewise indicates that equilibration was achieved (except at very low potentials) and also that FAD was not released from ETF during the course of the potentiometric titrations. The midpoint reduction potentials for $E_{1}$ and $E_{2}$ were obtained by fitting the data shown in Fig. 4A to Equation 1. These potentials are compared with values obtained by other workers for wild-type M. methylotrophus and mammalian ETFs in Table I.

**Potentiometric Titrations of aR237A ETF**—As with wild-type ETF, aR237A ETF remained soluble throughout the course of reductive and oxidative titrations, and no hysteresis was observed during the course of reduction by dithionite and reoxidation by ferricyanide. Representative spectra during the course of reductive titration with dithionite are shown in Fig. 4B, and plots of absorbance versus potential are shown in Fig. 4D.

The spectral changes observed during reductive titration (over 3–4 h) are again different from those observed in the spectroscopic characterization of the aR237A ETF shown in Fig. 3, B and C. In Fig. 3, the anionic semiquinone species is populated prior to full reduction to the dihydroquinone, whereas in potentiometric titrations full development of the anionic semiquinone signature at 370 nm is not observed.
(Fig. 4, C and D). Again, we attribute this to a kinetic limitation that prevents rapid reduction to the dihydroquinone in the absence of mediators (see also the value for $E'_2$ below). However, mutation of αArg-237 to Ala partially relieves the kinetic block, because the dihydroquinone clearly does form in the αR237A mutant enzyme during the reductive titration performed without mediators (Fig. 3C), unlike wild-type ETF, which is reduced only to the level of the flavin semiquinone (Fig. 3A). We conclude, therefore, that αArg-237 contributes to the kinetic block on reduction to the dihydroquinone seen in wild-type ETF.

During reductive titration of the αR237A mutant enzyme, small amounts of red anionic semiquinone are observed, as evidenced by very small increases in absorption at 370 nm during the early phase of reduction (Fig. 4D). The data at this wavelength fit to a two-electron Nernst function, and the two one-electron reduction steps are resolved as semiquinone ($E'_1$ (oxidized-semiquinone) = −43 ± 2 mV, with $E'_2$ (semiquinone-dihydroquinone) = −31 ± 2 mV). Clearly, there is a large (200 mV) perturbation of the reduction potential of the αR237A ETF compared with wild-type. Thus, the mutation appears to relieve both thermodynamic and kinetic blocks to full reduction of the flavin.

We are confident that equilibration is achieved in the potentiometric studies of the αR237A mutant ETF, as reflected in the stability of the potential readings, the well defined absorbance transitions (Fig. 4D), and the lack of hysteresis in reductive and oxidative titrations. Relative values for the redox couples in wild-type and mutant enzymes are presented in Table 1.

**TABLE I**

| Source of ETF | Midpoint potential of $E'_1$ (oxidized-semiquinone) couple | Midpoint potential of $E'_2$ (semiquinone-dihydroquinone) couple |
|---------------|----------------------------------------------------------|---------------------------------------------------------------|
| *M. methylotrophus* wild type (this work) | +153 ± 2 mV | <−250 mV |
| *M. methylotrophus* αR237A (this work) | −43 ± 2 | −31 ± 2 |
| *M. methylotrophus* wild type (Ref. 21) | +141 | ND |
| *M. methylotrophus* wild type (Ref. 17) | +196 | −197 |
| Human wild type (Ref. 37) | +22 | −42 |
| Human αR249K (Ref. 37) | −39 | −124 |
| Pig liver (Ref. 40) | +4 | −50 |

*a* Values taken from the fit of Equation 1 to absorbance (470 nm) versus potential plots for wild-type and αR237A ETF proteins.

*b* Values determined using the xanthine/xanthine oxidase method of Massey (39).

**Fig. 5. Reduction of wild-type and αR237A ETF by catalytic amounts of TMADH.** Reactions were performed under anaerobic conditions at 20 °C in 50 mM potassium phosphate buffer, pH 7.0. ETF concentration, 26 µM; trimethylamine concentration, 10 mM; TMADH concentration, 0.1 and 1 µM for the wild-type and αR237A ETF, respectively. Main panel (αR237A ETF), complete reduction was achieved only after 24 h of incubation. Inset (wild-type (wt) ETF), complete reduction was achieved in less than 5 min.

Reaction. This driving force can be calculated from the midpoint reduction potential of the 4Fe-4S center of TMADH (+102 mV; (22)) and the $E'_1$ and $E'_2$ couples of αR237A ETF (Table 1). The driving forces for electron transfer from the 4Fe-4S center of TMADH to the oxidized and semiquinone forms of αR237A ETF are +0.16 and +0.15 eV, respectively. Therefore, the equilibrium position for electron transfer is shifted toward oxidized ETF and reduced TMADH. Using (i) the robust engineering principles of Dutton and colleagues (27) for the design of electron transfer proteins and (ii) the known electron tunnelling distance of ~12 Å from the 4Fe-4S center to ETF (28), we were able to make these driving forces compatible with intrinsic endergonic tunnelling rates in the region of $10^{-4}-10^3$ s⁻¹. Using these arguments, it is perhaps surprising that the rate of reduction of αR237A ETF is severely compromised. However, as noted in our studies of electron transfer from TMADH to ETF (28), the structural rearrangement of ETF to form geometries compatible with electron transfer in an “induced fit” process with TMADH can become rate-limiting (especially with mutant forms of TMADH) for electron transfer.
The structural changes on complex formation can be followed conveniently by difference spectroscopy studies of the wild-type complex (18). For the wild-type complex, these spectral changes occur rapidly, i.e. within the mixing time (<10 s). Similar studies with native TMADH and the aR237A ETF also reveal that spectral changes accompany complex formation (Fig. 6). However, the time course for development of these spectral perturbations is protracted (~5 h), indicating that assembly of the productive electron transfer complex is severely compromised as a result of mutating aArg-237. These observations thus illustrate that the rate of rearrangement of ETF to form the electron transfer complex, and not the intrinsic rate (k_e) of endergonic electron tunnelling, is limiting for electron transfer from TMADH to aR237A ETF.

**DISCUSSION**

Developing a better understanding of the control of reduction potential of redox centers in proteins is pivotal to our appreciation of the mechanisms of biological electron transfer. Not surprisingly, the simple electron transfer proteins (e.g. the rubredoxins and flavodoxins) have proved to be tractable model systems for studies of this type. The control of reduction potential for large centers such as flavin is particularly challenging, given the number of potential interactions it can make with the protein. The large number of potential contacts between the protein and flavin probably accounts for the extensive range of potentials observed in flavoproteins. The structural simplicity of the flavodoxin family of flavoproteins has made them attractive models for establishing the relationship between oxidation-reduction properties of the flavin and its interactions with the apoprotein. The flavodoxins shuttle between the semiquinone and hydroquinone states, and they exhibit the lowest reduction potentials among the flavoprotein family, with values recorded as low as ~60 mV compared with ~124 mV for FMN in aqueous solution (29). Detailed potentiometric, mutagenesis, and structural studies have highlighted the role of key hydrogen bonding interactions with the flavin N(5) (30, 31) and N(3) (32), the role of aromatic residues close to the flavin isoalloxazine ring (33, 34), and conformational dynamics (35, 36) in modulating the potential of the one-electron couple in flavodoxins.

In this paper we have initiated a study of the control of redox potential in another simple flavoprotein, ETF. ETF is an attractive model system because of the exceptionally high potential of the oxidized-semiquinone couple (cf. the flavodoxins, where this couple is exceptionally low in potential) and the known large scale conformational dynamics of ETF, which probably affect the redox properties of this protein. The midpoint reduction potential of the E'_1 (oxidized-semiquinone) couple of *M. methylotrophus* (sp. W_{24}) ETF is the most positive couple for any known flavoprotein. Identification of the environmental effects around the flavin isoalloxazine ring that contribute to this extreme stabilization of the flavin anionic semiquinone is of major interest in terms of understanding the mechanism and from the viewpoint of engineering novel flavoenzymes with altered redox properties. Human ETF, which is highly related to *M. methylotrophus* ETF, does not stabilize the flavin semiquinone to anywhere near the same extent (Table I). In *P. denitrificans* ETF, the E'_1 (oxidized-semiquinone) couple is not widely separated from the E'_2 (semiquinone-dihydroquinone) couple, and the value of the two-electron oxidation-reduction potential has been determined as ~21 mV (11). The small difference in reduction potentials between the human and *Paracoccus* proteins reflects the close similarity of the flavin surroundings apparent in the crystallographic structures of these proteins (10, 11). Indeed, based on detailed sequence alignments of ETFs from different species, the flavin environment in ETFs is remarkably conserved across species (11). On the basis of the conservation of aArg-237 in all ETFs and the wide variation in measured potentials, Freeman and co-workers (37) have suggested that this residue is more likely to be essential to the basic chemistry of ETFs, rather than a modulator of potentials. Our mutagenesis work with *M. methylotrophus* ETF suggests that this may not be the case, because aArg-237 clearly plays a major role in modulating the potential of the E'_1 couple. However, the counterpart residue in human ETF (aArg-249) is not sufficient to elevate the potential of the E'_1 couple to the extent found in *M. methylotrophus* ETF. In searching for other determinants of redox potential, notable differences are apparent in the flavin environment of *M. methylotrophus* ETF and human ETF (Fig. 1). The human residues aAsn-259, aThr-266, bTyr-16, and bPro-40 are exchanged for lysine, serine, leucine, and glutamate, respectively, in *M. methylotrophus* ETF. Our data reveal that aR237A can more than account for the ~130-mV difference in the midpoint potential of E'_1 couples of human and *M. methylotrophus* ETFs. However, human ETF also contains an arginine at this position (as does *P. denitrificans* ETF). The possibility arises, therefore, that exceptional stabilization of the semiquinone in *M. methylotrophus* ETF may also require the flavin contact residues aLys-247, aSer-254, bLeu-13, and bGlu-37 in addition to aArg-237.

The role of flavin contact residues in human ETF in determining the values of the E'_1 and E'_2 couples has been investigated (37). aArg-249, the counterpart of aArg-237 in *M. methylotrophus* ETF, has been exchanged for Lys. This exchange leads to an ~60-mV destabilization of the flavin semiquinone (Table I). This destabilization of the E'_1 couple probably reflects a shortening of the side chain and thus the movement of the positive charge away from the dimethylbenzene subnucleus of the FAD, potentially to form a salt bridge with aAsp-253. The same mutation destabilizes the E'_2 couple by 80 mV, which contrasts with the >220-mV stabilization of the E'_2 couple in *M. methylotrophus* ETF following introduction of the aR237A mutation (Table I). Mutation of the other flavin contact residues, bY16A and aD253A, in human ETF produced only modest perturbations in the potentials of the E'_1 and E'_2 couples (37). Our modeling suggests that the kinetic
block of \textit{M. methylotrophus} ETF arises (at least in part) from an interdomain salt bridge between \textit{oArg}-237 and \textit{bGlu}-37 (which cannot form in human and \textit{P. denitrificans} ETF, where the corresponding residue is a Pro). This salt bridge might be broken upon reduction from FAD semiquinone to FAD dihydroquinone and thus also provides a possible explanation as to why reduction to the FAD dihydroquinone is relatively facile in \textit{oArg}-237A, unlike in the wild type. The \textit{~200-mV} destabilization of the FAD semiquinone in \textit{oArg}-237A could also be explained in terms of this interdomain salt bridge; in \textit{oArg}-237A the (stabilizing) positive charge is removed from the \textit{si} face of the flavin, and a (destabilizing; \textit{bGlu}-37) negative charge is effectively introduced. \textit{oArg}-237 clearly plays a key role in stabilizing the FAD semiquinone in \textit{M. methylotrophus} ETF. However, because an equivalent residue is present in other ETFs, other factors must give rise to the exceptional stabilization of the FAD semiquinone in \textit{M. methylotrophus} ETF. The modeling suggests a possible explanation for this; the positive charge on \textit{oArg}-247 (which is Asn in human and \textit{P. denitrificans} ETF) lies <6 Å away from the \textit{si} face of the pyrimidine subnucleus of FAD and could therefore stabilize the negative charge that builds up in the pyrimidine subnucleus on reduction of the flavin to the anionic semiquinone. The suggested role of \textit{oArg}-247 in the exceptional stabilization of the FAD semiquinone will need to be explored in future work.

The potentiometric and other reductive titrations we have performed highlight the importance of structural movement in the ETF protein, because kinetic as well as thermodynamic factors affect the reduction of the flavin. These data are entirely consistent with our \textit{x-ray} solution scattering studies of wild-type ETF that indicate that the molecule is highly dynamic (20) and that it populates an ensemble of conformations in which domain II (the flavin-binding domain) rotates around two hinge regions at its interface with domains I and III. By predicting the inferred (from our structural model of \textit{M. methylotrophus} ETF) salt link between \textit{oArg}-237 and \textit{bGlu}-37, a key restraint on domain II motion may be removed. This should enable \textit{oArg}-237A ETF to more readily explore these alternative conformations, thus perturbing the distribution between the different ETF conformers. The \textit{oArg}-237A ETF is clearly less kinetically restricted on being reduced to the dihydroquinone level, supporting this hypothesis.

An unusual feature of the flavin environment in human and \textit{P. denitrificans} ETF is the presence of an intraflavin hydrogen bond between the ribityl 4’ hydroxyl and the flavin N1 (10, 11). The role of this novel hydrogen bond in stabilizing the potential of the flavin has been probed by exchanging FAD for 4’-deoxy-FAD in human ETF (38). By incorporating 4’-deoxy-FAD in human ETF a destabilization of the oxidized-semiquinone couple by 0.116 V is observed, indicating that the novel hydrogen bond stabilizes the flavin semiquinone. In this altered form of human ETF, reduction to the dihydroquinone is slow and incomplete, suggesting a kinetic block on full reduction, analogous to that seen with \textit{M. methylotrophus} ETF, and to a lesser extent, pig liver ETF (13, 14). The kinetic limitation on reduction to the dihydroquinone probably reflects conformational differences in protein structure that occur during reduction of the FAD. Whether \textit{M. methylotrophus} ETF contains the unusual intraflavin hydrogen bond is uncertain and must await a crystallographic determination of the structure of the protein, but the potential absence of such a bond may account (at least in part) for the kinetic limitation on achieving full reduction in wild-type ETF. The interplay between residue \textit{oArg}-237 and possible interactions between the 4’-hydroxyl and flavin N1 atom in providing a kinetic block on full reduction to the dihydroquinone will need to be explored in future work. However, it is clear from the analyses presented in this paper that mutation of \textit{oArg}-237 to Ala goes a long way in relieving the kinetic limitation on full reduction of \textit{M. methylotrophus} ETF.

Concluding Remarks—Mutagenesis of \textit{oArg}-237 in \textit{M. Methylotrophus} ETF results in an \textit{~200-mV} destabilization of the \textit{E’} \textit{couple of the protein-bound FAD}, which is an unprecedented large perturbation in the midpoint reduction potential of a flavin redox center facilitated by a single point mutation. Exchange of this arginine residue partially removes a kinetic block and causes a large elevation (>280 mV) of the \textit{E’} \textit{couple, resulting in our ability to reduce the \textit{oArg}-237A ETF completely to the dihydroquinone (in the presence of mediators) with relatively little formation of the anionic semiquinone that becomes fully populated in wild-type ETF titrations. Our work underlines the unusual properties of ETF and the role of a single residue near the flavin in controlling the dynamic and redox properties of the molecule.