The Role of Tyr-169 of Trimethylamine Dehydrogenase in Substrate Oxidation and Magnetic Interaction between FMN Cofactor and the 4Fe/4S Center*

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Tyr-169 in trimethylamine dehydrogenase is one component of a triad also comprising residues His-172 and Asp-267. Its role in catalysis and in mediating the magnetic interaction between FMN cofactor and the 4Fe/4S center have been investigated by stopped-flow and EPR spectroscopy of a Tyr-169 to Phe (Y169F) mutant of the enzyme. Tyr-169 is shown to play an important role in catalysis (mutation to phenylalanine reduces the limiting rate constant for bleaching of the active site flavin by about 100-fold) but does not serve as a general base in the course of catalysis. In addition, we are able to resolve two kinetically influential ionizations involved in both the reaction of free enzyme with free substrate (as reflected in $k_{\text{lim}}/K_{d}$) and in the breakdown of the $E_{ox}$/$E_{\text{red}}$ complex (as reflected in $k_{\text{lim}}$). In EPR studies of the Y169F mutant, it is found that the ability of the Y169F enzyme to form the spin-interacting state between flavin semiquinone and reduced 4Fe/4S center characteristic of wild-type enzyme is significantly compromised. The present results are consistent with Tyr-169 representing the ionizable group of $pK_a \approx 9.5$, previously identified in pH-jump studies of electron transfer, whose deprotonation must occur for the spin-interacting state to be established.

Trimethylamine dehydrogenase (TMADH, EC 1.5.99.7), an iron-sulfur containing flavoprotein from the bacterium Methylobacterium extorquens (sp. W 3A1), catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde. The enzyme is a homodimer, and each subunit contains an unusual covalently linked 6-S-cysteinyl FMN cofactor and a bacterial ferredoxin-type 4Fe/4S center, as well as 1 equivalent of tightly bound ADP of unknown function (1–6). The physiological electron acceptor of TMADH is an electron-transferring flavoprotein, a 62-kDa heterodimer containing 1 equivalent each of FAD (7) and AMP (8). Electron-transferring flavoprotein is thought to oxidize reduced TMADH in two successive one-electron steps, cycling between the quinone and (anionic) semiquinone oxidation states. The availability of a high resolution structure for TMADH (6) and the cloned and overexpressed gene for the enzyme (10, 11) has made it possible to examine many aspects of the reaction mechanism by conventional site-directed mutagenesis. These have included studies of the role of (i) the 6-S-cysteinyl FMN in catalysis (11–13), (ii) cation–π bonding in substrate recognition (14), and (iii) residues on the surface of TMADH involved in electron transfer to electron-transferring flavoprotein (15).

The reaction of TMADH with trimethylamine exhibits three sequential kinetic phases (16–18): a fast phase that represents bleaching of the 6-S-cysteinyl FMN, an intermediate phase that reflects intramolecular electron transfer from dihydroflavin to the 4Fe/4S center to generate the flavin semiquinone and reduced 4Fe/4S center, and a slow phase that involves formation of an unusual spin-interacting state of the enzyme in which the unpaired magnetic moments of the reduced 4Fe/4S center and flavin semiquinone are strongly ferromagnetically coupled (18–21).

In the crystal structure of TMADH, Tyr-169 lies in van der Waals contact with the pyrimidine ring of the flavin cofactor, and is hydrogen-bonded to His-172 (which is also in van der Waals contact with the flavin). In order to ascertain the catalytic significance of Tyr-169 in TMADH, we have isolated a Y169F mutant enzyme and analyzed its kinetic behavior. We find that mutation of this residue to phenylalanine reduces the limiting rate constant for flavin reduction by a factor of approximately 100, but does not function as an active site base. The mutation also significantly reduces the ability of the flavin semiquinone to interact magnetically with the reduced 4Fe/4S center of the two-electron reduced enzyme. This is due to a substantial decrease in the equilibrium amount of enzyme possessing flavin semiquinone and reduced 4Fe/4S center, presumably by perturbing the semiquinone/hydroquinone half-potential of the active site flavin, and a decrease in the magnetic interaction between the centers in mutant enzymes possessing this electron distribution. The result suggests that Tyr-169 in all likelihood represents the ionizable group of $pK_a \approx 9.5$, previously identified in pH-jump studies of electron transfer (22), whose deprotonation must occur for the spin-interacting state to be established.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—Complex bacteriological media were from Unipath and all media were prepared as described by Sambrook et al. (23). Trimethylamine, 2,6-dichlorophenoldiphenol, phenazino methosulfate, tetramethylammonium chloride (TMAC), and all buffers were from Sigma. Sodium dithionite was obtained from Virginia Chemicals. Perdeuterated trimethylamine HCl (99.7% D) was from CK Gas Products Ltd. All other chemicals were of analytical grade where possible. Wild-type TMADH was purified from M. methylotrophus as described...
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Fig. 1. Steady-state kinetic analyses of the reaction of wild-type and Y169F TMADH with trimethylamine. Open circles, data for wild-type TMADH; closed circles, data for Y169F TMADH. Data for the wild-type were fitted using the equation described by Falzon and Davidson (30); Y169F data were fitted using the standard Michaelis-Menten equation.

Fig. 2. Reduction of the 6-S-cysteinyl FMN in Y169F TMADH at different pH values, as monitored by the decrease in absorbance at 443 nm. TMADH (4 μM) was mixed with trimethylamine in 100 mM buffer of the appropriate pH (see “Experimental Procedures”) at 5 °C. Kinetic transient A is fitted to the expression for a monophasic reaction (Equation 1) and transients B and C are fitted to a biphasic expression (Equation 2). Substrate concentrations were: transient A, 5 mM; transient B, 20 mM; transient C, 100 mM. Inset, plot of contribution made by the fast phase to the total amplitude change at 443 nm versus solution pH. Data were fitted to the equation describing a single ionization (p Ka, 6.2 ± 0.2).

incomplete flavinylation in this mutant protein to facilitate direct comparisons in the data. UV/visible spectra of each sample were recorded before and after each addition using a special spectrophotometer cell holder, which accommodates EPR tubes. Samples were then thoroughly mixed and slowly frozen by hand in liquid nitrogen. EPR spectra were recorded at both half- and high-field (in separate sweeps) for each sample.

Kinetic Measurements—Steady-state kinetic measurements were performed with a 1-cm light path in a final volume of 1 ml. The desired concentrations of trimethylamine, phenazine methosulfate, and 2,6-dichlorophenolindophenol were obtained from stock solutions to the assay mixture. Assays were performed using an Applied Photophysics SX.17MV stopped-flow spectrophotometer. All data were collected at 30 °C. Data were fitted to the appropriate rate equation using the fitting program Grafit (28).

Rapid kinetic experiments were performed using an Applied Photophysics SX.17MV stopped-flow spectrophotometer. Time-dependent reduction of TMADH by trimethylamine at pH 6.5 and 7.0 was performed using a photodiode array detector. Spectral deconvolution was performed by global analysis and numerical integration methods using PROKIN software (Applied Photophysics). For single wavelength studies, data collected at 443 nm were analyzed using nonlinear least
concentrations (up to 45 mM; Fig. 1). The kinetic parameters
substrate inhibition was seen even at very high substrate con-
steady-state kinetics of wild-type TMADH exhibit excess sub-
Data were then fitted to obtain related
$s$
and 6-fold lower, respectively, than those determined for the
The observed rate constants were found to exhibit hyperbolic depend-
the kinetic behavior. To explore this possibility, the reaction
wild-type enzyme (13.7 ± 1.7 μM and 15.6 ± 2.4 s⁻¹, respecti-
the reaction with trimethylamine. The reaction of Y169F TMADH
The Reaction of Y169F TMADH with TMA—Reduction of the
results suggest that Tyr-169 plays only a relatively small role in the overall catalytic efficiency of the
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FIG. 3. Deconvoluted spectra for the Y169F TMADH in the

FIG. 4. pH dependence of flavin reduction in Y169F TMADH. A, plot of limiting electron transfer rate constant, $k_{lim}$ as a function of pH. Data are fitted to the expression for a double ionization. Macroscopic $pK_a$ values are 6.7 ± 0.2 and 9.5 ± 0.3. B, plot of $k_{lim}/K_d$ as a function of pH. Data are fitted to the expression for a double ionization. Macro-

steady-state analyses—As reported previously, the steady-state kinetics of wild-type TMADH exhibit excess sub-
exhibit well behaved steady-state behavior: no evidence for
up to 45 mM; Fig. 1). The kinetic parameters $K_{TMA}$ and $K_{mut}$ for the mutant protein are 63 ± 3 μM and 2.6 ± 0.03
s⁻¹ at pH 8.5 and 30 °C, which are approximately 5-fold higher and 6-fold lower, respectively, than those determined for the

The observed rate constants were found to exhibit hyperbolic depend-
substrate concentration and the reaction sequence was modeled
as shown in the general scheme,

\[ K_d \]

\[ TMADH_{ox} + TMA \rightleftharpoons TMADH_{ox} \cdot TMA \rightleftharpoons TMADH_{red} + P \]  

Data were then fitted to obtain related $K_d$ and $k_{lim}$ values using $k_{obs} = k_{lim}[S]/(K_d + [S])$ (29).

RESULTS

Steady-state Kinetic Analyses—As reported previously, the
steady-state kinetics of wild-type TMADH exhibit excess sub-
substrate inhibition was seen even at very high substrate concen-
upto 45 mM; Fig. 1). The kinetic parameters $K_{TMA}$ and $K_{mut}$ for the mutant protein are 63 ± 3 μM and 2.6 ± 0.03
s⁻¹ at pH 8.5 and 30 °C, which are approximately 5-fold higher and 6-fold lower, respectively, than those determined for the
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Table I

Limiting rate and equilibrium constants for wild-type and Y169F TMADH reductive half-reactions at 25 °C, pH 7.0, and pH 6.5

For Y169F TMADH at pH 7.0, buffer conditions were 20 mM potassium phosphate to enable expression of the biphasic nature of the 443 nm transients throughout the entire range of substrate concentration. All other data are for enzyme contained in 100 mM potassium phosphate at the respective pH value.

|         | Wild-type | Y169F (fast phase) | Y169F (slow phase) |
|---------|-----------|--------------------|-------------------|
|         | $k_{\text{lim}}$ | $K_d$ | $k_{\text{lim}}$ | $K_d$ | $k_{\text{lim}}$ | $K_d$ |
| pH 7   | $s^{-1}$  | $\mu M$ | $s^{-1}$  | $\mu M$ | $s^{-1}$  | $\mu M$ |
| TMA    | 903 ± 50 | 6.6 ± 0.5 | 43.0 ± 1.7 | 34.7 ± 3.4 | 5.4 ± 0.26 | 38.0 ± 4.3 |
| Perdeuterated TMA | 185 ± 21 | 12.4 ± 1.8 | 5.7 ± 0.17 | 49.4 ± 3.3 | 0.74 ± 0.048 | 43.3 ± 7.4 |
| TMA and ferricenium | 596 ± 58 | 5.4 ± 0.8 | 46.9 ± 2.8 | 30.6 ± 5.1 | 11.3 ± 0.8 | 40.4 ± 6.9 |
| Inactivated enzyme | 679 ± 44 | 26.9 ± 2.5 | 9.0 ± 0.8 | 55.5 ± 9.9 | 0.092 ± 0.004 |
| pH 6.5 | $s^{-1}$  | $\mu M$ | $s^{-1}$  | $\mu M$ | $s^{-1}$  | $\mu M$ |
| TMA    | 97.5 ± 7 | 31.6 ± 2.5 | 1.37 ± 0.14 | 79.0 ± 17 | 0.0286 ± 0.005 |

* Rates were independent of substrate concentration. Data shown are the average of all rates measured over the range of substrate concentration investigated (0 to 120 mM trimethylamine).

substrate concentration over which biphasic kinetic behavior is seen (probably by influencing a kinetically relevant ionization; see below) rather than influencing the limiting rate of flavin reduction. An analysis of the amplitudes for each of the two phases seen at pH 7.0 and below indicates that the slower kinetic phase becomes increasingly prominent as pH decreases. The two kinetic phases also become more clearly resolved, principally due to a decrease in the rate constant for the slower phase. The pH dependence of the amplitudes for the two phases indicates the presence of a kinetically influential ionization of apparent pK_a 6.2 ± 0.2 (Fig. 2, inset). While there will be small differences in ionic strength across the pH range used for the determination of this value, these are not expected to compromise the analysis significantly.

Spectral Changes Associated with the Reaction of Y169F TMADH with Substrate—The reductive half-reaction of wild-type TMADH with TMA is triphasic (18). However, at the end of this half-reaction, the distribution of the two electrons derived from substrate in the enzyme is affected by pH (22, 32): at high pH (pH 7.5 and above), formation of flavin semiquinone and reduced 4Fe/4S center is favored; at low pH (e.g. pH 6.5), dihydroflavin and oxidized 4Fe/4S center is preferred, reducing the overall kinetics to nearly monophasic behavior. Similar pH effects on electron distribution were also seen for the Y169F TMADH. To simplify the analysis of the absorbance change associated with flavin reduction in Y169F TMADH, the experiment was performed at pH 6.5, thereby effectively eliminating the two slower phases associated with intramolecular electron transfer observed at higher pH values.

Upon completion of the faster phase of flavin reduction the spectrum resembles that of a mixture of oxidized enzyme and enzyme in the dihydroflavin form (Fig. 3). Following completion of the slower phase, the spectrum is that of the dihydroflavin form. The spectral form seen at the end of the faster phase rules out a sequential two-step reduction process involving a flavin semiquinone intermediate since this would give rise to a characteristic flavin semiquinone spectrum.2 At 1000 s after initial mixing of enzyme with substrate, the observed spectrum indicates that electron transfer to the 4Fe/4S center is indeed far from complete (data not shown). To further confirm that internal electron transfer to the 4Fe/4S center is not implicated in the biphasic behavior seen here, the 4Fe/4S center in the enzyme was selectively inactivated by ferricenium-PF_6 (see “Experimental Procedures”), which is known to render the 4Fe/4S center redox inert. The reductive half-reaction of the modified enzyme was studied at pH 6.5, 7.0, and 10.0. Again, the reaction is biphasic at pH 6.5 and 7.0 and monophasic at pH 10.0, as seen in the untreated Y169F TMADH, while ferricenium-PF_6 inactivated wild-type enzyme exhibits monophasic behavior at all pH values examined (data not shown).

Substrate Concentration Dependence and Kinetic Isotope Effects for Substrate Oxidation—The substrate concentration dependence of flavin reduction with Y169F TMADH has been investigated at pH 7.0 (using 20 mM phosphate buffer so that the biphasic behavior could be resolved throughout the entire substrate concentration range) and pH 6.5 (using 100 mM buffer).3 At pH 7.0, both phases for flavin reduction in Y169F TMADH exhibit hyperbolic dependence on [TMA]. The limiting rate constant for the faster phase (43 s^{-1} ± 1.7) was about 21-fold less than that seen with wild-type enzyme (903 s^{-1} ± 50) (Table I). The dissociation constants calculated for the two phases seen with the mutant protein are 35 ± 3.4 and 38 ± 4 mM, respectively, considerably larger than that seen with wild-type TMADH (6 mM, Table I). At pH 7.0, both phases were found to be sensitive to a kinetic isotope effect of approximately 7, as seen with wild-type TMADH, when perdeuterated TMA was used (Table I) indicating that the observed kinetics involve C-H bond cleavage. At pH 6.5, the faster phase of the reaction exhibits hyperbolic dependence on [TMA], with $k_{\text{lim}}$ and $K_d$ of 9 ± 0.8 s^{-1} and 55 ± 10 mM, respectively. The slower phase, however, is essentially independent of [TMA] over the concentration range studied (except in the very low substrate concentration regime), with an observed rate constant of 0.03 s^{-1}. There is also an associated loss of primary kinetic isotope effect on the reaction (Table I), indicating that cleavage of the C-H bond is no longer rate-limiting. Given the additional observation that the slow phase of the reaction seen at pH 7.0 is significantly slower than $k_{\text{cat}}$, we have not pursued the nature of this slow phase further in the present work.

Kinetically Influential Ionizations in Y169F—The reaction of trimethylamine is predominantly protonated at the pH values employed here and the wide substrate concentration range used in the experiments (0 to 120 mM), control experiments have been performed in the absence and presence of 0.2 mM potassium chloride to study the effect of ionic strength on kinetics. Additionally, in separate experiments and at selected pH values (6.5, 7.0, and 7.5), ionic strength was kept constant by balancing the substrate and potassium chloride concentrations over the entire substrate concentration range studied. In all cases, the values for the limiting rate constants for both phases and enzyme-substrate dissociation constants were found to be identical (within experimental error). The data therefore demonstrate that ionic strength influences only the relative spectral change associated with each of the two kinetic phases (presumably by perturbing the apparent pK_a of about 6.2) and does not affect the observed rate constants for each phase or the corresponding dissociation constant for the $E_S$ complex.
Y169F TMADH with trimethylamine has been investigated as a function of pH at 5 °C. The pH dependence of $k_{\text{lim}}$ seen with Y169F enzyme reveals two reasonably well resolved $pK_a$ values ($pK_a$ 6.7 ± 0.2 and 9.5 ± 0.3; Fig. 4A), whereas only one ionization is observed for the wild-type enzyme (18). A plot of $k_{\text{lim}}/K_a$ versus pH gives a bell-shaped curve (Fig. 4B), as seen in wild-type enzyme (18), with two $pK_a$ values of 9.7 ± 0.1 and 11.0 ± 0.1 attributable to the ionization of free enzyme and free substrate ($pK_a$ of TMA is 9.81), respectively. As in the case of wild-type enzyme, substrate is found to bind preferentially in the cationic form (14, 18). Comparison of the pH profiles for Y169F and wild-type enzymes indicates that the $pK_a$ values of Tyr-169 are slightly perturbed on mutating Tyr-169 to Phe. However, all kinetically influential ionizations seen in the wild-type enzyme remain in the Y169F mutant enzyme, indicating that Tyr-169 in the wild-type enzyme either does not ionize over the pH range investigated, or that its ionization is not kinetically influential for flavin reduction.

**UV/Visible Spectra of Y169F TMADH**—The UV/visible absorption spectra for the oxidized and substrate-reduced forms of wild-type and Y169F TMADH, along with the corresponding [oxidized] minus [substrate-reduced] difference spectra are shown in Fig. 5. Oxidized wild-type protein exhibits an $\Delta A_{344}$ of 0.8 absorbance ratio of about 1.3, whereas that for the Y169F TMADH gives a ratio around 1.03 due to incomplete flavinylation when expressed in *Escherichia coli*. Interestingly, the absorption change elicited by reduction with excess substrate for this mutant protein is different from that seen with wild-type protein under the same conditions (which we have previously shown to be identical in native and recombinant wild-type enzyme; Ref. 11). In particular, the difference maximum at 365 nm (reflecting accumulation of the flavin semiquinone form) is absent in the mutant. The observed spectral change seen with the Y169F mutant is in fact quite reminiscent of that generated by reduction of wild-type protein to the two-electron reduced level using sodium dithionite at pH 8.0, where the enzyme principally contains flavin semiquinone and reduced 4Fe/4S center but their magnetic moments do not interact (22). This interpretation is further supported by the EPR spectroscopic studies discussed below. The implication is that the distribution of reducing equivalents between the 4Fe/4S and flavin centers in the two proteins are different; a larger portion of Y169F TMADH exists as flavin hydroquinone and oxidized 4Fe/4S center rather than flavin semiquinone and reduced 4Fe/4S center, especially at pH 7.0.

Full reduction of wild-type TMADH, which requires 3 reducing equivalents, is observed when titrated with sodium dithionite, however, the enzyme takes up only two electrons when reduced with excess substrate or reduced by sodium dithionite in the presence of TMAC (a substrate analog and inhibitor of the enzyme cannot be reduced by substrate. In determining the spectrum seen with the two forms of the enzyme, as only that portion of the mutant protein possessing flavin can become reduced by substrate. All discussion and conclusions here are based on these substrate-induced difference spectra and not on the absolute spectra themselves. Use of substrate as reductant ensures that the entirety of the spectral change seen with the mutant arises from enzyme that possesses the full complement of redox-active cofactors, as the deflavo form of the enzyme cannot be reduced by substrate. In determining the concentration of the Y169F protein, we use an effective extinction coefficient that gives the concentration of the fully functional, flavin-containing portion of the enzyme, not simply the total concentration of polypeptide. In both Figs. 5 and 6, spectra of native and Y169F enzyme are presented that have been normalized on a per-flavin basis so that a direct comparison can be made in extinction coefficient and EPR intensity.
TMADH) (19, 31): binding of the substrate analog perturbs the reduction potential of the flavin semiquinone/hydroquinone couple such that full reduction of the enzyme does not occur. When Y169F TMADH is reduced with sodium dithionite in the presence of TMAC at pH 7.0, however, the final difference absorption spectrum resembles that for three-electron reduction of wild-type enzyme (data not shown). This indicates that full reduction has occurred, consistent with the EPR studies described below. The results indicate that the oxidation-reduction properties of the mutant protein are perturbed and that the ability of the Y169F mutant to form the spin-interacting state is compromised.

**DISCUSSION**

Tyr-169 is one of three amino acids comprising a novel Tyr-His-Asp triad in the active site of TMADH. Our data for the Y169F mutant clearly indicate that C-H bond cleavage and FMN reduction occur in the mutant enzyme, albeit at a limiting rate that is approximately 100-fold slower than is seen with

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**Fig. 6.** EPR spectra of wild-type and Y169F TMADH at pH 7.0. A, half-field EPR spectrum of substrate-reduced wild-type TMADH. B, high-field EPR spectrum of substrate-reduced wild-type TMADH. C, half-field EPR spectrum of substrate-reduced Y169F TMADH. D, high-field EPR spectrum of substrate-reduced Y169F TMADH. E, half-field EPR spectrum of Y169F TMADH reduced with sodium dithionite in the presence of TMAC. F, high-field EPR spectrum of Y169F TMADH reduced with sodium dithionite in the presence of TMAC.
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wild-type enzyme. Tyr-169 is in van der Waals contact with the flavin isoalloxazine ring, and local adjustments in active site structure (both physical and electronic) as a result of the mutation are likely to be responsible, at least in part, for the slower rates observed in the flavin reduction of Y169F TMADH. The pH-dependence profiles for Y169F are similar to those for wild-type enzyme, indicating that Tyr-169 is not the group whose ionization facilitates substrate oxidation and/or substrate binding in wild-type enzyme. However, ionization of substrate is controlled by an additional ionization with pKα of 6.2 in Y169F TMADH, not observed in the wild-type enzyme. At pH values below this pKα, enzyme reduction occurs as two kinetically resolvable steps, only the faster of which appears to be catalytically significant. The identity of the amino acid residue responsible for the additional ionization (pKα value 6.2) that controls the expression of the biphasic reductive transients in Y169F at low pH remains to be determined, however, it cannot be His-172 (which H-bonds to Tyr-169 in wild-type enzyme), since recent studies of a mutant H172Q TMADH suggest that ionization of this residue occurs around pH 8.5. Similarly, this work indicates that none of these residues is likely to be involved in abstraction of a proton from substrate to form a carbanion intermediate (18). Indeed the base catalysis accounts for only a quite modest portion of the enzyme-catalyzed rate acceleration for substrate oxidation (17, 18). The lack of an obvious base to support a carbanion mechanism provides indirect support for homolytic C-H bond cleavage analogous to the mechanism that has been proposed for the mechanism of monoamine oxidase (as discussed in Ref. 18).

The present work clearly demonstrates that Tyr-169 also plays an important role in mediating the spin-interaction between the flavin semiquinone and reduced 4Fe/4S center in TMADH. Previous work has shown that formation of the spin-interacting state of TMADH is governed by a basic residue located at or near the active site, with pKα value around 9.5 (22), and our results implicate Tyr-169 as this basic residue. Although Tyr-169 lies opposite the flavin ring from the iron-sulfur in TMADH, the importance of this residue in forming the spin-interaction between the two centers can be rationalized in the context of the x-ray crystal structure of TMADH (6). Tyr-169 is located near the C(2) = O group of the flavin isoalloxazine ring and its van der Waals surface is in contact with that of the flavin ring. When a negative charge is developed on the hydroxyl group of Tyr-169 side chain, due to electrostatic repulsion the unpaired electron density on the flavin isoalloxazine ring is reasonably expected to be forced to redistribute away from this residue toward the 4Fe/4S center, effectively reducing the spin-spin distance. This may also induce a larger dipole moment on the flavin isoalloxazine ring, which could be important in promoting the formation of the spin-interacting state.

The present work also indicates that the reduction potential of the semiquinone/hydroquinone flavin couple is perturbed in the Y169F enzyme, as reflected in the shift in oxidation-reduction equilibrium inferred from the UV/visible spectra. This is supported by the steady-state kinetic study demonstrating that substrate inhibition is absent in the Y169F TMADH, and the spectroscopic studies showing that full reduction of Y169F is achieved with dithionite even in the presence of TMAC. As described in Ref. 18, substrate inhibition in wild-type TMADH is accounted for by perturbation of the semiquinone/hydroquinone flavin couple upon substrate binding to partially reduced enzyme. Potentiometric studies on Y169F will soon help to further illustrate this point.

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REFERENCES

1. Steenkamp, D. J., and Mallinson, J. (1976) Biochim. Biophys. Acta 429, 705–719
2. Hill, C. L., Steenkamp, D. J., Holm, R. H., and Singer, T. P. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 547–551
3. Steenkamp, D. J., Kenney, W. C., and Singer, T. P. (1978) J. Biol. Chem. 253, 2812–2817
4. Steenkamp, D. J., McIntyre, W. S., and Kenney, W. C. (1978) J. Biol. Chem. 253, 2818–2824
5. Kasprazak, A. A., Papas, E. J., and Steenkamp, D. J. (1983) Biochem. J. 211, 535–541
6. Lim, L. W., Shamala, N., Mathews, F. S., Steenkamp, D. J., Hamlin, R., and Xuong, N. (1986) J. Biol. Chem. 261, 15140–15146
7. Steenkamp, D. J., and Gallup, M. (1978) J. Biol. Chem. 253, 4086–4089
8. Dupertuis, R. R., Rohlis, R. J., Hille, R., and Thorpe, C. (1994) Biochem. Mol. Biol. Int. 32, 195–199
9. Deleted in proof
10. Boyd, G., Mathews, F. S., Packman, L. C., and Scrutton, N. S. (1992) FEBS Lett. 308, 271–276
11. Scrutton, N. S., Packman, L. C., Mathews, F. S., Rohlis, R. J., and Hille, R. (1994) J. Biol. Chem. 269, 13942–13950
12. Huang, L., Scrutton, N. S., and Hille, R. (1996) J. Biol. Chem. 271, 13401–13406
13. Mewies, M., Bastran, J., Hille, R., and Scrutton, N. S. (1997) Biochemistry 36, 7162–7168
14. Bastran, J., Mewies, M., Mathews, F. S., and Scrutton, N. S. (1997) Biochemistry 36, 13899–13903
15. Wilson, E. K., Huang, L., Sutcliffe, M. J., Mathews, F. S., Hille, R., and Scrutton, N. S. (1997) Biochemistry 36, 41–48
16. Steenkamp, D. J., and Beinert, H. (1982) Biochem. J. 207, 241–252
17. Rohlis, R. J., and Hille, R. (1994) J. Biol. Chem. 269, 30869–30879
18. Jang, M. H., Bastran, J., Scrutton, N. S., and Hille, R. (1999) J. Biol. Chem. 274, 13147–13154
19. Steenkamp, D. J., Singer, T. P., and Beinert, H. (1978) Biochem. J. 169, 361–369
20. Steenkamp, D. J., Beinert, H., McIntyre, W. S., and Singer, T. P. (1978) in Mechanisms of Oxidizing Enzymes (Singer, T. P., and Ondarza, R. N., eds) pp. 127–141, Elsevier North-Holland Inc., New York
21. Singer, T. P., Steenkamp, D. J., Kenney, W. C., and Beinert, H. (1980) in Flavins and Flavoproteins (Yagi, K., and Yamamoto, T., eds) pp. 277–287, Japanese Scientific Societies Press, Tokyo
22. Rohlis, R. J., and Hille, R. (1991) J. Biol. Chem. 266, 15244–15252
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24. Wilson, R. K., Mathews, F. S., Packman, L. C., and Scrutton, N. S. (1995) Biochemistry 34, 2584–2591
25. Packman, L. C., Mewies, M., and Scrutton, N. S. (1995) J. Biol. Chem. 270, 13186–13195
26. Mewies, M., Packman, L. C., Mathews, F. S., and Scrutton, N. S. (1996) Biochem. J. 317, 267–272
27. Kasprazak, A. A., Papas, E. J., and Steenkamp, D. J. (1983) Biochem. J. 211, 535–541
28. Leatherbarrow, R. J. (1990) “Graftit” version 2.0, Ehrlich Software Ltd., Staines, United Kingdom
29. Strickland, S., Palmer, G., and Massey, V. (1975) J. Biol. Chem. 250, 4048–4052
30. Falzen, L., and Davidson, V. L. (1996) Biochemistry 35, 2445–2452
31. Steenkamp, D. J., and Beinert, H. (1982) Biochem. J. 207, 233–239
32. Rohlis, R. J., Huang, L., and Hille, R. (1995) J. Biol. Chem. 270, 22196–22207

5 J. Basran, M. J. Sutcliffe, R. Hille, and N. S. Scrutton, unpublished data.