Prototrophic Control of Intramolecular Electron Transfer in Trimethylamine Dehydrogenase*

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Ronald J. Rohlf, Liuxin Huang, and Russ Hille†
From the Department of Medical Biochemistry, Ohio State University, Columbus, Ohio 43210

The pH dependence of static optical/EPR spectra of trimethylamine dehydrogenase reduced to the level of two equivalents (TMADH$_{2eq}$) has been examined and indicates the existence of three different states for this iron-sulfur flavoprotein. At pH 6, TMADH$_{2eq}$ exists principally in a form possessing flavin mononucleotide hydroquinone, with its iron-sulfur center oxidized. At pH 8, the enzyme principally contains flavin mononucleotide semiquinone and reduced iron-sulfur, but despite the proximity of the two centers to one another, their magnetic moments do not interact. At pH 10, TMADH$_{2eq}$ exhibits the EPR spectrum that is diagnostic of a previously characterized spin-interacting state in which the magnetic moments of the flavin semiquinone and reduced iron-sulfur center are strongly ferromagnetically coupled. The kinetics of the interconversion of these three states have been investigated using a pH jump technique in both H$_2$O and D$_2$O. The observed kinetics are consistent with a reaction mechanism involving sequential protonation/deprotonation and intramolecular electron transfer events. All reactions studied show a normal solvent kinetic isotope effect. Proton inventory analysis indicates that at least one proton is involved in the reaction between pH 6 and 8, which principally controls intramolecular electron transfer, whereas at least two protons are involved between pH 8 and 10, which principally control formation of the spin-interacting state. The results of these and previous studies indicate that for TMADH$_{2eq}$ between pH 10 and 6, at least three protonation/deprotonation events are associated with intramolecular electron transfer and formation of the spin-interacting state, with estimated pH$_a$ values of 6.0, 8.0, and 9.5. These pH$_a$ values are attributed to the flavin hydroquinone, flavin semiquinone, and an undesignated basic group on the protein, respectively.

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† To whom correspondence and reprint requests should be addressed.
Tel.: 614-292-4964; Fax: 614-292-4118.
‡ The abbreviations used are: TMADH, trimethylamine dehydrogenase; TMADH$_{2eq}$, trimethylamine dehydrogenase reduced to the level of 2 equivalents/subunit; FMN, flavin mononucleotide; 4Fe/4S, four iron-four sulfur center; FeS$_{2eq}$, oxidized iron-sulfur center; FeS$_{red}$, reduced iron-sulfur center; EPR, electron paramagnetic resonance; FMNH$_2$, FMN hydroquinone.

1 Since the two subunits of trimethylamine dehydrogenase appear to behave independent of each other, a single subunit will be referred to as one enzyme throughout this paper.
2 L. Huang and R. Hille, unpublished results.
Preparation of TMADH$_{2eq}$ and D$_2$O Solutions—In order to prepare two-electron reduced trimethylamine dehydrogenase for pH(D) jump measurements, concentrated samples of oxidized enzyme were passed through a Sephadex G-25 column (1.5 × 25 cm) equilibrated with a 10 mM solution of an appropriate buffer and adjusted to the desired initial pH. Enzyme in D$_2$O was obtained by passage through a separate Sephadex G-25 column equilibrated with D$_2$O buffer (initial swelling of the dried gel material having also been performed with D$_2$O). 10 mM buffers adjusted to the initial pH(D) also contained 0.1 M potassium chloride, 0.5 μM phenazine ethosulfate, and 0.5 μM benzyl viologen. Potassium chloride was included to maintain solvent ionic strength throughout the experiment. The dyes were included to facilitate initial enzyme reduction and, at the low concentrations used, have been shown to have no effect on the observed kinetics (11). Solutions of 0.1 M buffer at the desired mol fraction of D$_2$O and desired final pH(D) were added to 20-ml glass syringes, bubbled for 30 min with anhydrous, O$_2$-free argon, and sealed with rubber septa. For experiments in mixed solvent, enzyme was diluted with the appropriate buffer to give the desired mol fraction D$_2$O and a final enzyme concentration of 50–100 μM (25–50 μM dimers) and placed with a tonometer equipped with a ground glass joint for the dithionite titration syringe, a side arm cuvette, and a three-way stopcock valve with a male Luer connector. The sample was made anaerobic by repeated evacuation and flushing with anhydrous, O$_2$-free argon, after which the tonometer was fitted with an anaerobic syringe containing a dithionite solution, and the enzyme was titrated to the level of two equivalents/subunit with sodium dithionite. No differences were observed between enzyme solutions prepared in D$_2$O and used immediately and enzyme solutions prepared in D$_2$O and allowed to stand overnight before use.

Earlier studies have shown that the bisulfite product of dithionite oxidation reacts with oxidized trimethylamine dehydrogenase at pH = 7 (11). As an alternative to dithionite, low pH enzyme samples used in optical/EPR measurements were reduced with titanium citrate solutions (see below). This method of reduction is inappropriate for the preparation of pH jump samples; however, since titanium citrate requires strongly buffered (0.1–0.2 M) citrate solutions to remain in solution. The bisulfite adduct problem was avoided in the earlier pH jump study by first reducing the enzyme in 1 mM borate buffer, pH 10, and then bringing the solution to 10 mM phosphate at the desired initial pH by the addition of concentrated phosphate buffer (11). However, for the purposes of this study, the complexity of this procedure would likely lead to H$_2$O contamination of D$_2$O solutions and was not used. Since formation of the bisulfite adduct renders the FMN redox-inert, any enzyme molecules that have reacted with bisulfite (estimated to be no greater than 10–20% in any case) are assumed not to contribute to the absorbance change on the timescale of the pH(D) jump experiment and merely add to the total background absorbance. Any substantial breakdown of a sulfite adduct under the present experimental conditions would be expected to give rise to extremely slow spectral changes in the absence of such slow spectral changes in the transients observed in the course of the work presented here indicates that sulfite adduct formation presents a negligible problem in the present studies.

Rapid Reaction Studies—Rapid reaction studies were carried out as described previously, using a stopped-flow apparatus whose calibrated dead time was determined to be 600 μs (11). Time courses were fitted to either single or double exponential expressions (ΔA(t) = ΔA∞ exp(−kt) + ΔA) using an iterative nonlinear least squares Levenberg-Marquardt algorithm (17) for the parameters ΔA∞, and k, representing the total absorbance change and observed rate constant, respectively, exhibited by the nth kinetic phase.

Optical/EPR Titrations—Optical/EPR titrations were performed using an aerobically cuvette equipped with a quartz observation cell and a port sealed with a rubber septum. Anaerobic samples of oxidized enzyme were prepared at the desired pH through the use of a Sephadex G-25 column and titrated with reductant as described above. For samples at pH > 8, sodium dithionite was used as the reductant; enzyme samples at pH < 8 were reduced with titanium citrate according to Zehnder et al. (18) by anaerobic addition of a 1.9 M solution of TiCl$_3$ in 2.0 M hydrochloric acid (from Aldrich) to an appropriate volume of 0.1 M sodium citrate (from Pierce), followed by adjustment of the pH to 7.0. After reduction to an appropriate level, 400-μl enzyme samples were removed using a long needle Hamilton syringe and placed in quartz EPR tubes which had been previously flushed with O$_2$-free argon and finally frozen in liquid nitrogen. X-band EPR spectra were recorded at 25 K using a Bruker ER 300 EPR spectrometer equipped with a ER035M gaussmeter and a Hewlett-Packard 5352B microwave spectrometer.
frequency counter. Instrument parameters are given in the appropriate figure legends. A total of 20–50 40-second scans were accumulated for each sample to improve the signal-to-noise ratio. EPR signal intensities due to flavin semiquinone and reduced iron-sulfur were determined by double integration of spectra using Bruker Instruments software. Intensities of the half-field signal indicative of the spin-interacting state were determined using the peak-to-trough amplitude of the signal at \( g = 4 \).

RESULTS

pH Dependence of TMADH\(_{2eq}\) Optical/ EPR Spectra—Optical/EPR reductive titrations of trimethylamine dehydrogenase at pH 6 and pH 10 have established the pH dependence of the intramolecular electron distribution between the FMN and 4Fe/4S centers in partially reduced enzyme (11). It has also been shown that the FMN semiquinone seen in partially reduced enzyme ionizes over this pH range; the neutral form is observed at pH 6, and the anionic form is observed at pH 10. The visible absorbance spectrum for TMADH\(_{2eq}\) at pH 10 exhibits absorbance peaks at 365, 440, and 510 nm (Fig. 1, – – – – ), and is nearly identical to that seen at pH 8.0 with enzyme reduced by excess substrate or by dithionite in the presence of the inhibitor trimethylammonium chloride (7, 8, 11). Previous work has shown that this three-banded spectrum arises from the form of the enzyme that exhibits the complex high field EPR spectrum and half-field (\( g = 4 \)) signal indicative of the spin-interacting state (Fig. 2, A and B; Refs. 5–12). Since these EPR features reflect magnetic interaction of the unpaired electron spins on the two centers, TMADH\(_{2eq}\) at pH 10 must possess (anionic) flavin semiquinone and a reduced iron-sulfur center. At pH 6, on the other hand, the reductive titration data (11) indicate that much of the flavin exists as the hydroquinone in TMADH\(_{2eq}\) (Fig. 1, – – – – ). At no time during the pH 6 reductive titration are the EPR features characteristic of the spin-interacting state observed.

The visible absorbance spectrum of TMADH\(_{2eq}\) at pH 8 is intermediate between that seen at pH 6 and 10, showing some of the features present in the pH 10 spectrum and suggesting that two-electron reduced enzyme contains mostly flavin semiquinone and reduced iron-sulfur center at pH 8 (Fig. 1, – – – – ). However, the features at 365 and 440 nm are not as well defined at pH 8 as they are at pH 10, and the peak at 510 nm seen in the pH 10 spectrum is virtually absent at pH 8, indicating that a significant difference exists in TMADH\(_{2eq}\) at these two pH values. These differences appear to reflect changes in ionization of the flavin semiquinone and, to a lesser degree, a shift in the electron distribution toward further iron-sulfur reduction at the higher pH. Differences between TMADH\(_{2eq}\) at pH 8 and 10 is most pronounced when examined by EPR. At pH 10, the EPR spectrum of TMADH\(_{2eq}\) reflects formation of the spin-interacting state (Fig. 2, A and B). At pH 8, however, the high-field region of the EPR spectrum reflects a simple combination of flavin semiquinone and reduced 4Fe/4S signals (Fig. 2C), and no half-field feature indicative of spin-interaction is observed (Fig. 2D). EPR spectra of samples at pH 8 were recorded at several microwave power levels in order to facilitate quantitation of these signals (the flavin semiquinone readily power saturates at the low temperatures required to observe the reduced 4Fe/4S center). Spin integration of the axial flavin semiquinone and rhombic 4Fe/4S

Fig. 2. EPR spectra of TMADH\(_{2eq}\) at pH 10.0 and 8.0. Enzyme in 0.1 M potassium borate, pH 10.0, or 0.1 M sodium pyrophosphate, pH 8.0, buffer containing 0.5 \( \mu \) M benzyl viologen was reduced with dithionite to 2 equivalents/subunit. EPR parameters were as follows: microwave frequency, 9.4955 GHz; microwave power, 1.00 milliwatts; modulation amplitude, 10.084 gauss; 15 K. Panel A is the high field region of the spectrum of TMADH\(_{2eq}\) at pH 10.0. Panel B is the half-field region of the spectrum of TMADH\(_{2eq}\) at pH 10.0. Panel C is the high field region of the spectrum of TMADH\(_{2eq}\) at pH 8.0. Panel D is the half-field region of the spectrum of TMADH\(_{2eq}\) at pH 8.0.
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The pH dependence of the EPR half-field signal intensity and extinction coefficient at 365 nm. Panel A, relative g = 4 signal intensity is plotted versus the pH at which the experiments were performed. The filled circles represent the amount of g = 4 signal observed at a given pH, normalized to the amount seen at pH 10.0. The solid line represents a fit of the data to a single pK\textsubscript{a} expression, yielding a value of 9.4. Panel B, the maximum extinction coefficient at 365 nm (filled circles) during reductive titration of TMADH with dithionite (at pH < 8.0) is plotted versus pH. The filled circles represent the extinction coefficients. The solid line represents a fit of the data to a double pK\textsubscript{a} expression, yielding values of 8.0 and 9.7.

EPR signals for TMADH\textsubscript{2eq} at pH 8 extrapolated to very low microwave power (<1 microwatts, so as not to saturate the flavin semiquinone signal) confirms a 1:1 stoichiometry, i.e., an electron distribution in which one reducing equivalent is on the FMN and one is on the iron-sulfur center. This important result indicates that an intramolecular electron distribution consisting of a flavin semiquinone and a reduced iron-sulfur center is necessary, but not sufficient, to produce the spin-interacting form of the enzyme.

One or more ionizable groups with pK\textsubscript{a} values > 8 must be responsible for induction of the spin-interacting state in TMADH\textsubscript{2eq} on raising the pH from 8 to 10. The pH dependence of the g = 4 signal intensity at 15 K is shown in Fig. 3A, and indicates that formation of the spin-interacting state of the enzyme is controlled by a single ionizable group exhibiting a pK\textsubscript{a} of 9.4. In an effort to correlate formation of the spin-interacting state with ionization of the neutral flavin semiquinone and to determine directly the pK\textsubscript{a} for ionization of the semiquinone, the pH dependence of the absorbance at 365 nm of TMADH\textsubscript{2eq} has been examined. The anionic form of flavin semiquinone displays an absorbance band at this wavelength (19), and the case of trimethylamine dehydrogenase is relatively uncomplicated by the spectral contribution of its iron-sulfur center. The pH dependence of the 365 nm absorbance in TMADH\textsubscript{2eq} is shown in Fig. 3B, where it is evident that the absorbance grows in over too great a pH range to be attributable to a single ionization. A fit of the data using a two-pK\textsubscript{a} equation suggests that full formation of the semiquinone anion requires the ionizations of two groups exhibiting pK\textsubscript{a} values of 8.0 and 9.7, the latter being within experimental error of that determined from the pH dependence of the g = 4 signal. One of these ionizations must derive from the N-5 position of the flavin semiquinone, while the other presumably derives from another site in the protein, possibly hydrogen-bonded or otherwise associated with the iron-sulfur center. The data indicate that three conditions must be met for formation of the spin-interacting state in TMADH\textsubscript{2eq}: 1) the distribution of reducing equivalents within the active site must give flavin semiquinone and reduced iron-sulfur center; 2) the semiquinone must be ionized; and 3) a third group within the active site must also be deprotonated. All of these requirements are met at pH 10, but only the first (and possibly the second, to a greater or lesser extent) is met at pH 8.

pH(D) Jump Kinetics—(High pH) minus (low pH) kinetic difference spectra for TMADH\textsubscript{2eq} show maxima at 365, 410, and 520 nm (11) that agree well with the static absorbance changes reported in Fig. 1. This indicates that it should be possible to separately monitor intramolecular electron transfer and formation/decay of the spin-interacting state of TMADH\textsubscript{2eq} subsequent to a pH jump. Accordingly, pH(D) jump experiments between pH(D) 8 and 6 (following principally intramolecular electron transfer between the flavin and iron-sulfur center), between pH(D) 10 and 8 (principally following formation of the spin-interacting state), and between pH(D) 10 and 6 (which monitors both processes) have been undertaken. Time courses for the 10 → 6 and 10 → 8 pH jump reactions with TMADH\textsubscript{2eq}, as carried out by stopped-flow in H\textsubscript{2}O, exhibit monophasic behavior and are successfully fit using a single exponential expression (Fig. 4, A and B). The kinetic behavior and fitted rate constants obtained are consistent with the results of previous work (11), with k\textsubscript{obs} = 440 s\textsuperscript{-1} and 420 s\textsuperscript{-1} for the 10 → 6 and 10 → 8 pH jump reactions, respectively (all rate constants with standard deviations are summarized in Table I). These values are independent of observation wavelength within the uncertainty associated with these measurements. In D\textsubscript{2}O, the 10 → 6 pD jump reaction also exhibits well behaved monophasic kinetics with k\textsubscript{obs} = 56 s\textsuperscript{-1} (Fig. 4C). A comparison of the observed rate constants in H\textsubscript{2}O and D\textsubscript{2}O yields an observed solvent kinetic isotope effect of 7.9 for the pH(D) 10 → 6

signal intensity of the EPR signal at 150 K decreases significantly above pH 9 as the spin-interacting state accumulates, suggesting that the magnetic interaction between the unpaired electron spins of the flavin semiquinone and reduced iron-sulfur cluster (manifested directly in the EPR signal at 15 K) modulates the signal intensity of the EPR signal of the flavin radical observed at 150 K. The upshot is that the pK\textsubscript{a} of the flavin semiquinone cannot be sufficiently accurately determined from EPR line widths for the present purposes.

The rate constants presented with the kinetic time courses in Fig. 4, A–M, represent the average of at least six independent measurements for a given set of conditions. The errors associated with these values were determined as the standard deviation of the mean. In most cases, this uncertainty is approximately 20–25% of the value of the rate constant. However, the error (expressed as a percentage of the value of the rate constant) increases for kinetic time courses, which exhibit either extremely large fitted rate constants (e.g. Fig. 4, E and F) or a low signal-to-noise ratio due to a small absorbance change against a large background absorbance (e.g. 365 nm traces in Fig. 4, I and L). In these cases, the uncertainty may increase to approximately 40% of the value of the associated observed rate constants (see also Table I in Ref. 11).
Fig. 4. Time courses observed for the TMADH_{eq} pH(D) jump reaction in 100% H$_2$O and 100% D$_2$O. Samples of two-electron reduced trimethylamine dehydrogenase were prepared, and stopped-flow rapid mixing experiments were performed as described under "Material and Methods." The final enzyme concentrations range from 50 to 100 μM. Absorbance changes observed at 365, 410, and 520 nm after mixing are plotted versus time. The solid lines represent fits of the data to exponential expressions. The values of the observed rate constants for each wavelength shown in the figure represent the average of at least six independent measurements at each wavelength for a given set of conditions. Panel A, pH 10 → 6 (100% H$_2$O). Data are fitted to a single exponential expression. Panel B, pH 10 → 8 (100% H$_2$O). Data are fitted to a single exponential expression. Panel C, pD 10 → 6 (100% D$_2$O). Data are fitted to a single exponential expression. Panel D, pD 10 → 8 (100% D$_2$O). Data collected at time t ≥ 20 ms are fitted to a single exponential expression in order to account for the lag phase. Panel E, pH 6 → 10 (100% H$_2$O). Data are fitted to a single exponential expression. Panel F, pH 8 → 10 (100% H$_2$O). Data are fitted to a single exponential expression. Panel G, pD 6 → 10 (100% D$_2$O). Data are fitted to a double exponential expression, and the fraction of absorbance change attributed to each kinetic phase is given next to the value of the observed rate constant. Panel H, pD 8 → 10 (100% D$_2$O). Data are fitted to a double exponential expression, and the fraction of absorbance change attributed to each kinetic phase is given next to the value of the observed rate constant. Panel I, pH 8 → 6 (100% H$_2$O). Data
reaction.

By contrast to the well behaved monophasic behavior exhibited by the reactions described above, the 10 → 8 pH jump reaction with TMADH<sub>2eq</sub> in D<sub>2</sub>O exhibits complex kinetic behavior (Fig. 4D). The time courses show a distinct lag phase at all three wavelengths monitored, followed by monophasic kinetic behavior. Ignoring the lag phase, rate constants for these time courses have been obtained by fitting the data points collected at times ≥20 ms after mixing to a single exponential expression. The results (Fig. 4D, solid lines) give an observed rate constant for the 10 → 8 pH jump reaction of 60 s<sup>-1</sup>. Comparison with the corresponding rate constant in H<sub>2</sub>O gives an observed solvent kinetic isotope effect of 7.0 for the pH(D) 10 → 8 reaction. The emergence of the lag phase in the 10 → 8 pH jump time courses as the mol fraction of D<sub>2</sub>O present in the solvent increases (not shown) suggests a multi-step mechanism for loss of the spin-interacting state on jumping the pH(D) from 10 to 8.

pH jump experiments in the reverse direction (i.e. pH(D) 6 → 10 and pH(D) 8 → 10) have also been performed. When performed in H<sub>2</sub>O, the results are consistent with those obtained from the previous trimethylamine dehydrogenase pH jump study (11), with observed time courses that exhibit fitted rate constants of 1000 s<sup>-1</sup> for the 8 → 10 reaction and 930 s<sup>-1</sup> for the 6 → 10 reaction. Again, well behaved monophasic time courses are observed, with wavelength-independent rate constants (Fig. 4, E and F). In D<sub>2</sub>O, however, the observed time courses are distinctly biphasic (Fig. 4, G and H), and a two-
exponential expression is required to fit both the 6 → 10 and 8 → 10 pD jump time courses satisfactorily (Fig. 4, G and H, solid lines). For the 6 → 10 pD jump reaction, the rate constant for the fast kinetic phase is 310 s⁻¹ (which contributes approximately half of the total observed absorbance change at all three wavelengths monitored). The rate constant obtained for the slow kinetic phase of the reaction, on the other hand, is wavelength-dependent, ranging from 76 s⁻¹ at 365 nm to 47 s⁻¹ at 410 nm. This behavior suggests that the slow phase of the reaction consists of multiple components having distinct spectral changes, but which are kinetically unresolved. Multiphasic kinetics makes the determination of an observed solvent kinetic isotope effect for the pH(D) 6 → 10 jump problematic, but the effect is clearly significant in the range of 3-15 (depending on whether rate constants from the fast or slow phase of the reaction in D₂O are used in the calculation). As in the case for the D₂O 10 → 8 pD jump experiment in D₂O, the multiphasic behavior exhibited by the 6 → 10 pD jump reaction suggests a complex reaction mechanism.

The 8 → 10 pD jump reaction also gives biphasic and wavelength-dependent time courses (Fig. 4H). At 365 and 520 nm, the fast phase of the reaction gives a rate constant of 260 s⁻¹ and accounts for about half of the total absorbance change observed at these wavelengths; the slow kinetic phase gives a rate constant of 46 s⁻¹. At 410 nm, the observed rate constant is 550 s⁻¹, significantly larger than those observed at 365 and 520 nm; the rate constant for the slow kinetic phase of the reaction is 65 s⁻¹. Again, the implication is that the spectral change associated with each of the two kinetic phases of the reaction consist of multiple components. Calculating the solvent kinetic isotope effect for the 8 → 10 pH(D) jump reaction is again complicated by the multiphasic kinetics observed in D₂O but is in the range of 4-15.

For the 8 → 6 and 6 → 8 pH jump experiments in H₂O, the results are significantly different than observed in the other pH jump experiments. In each of the other cases (pH 8 → 10, 10 → 8, 6 → 10, and 10 → 6) the time courses are monophasic and observed rate constants wavelength-independent for a given reaction. Wavelength-dependent rate constants are observed only in the biphasic low-to-high pH(D) jump time courses in D₂O (Fig. 4, G and H). In the case of the 8 → 6 pH jump in H₂O, simple monophasic behavior is observed, but the observed rate constants are wavelength-dependent, ranging from 500 s⁻¹ at 365-200 s⁻¹ at 410 nm (Fig. 4I). ⁶ For the 6 → 8 pH jump reaction, time courses at 410 and 520 nm are monophasic with an observed rate constant of 400 s⁻¹ (Fig. 4J). At 365 nm, however, no kinetic absorbance change is observed, although an increase is expected from the static difference spectrum (Fig. 1). It appears that the spectral change at this wavelength occurs so rapidly that the absorbance change is lost in the 600-µs dead time of the apparatus. Again, the implication is that there are multiple components to the overall spectral change associated with the reaction.

In all of the pH(D) jump experiments performed up to this point, absorbance decreases at 365 and 520 nm are observed along with an absorbance increase at 410 nm when the pH(D) is decreased and the reverse observed when the pH(D) is increased (Fig. 4; Ref. 11). In both the 8 → 6 and 6 → 8 pD jump experiments in D₂O, by contrast, the direction of absorbance change at 365 nm is in the same direction as that seen at 410 nm (Fig. 4, K and L) and opposite to that observed at 365 nm in the corresponding H₂O experiment. At 520 nm, the transient exhibits rise-fall behavior (Fig. 4M). For the 8 → 6 pD jump reaction, the 365-nm kinetic transients collected at intermediate D₂O mol fractions exhibit rise-fall behavior with contributions from absorbance changes in both directions (not shown). For the 6 → 8 pH(D) jump reaction, a discernible absorbance change is observed at 365 nm only in 100% D₂O, and is too small to permit a precise evaluation of the observed rate constant (Fig. 4L).

A multi-step kinetic scheme must be invoked to account for the above observations. It appears that absorbance changes at 365 nm due to very rapid reactions, which are lost in the dead time when performed in H₂O, become slow enough to observe in D₂O. As the mol fraction of D₂O increases, two distinct kinetic processes are observed with absorbance changes in opposite directions at both 365 and 520 nm. Given the nature of the experiments, the data most likely reflect a protonation/deprotonation event (which is too rapid to observe in H₂O), followed by subsequent intramolecular electron transfer.

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### Table I

| pH(D) jump (initial → final) | 365 nm | 410 nm | 520 nm |
|-------------------------------|--------|--------|--------|
|                               | k_{fast} | k_{slow} | k_{fast} | k_{slow} | k_{fast} | k_{slow} |
| 100% H₂O                     | 400 ± 30 | 100% D₂O | 420 ± 50 | 930 ± 200 | 997 ± 200 | 400 ± 90 |
| 6 → 10                        | 500 ± 110 | 200 ± 19 | 250 ± 9 |
| 100% D₂O                     | 56 ± 2 | 60 ± 3 | 290 | 47 |
| 6 → 8                         | 320 | 76 | 360 | 64 |
| 6 → 10                        | 260 | 45 | 550 | 65 |
| 8 → 10                        | 280 | 103 ± 12 | 260 | 47 |
| 8 → 6                         | 190 | 120 ± 8 | 170 | 23 ± 10 |

⁶ The difference between the values of the observed rate constants obtained at 410 nm (k_{fast} = 200 s⁻¹) and 520 nm (k_{slow} = 250 s⁻¹) for the 8 → 6 pH jump reaction considered significant as it is just beyond the limit of experimental error associated with these measurements.
function of the solvent mol fraction D$_2$O and plot the parameter versus mol fraction of D$_2$O (22, 23). The shape of such "proton inventory" plots is described by the Gross-Butler equation (24, 25), and it is possible in principle to determine the exact number of protons involved in the reaction mechanism in this way. The simplest distinction is made between a reaction mechanism involving a single proton, which yields a linear plot, or multiple protons, in which case the plot is bowed. However, the precision in experimental data required to make such a determination can be prohibitive (22), increasing as the overall solvent kinetic isotope effect decreases or as one tries to distinguish between mechanisms involving one- and two-, two- and three-, three- and four-proton, etc.

A proton inventory analysis for the pH jump experiments with TMADH$_{2eq}$ is straightforward only for the 10 → 6 pH(D) jump reaction, since this is the only case where the time courses exhibit monophasic kinetics and wavelength-independent observed rate constants at all solvent mol fractions of D$_2$O. In order to obtain rate constants for the 10 → 8 pH(D) jump reaction, it is necessary to ignore a plainly visible lag phase from the kinetic transients (Fig. 4D). In the case of the 8 → 6 and 6 → 8 pH(D) jump reactions, a proton inventory is possible if only the data obtained at 410 and 520 nm are considered. Finally, the 6 → 10 and 8 → 10 pH(D) jump reactions display increasingly biphasic time courses as the solvent mol fraction of D$_2$O increases, and neither the determination of an overall solvent kinetic isotope effect nor a proton inventory analysis is justified for these reactions.

With these limitations in mind, a proton inventory analysis has been performed for the 10 → 6, 10 → 8, 8 → 6, and 6 → 8 pH(D) jump reactions (Fig. 5). The proton inventory plot for the 8 → 6 pH(D) jump reaction is linear, consistent with the involvement of only a single proton in the reaction (Fig. 5A, circles); the overall observed solvent kinetic isotope effect is 1.7. The proton inventory plot for the pH(D) jump in the reverse direction (6 → 8) is also linear, with an observed overall solvent kinetic isotope effect of 3.2. While the relative error in these stopped-flow rapid mixing experiments, defined as the standard deviation of the mean of at least six determinations, is too great to discriminate between one- and two-proton mechanisms, it is clear that both reactions involve at least one proton.

For both the 10 → 6 and 8 → 6 pH(D) jump reactions, the proton inventory plots are distinctly convex downward (Fig. 5B). In both cases, the precision of the kinetic data are sufficient to conclude that a minimum of two protons are involved (22). This interpretation is consistent with the static optical/EPR titration results (see above), which suggest that there are at least two ionizable groups that exhibit pK$_a$ values > 8 and are important in controlling formation or breakdown of the spin-interacting state. The precision of the kinetic data is not high enough, however, to distinguish between two- and three-proton reaction mechanisms. Taken together, the proton inventory data indicate that there is at least one proton involved in the reaction mechanism between pH(D) 6 and pH 8, and at least two protons involved in the reaction mechanism between pH(D) 8 and 10. Thus there must be at least three protons involved in the overall pH(D) jump reaction mechanism between pH(D) 6 and 10.

**DISCUSSION**

The pH dependence of the static visible and EPR spectra from this and previous studies indicate that three identifiable states of TMADH$_{2eq}$ are most easily observed at pH 6, 8, and 10. At pH 6, TMADH$_{2eq}$ appears to consist principally in the form possessing flavin hydroquinone and oxidized iron-sulfur center. This is consistent with the pK$_a$ value of 6 estimated for the N-1 position of flavin hydroquinone from reductive half-reaction studies (26). At pH 8, TMADH$_{2eq}$ possesses mainly flavin semiquinone and reduced iron-sulfur center, but the magnetic moments of the unpaired spins do not interact at this pH. It is likely that pH 8 is close enough to the pK$_a$ value for the neutral/anionic flavin semiquinone equilibrium that significant amounts of both forms are present. Finally, at pH 10, trimethylamine dehydrogenase reduced by two equivalents possesses anionic flavin semiquinone and reduced iron-sulfur center, with the magnetic moments of the unpaired spins interacting strongly.

The data describe a situation in which the relative reduction potentials of FMN and the iron-sulfur center are such that an intramolecular electron distribution consisting of flavin hydroquinone and oxidized iron-sulfur is preferred at low pH, with the flavin hydroquinone predominantly protonated at the N-1 position of the isoalloxazine ring. Recent work on the reductive half-reaction of trimethylamine dehydrogenase with the alternative substrate diethylmethylamine suggests that the N-1 position of the flavin hydroquinone exhibits a pK$_a$ value of approximately 6 (26). This value is comparable with the pK$_a$ value for the N-1 position of free flavin hydroquinone, which has been shown to exhibit a pK$_a$ value near 6.5 (27). This value
is reasonably expected to be lowered if the protein structure causes strain that introduces a bend in the flavin about a line connecting atoms N-5 and N-10 of the isoalloxazine ring, as is known to be the case for trimethylamine dehydrogenase (28). As the pH increases, loss of this proton to produce flavin hydroquinone apparently decreases the reduction potential for the hydroquinone/semiquinone couple below that for the iron-sulfur center, so that by pH 8.0 the intramolecular electron distribution favors flavin semiquinone and reduced iron-sulfur center over the distribution consisting of anionic hydroquinone and oxidized iron-sulfur by a factor of approximately four (26). This distribution is reflected in the EPR spectrum of trimethylamine dehydrogenase reduced by two equivalents at pH 8, which appears to be a simple combination of an axial flavin semiquinone radical signal and a rhombic signal due to reduced iron-sulfur center (Fig. 2C). At pH 8, the FMN semiquinone appears to be present mostly in the neutral form, as judged by the linewidth of its EPR signal. A further increase in pH leads to ionization at the N-5 position of the flavin semiquinone (which exhibits a pK_a of 8.0) to give the semiquinone anion. The semiquinone anion is unable to become reduced by back electron transfer without uptake of a proton (to do so would give rise to the very unstable dianionic flavin hydroquinone), and at sufficiently high pH the distribution shifts further in favor of iron-sulfur reduction over formation of the flavin hydroquinone. The static optical/EPR titration and stopped-flow rapid mixing kinetic data support the existence of still another ionizable group within trimethylamine dehydrogenase, which exhibits a pK_a of approximately 9.5 (Fig. 3). Deprotonation of this ionizable group as the pH is increased from 8 to 10 results in interaction of the magnetic moments of the unpaired spins present on the flavin semiquinone anion and reduced iron-sulfur center. Thus at sufficiently high pH, TMADH_{2eq} is predominantly in the form giving rise to the spin-interacting state, as evidenced by the EPR signal exhibited by the enzyme (Fig. 2, A and B).

Low pH favors an electron distribution in TMADH_{2eq} in which the flavin is largely reduced and iron-sulfur center is oxidized, whereas high pH favors formation of the flavin semiquinone and reduced iron-sulfur center. This is consistent with the expected pH dependence of the three reduction potentials of the system, with the observed pH dependence of the intensity of the half-field EPR signal (attributable to the spin-interacting state in which the iron-sulfur center must be reduced), and with the intensity of the absorbance at 365 nm (attributable to the anionic flavin hydroquinone) that is observed with TMADH_{2eq}. The semiquinone form of FMN can exist in either the neutral (protonated) or anionic (deprotonated) form depending on the ionization state of the N-5 position of the isoalloxazine ring. Trimethylamine dehydrogenase has been shown to be unusual among flavoproteins in that it can accommodate either of these forms of flavin semiquinone depending on solvent pH, with a pK_a of approximately 8.0 (Fig. 3B; Ref. 11). Similarly, the hydroquinone of flavin can exist as either the neutral or anionic form depending on the ionization state of the N-1 position. The ionization of N-1 of the hydroquinone has been inferred from the pH dependence of the reductive half-reaction of trimethylamine dehydrogenase with diethylylmethylamine (26). The reduction potentials of both the partially and fully reduced forms of FMN increase with decreasing solvent pH since protonation of the isoalloxazine ring neutralizes a negatively charged electron. In the simplest formulation, the possible pH dependence of the iron-sulfur center reduction potential has not been considered. The reduction potential of the iron-sulfur center might also be expected to increase with decreasing solvent pH for the same reason (e.g. protonation of a site near the 4Fe4S cluster is expected to increase the reduction potential). However, in determining the pH dependence of TMADH_{2eq} intramolecular electron distribution, it is the relative reduction potentials of the two centers that are important. Since the results show that low pH favors flavin hydroquinone and oxidized iron-sulfur center, whereas high pH favors flavin semiquinone and reduced iron-sulfur center, the flavin reduction potential must increase relative to the iron-sulfur reduction potential with decreasing solvent pH.

The kinetic results presented here, particularly of the pH 6 → 8 and 8 → 6 experiments, indicate that the reequilibration of reducing equivalents within TMADH_{2eq} is a kinetically complicated process. We have previously reported both static and kinetic difference spectra for the 10 → 7 and 7 → 10 pH jumps (11). All of the spectral features observed in the pH 10 minus pH 7 static difference spectrum for TMADH_{2eq} are quantitatively reproduced in the corresponding kinetic difference spectra obtained from both the 10 → 7 and 7 → 10 pH jump reactions (although the two kinetic difference spectra are necessarily opposite in sign). Since the static and kinetic difference spectra agree so well in these experiments, we have concluded that there are no dead-time spectral changes in the course of these reactions. This contrasts with the results of the 6 → 8 pH jump experiments reported here, particularly in the region around 365 nm, where there is a substantial discrepancy between the kinetic spectral changes observed kinetically (Fig. 4) and those anticipated on the basis of the static spectra at these two pH values (Fig. 1, dashed and dotted lines), presumably due to a significant dead time spectral change in the kinetic experiments. The apparent discrepancy between the 7 → 10 and 6 → 8 experiments is resolved when one considers the difference in the state of TMADH_{2eq} at pH 6 versus pH 7; at pH 6 a substantial portion of the flavin present as the hydroquinone is protonated, whereas at pH 7 it exists predominantly as the anionic FMNH^- . If, during the low-to-high pH jump, intramolecular electron transfer must be preceded by the ionization of the N-1 position of flavin hydroquinone and this ionization exhibits a pK_a of 6 (both of these assertions are supported by recent studies of the reductive half-reaction; Ref. 26), then approximately 90% of TMADH_{2eq} at pH 7 already exists in the form (anionic flavin hydroquinone and oxidized iron-sulfur center) observed at the conclusion of the 6 → 8 pH jump reaction. In other words, the absorbance increase at 365 nm, which is lost in the dead time of the mixing apparatus in the 6 → 8 pH jump, is undetectable as a dead time spectral change in the 7 → 10 pH jump reaction since only 1% of the enzyme molecules undergo this process.

The multiphasic kinetic behavior and observation wavelength dependent rate constants observed in the 10 → 8, 8 → 10, and 6 → 10 100% D_2O pD jump time courses coupled with the moderate D_2O-dependent increase in absorbance change observed in the 8 → 6 and 6 → 8 100% D_2O pD jump time courses eliminate the possibility that the protonation/deprotonation and intramolecular electron transfer events occur concomitantly and indicate instead a reaction mechanism that entails discrete protonation/deprotonation and electron transfer steps. Scheme 1 represents the simplest overall reaction mechanism for prototropically controlled electron transfer within TMADH_{2eq} that is consistent with the known kinetic behavior of the enzyme.

The mechanism consists of discrete equilibria involving three prototropic equilibria and an intramolecular electron transfer step. Two of the three prototropic equilibria shown in Scheme 1 involve ionizations of the flavin N-1 of hydroquinone.
and position N-5 of the semiquinone. The third prototropic equilibrium is between the protonated and unprotonated forms of an as yet unidentified ionizable group whose ionization is required for formation of the spin-interacting state. The intramolecular electronic equilibrium involves electron transfer from anionic flavin hydroquinone to oxidized iron-sulfur center (to give neutral flavin semiquinone and reduced iron-sulfur center) and the reverse reaction.

In the course of a 10 → 6 pH jump experiment, the unknown group and the N-5 position of anionic flavin semiquinone are first protonated, disrupting the interaction of the unpaired spins and forming the neutral flavin semiquinone. The order of protonation might be the reverse of that shown in Scheme 1, or protonation of these sites may occur simultaneously. Initial protonation of the unknown group followed by protonation of the anionic flavin semiquinone as drawn, however, is consistent with the observation of a lag phase in the 100% D₂O 10 → 8 time courses, and also with the results of reductive half-reaction studies with the slow substrate diethylmethylamine, which indicate that formation of the spin-interacting state is kinetically distinct from intramolecular electron transfer, with little or no absorbance change associated with it (26). Subsequent to these two protonations, electron transfer from the iron-sulfur center to FMNH forms the anionic hydroquinone, which finally protonates (at sufficiently low pH) to give the neutral hydroquinone.

In principle, all of the active sites in a sample of TMADH₂eq will possess the neutral flavin hydroquinone at sufficiently low pH. At pH 6 (the lowest pH used in this and previous work and approximately the pKₐ of the hydroquinone) approximately 50% of the enzyme possesses the neutral form of the flavin hydroquinone (26). The remaining 50% of the active sites should consist of a mixture of (anionic flavin hydroquinone with oxidized iron-sulfur center), and (neutral flavin semiquinone and reduced iron-sulfur center) in a ratio of ~4:1. The visible absorbance spectrum of the enzyme species at the far right equilibrium of Scheme 1 gives Scheme 2, in which the several microscopic rate constants for the interconversion of all intermediates are given explicitly.

Since the pH remains constant subsequent to the pH jump, the time rate of change for [H⁺] is zero, and each of the protonation steps can be considered pseudo-first-order. Scheme 2 is of the form A ⇌ B ⇌ C ⇌ D. In situations in which the appearance of final product (either A or D depending on the direction of the pH jump) follows first-order kinetics, as is observed in the present pH jump reactions in H₂O, an expression can be derived for the observed first-order rate constant (kobs) as a function of pH and the microscopic rate constants that describe the three equilibria (making a steady state approximation for the rate of change of intermediates B and C; i.e. dB/dt = dC/dt = 0). Making these assumptions, the observed rate constant for intramolecular electron transfer as a function of pH is given by:

\[
\frac{d[A]}{dt} = k_{1} \left[ \text{FMNH}_2 \right] - k_{2} \left[ \text{FMNH}_2 \right] + H^+ \\
\frac{d[B]}{dt} = k_{3} \left[ \text{FMNH}_2 \right] - k_{4} \left[ \text{FMNH}_2 \right] + H^+ \\
\frac{d[C]}{dt} = k_{5} \left[ \text{FMNH}_2 \right] - k_{6} \left[ \text{FMNH}_2 \right] + H^+ \\
\frac{d[D]}{dt} = k_{7} \left[ \text{FMNH}_2 \right] - k_{8} \left[ \text{FMNH}_2 \right] + H^+ \\
\frac{d[A]}{dt} = \text{constant}.
\]

\[
\text{Rate of change of } [A] = \text{constant}.
\]

\[
\text{Rate of change of } [B] = \text{constant}.
\]

\[
\text{Rate of change of } [C] = \text{constant}.
\]

\[
\text{Rate of change of } [D] = \text{constant}.
\]
of proton concentration is given by the following equation.\(^9\)

\[
\frac{k_{obs}}{k_{obs}} = \frac{k_1 k_5 + k_2 k_7 + k_3 k_8 + k_4 k_9}{k_1 + k_2 + k_3 + k_4 + k_5 + k_6 + k_7 + k_8 + k_9} \quad \text{(Eq. 1)}
\]

The rate constants for the protonation of the flavin hydroquinone (\(k_1\)) and semiquinone (\(k_2\)) anions are equal to the corresponding bimolecular association rate constants multiplied by the proton concentration (i.e., \(k_1 = k_{\text{hydroquinone}} \times [H^+]\) and \(k_2 = k_{\text{semiquinone}} \times [H^+]\)). The rate constants for deprotonation (\(k_3\) and \(k_4\)) are then defined in terms of the corresponding pseudo first-order protonation rate constants, the \(pK_a\) values of the appropriate flavin species, and the final pH value used in the experiment (i.e., \(k_3 = k_{\text{hydroquinone}} \times [H^+]\) and \(k_4 = k_{\text{semiquinone}} \times [H^+]\)).

The rate constants for electron transfer (\(k_5, k_6, k_7, k_8, k_9\)) are considered pH-independent parameters, reflecting the intrinsic rates of electron transfer from the reduced iron-sulfur center to the neutral semiquinone and from the anionic hydroquinone to the oxidized iron-sulfur center, respectively.

In fitting the \(k_{obs}\) versus final pH data to the above equation (Fig. 6, solid line), \(pK_a\) values of 6.0 for the flavin hydroquinone and 8.5 for the semiquinone were used. The former value is consistent with recent reductive half-reaction studies of trimethylamine dehydrogenase (26). In the case of the \(pK_a\) for the semiquinone, several attempts were made to fit the data using different values for the semiquinone \(pK_a\), and it was found that a value of 8.5 yielded the best result. This value is within the range established by the optical/EPR spectral data (11) and the present kinetic results. The lower limit for the rate constants associated with the intramolecular electron transfer steps (\(k_5, k_6, k_7, k_8, k_9\)) is 1000 s\(^{-1}\); fits of the \(k_{obs}\) versus pH data to Equation 1 at different fixed values of \(k_3, k_4, k_5, k_6, k_7, k_8, k_9\) below 1000 s\(^{-1}\) were unsatisfactory (data not shown). From the parameters giving the best fit to the pH dependence of \(k_{obs}\), the bimolecular rate constants for protonation of the hydroquinone and semiquinone are calculated to be 2.6 \(\times\) 10\(^{-1}\) M\(^{-1}\) s\(^{-1}\) and 1.0 \(\times\) 10\(^9\) M\(^{-1}\) s\(^{-1}\), respectively.

When considering the interrelationship between prototropic equilibria and intramolecular electron transfer in trimethylamine dehydrogenase, the protonation/deprotonation and electron transfer events may either occur concomitantly or as discrete chemical steps. Evidence for a discrete mechanism has been found in the kinetic and thermodynamic behavior of medium chain acyl-CoA dehydrogenase (29), while clear evidence for a concomitant mechanism has been found in the case of electron transfer within xanthine oxidase (30). The present results indicate that electron transfer within trimethylamine dehydrogenase operates via a mechanism involving discrete ionization and electron transfer steps. The kinetic transients under many reaction conditions are complex (with either lags or multiple phases); the proton inventories indicate multiple ionizations involved in a pH 6 \(\rightarrow\) 10 jump or the reverse and the pH dependence of the reaction in H\(_2\)O are distinctly nonlinear.

Xanthine oxidase, by contrast, exhibits well-behaved kinetics under all conditions, linear proton inventory plots indicative of the involvement of only a single proton in the electron transfer process, and a linear dependence of \(k_{obs}\) on pH. These results are not contradictory but are simply a reflection of different systems operating by different mechanisms.

Two factors in all likelihood combine to determine which type of mechanism is found in a given system. The first of these is the flavin redox couple participating in electron transfer; this is the quinone/semiquinone couple in xanthine oxidase and the semiquinone/hydroquinone couple in trimethylamine dehydrogenase. Since both semiquinone and hydroquinone, but not oxidized quinone, oxidation states of the flavin have ionizable protons, there is clearly greater likelihood for participation of multiple protons in the latter case than in the former. The second factor is the \(pK\) of the reduced form of the flavin that participates in the reaction. Thorpe and co-workers (29) point out that during the oxidation of a neutral semiquinone or hydroquinone, deprotonation to form the corresponding anionic species must precede electron transfer so as to avoid formation of an unfavorable cationic flavin species. Similarly, protonation of an anionic semiquinone should occur prior to reduction in order to avoid formation of an unfavorable dianionic hydroquinone. The protonation state of the flavin thus controls the kinetics as well as the thermodynamics of intramolecular electron transfer. For these reasons, a discrete mechanism for coupled proton/electron transfer might be expected for a given system in the absence of other considerations, as is observed in the cases of acyl-CoA dehydrogenase and trimethylamine dehydrogenase. However, should the polypeptide preferentially destabilize the deprotonated form of the flavin by virtue of its hydrogen bonding and other interactions with the isoalloxazine ring, then a discrete pathway might not necessarily reflect the lowest energy path from the initial to the final states in the electron transfer process, in which case concomitant electron/proton transfer is preferred. This is apparently the case with xanthine oxidase.

The present results extend previous work indicating that electron transfer from the flavin hydroquinone of trimethylamine dehydrogenase to the iron-sulfur center of the enzyme is quite fast and is not intrinsically rate-limiting in catalysis. Furthermore, evidence is found that formation of the spin-interacting state observed at the completion of the reaction of oxidized enzyme with substrate is governed by an ionizable group having a \(pK\) of approximately 9.5. Ionization of the flavin semiquinone to its anionic form also occurs in the course of formation of the spin-interacting state, with a \(pK\) of approximately 8.0. Given that only two redox-active centers are present in trimethylamine dehydrogenase, the enzyme might be considered a relatively simple system in which to examine electron transfer. Despite this apparent simplicity, the present results indicate a rather complicated reaction mechanism for the internal equilibration of reducing equivalents between the two centers that involves at least three discrete ionizations. These results combined with those obtained from other studies further indicate that two of these three ionizable groups are associated with the FMN coenzyme (in the semiquinone and hydroquinone oxidation state, respectively), while the third is

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\(^9\) We emphasize that this analysis is valid only for the results obtained in H\(_2\)O, where the observed transients are well represented as single-exponential processes. In addition, the data plotted in Fig. 6 are exclusively from experiments in which the initial pH was either 6 or 10, although the results of experiments jumping either to or from the intermediate pH 8 are not inconsistent with the proposed mechanism. It may seem paradoxical that the pH 6 \(\rightarrow\) 8 kinetics (\(k_{obs} \approx 400\) s\(^{-1}\)) are slower than for the pH 10 \(\rightarrow\) 10 experiment (\(k_{obs} = 1000\) s\(^{-1}\)), but it must be remembered that the observed kinetics are intrinsically dependent on the hydrogen ion concentration. In the pH 6 \(\rightarrow\) 10 experiment, there is no reason why the approach to the final state achieved at pH 8 cannot be faster than in the pH 6 \(\rightarrow\) 8 experiment, as is in fact empirically observed and also predicted by the model. For the reverse change in pH, it is difficult to rationalize a priori why the pH 8 \(\rightarrow\) 6 kinetics are slower than the pH 10 \(\rightarrow\) 6 kinetics at two of the three observation wavelengths followed here. Given the pronounced wavelength dependence of the observed rate constant in these experiments and the evidence presented for dead-time spectral changes, it is in any case not appropriate to include these data in the analysis of the pH dependence of \(k_{obs}\). We cannot exclude the possibility of a more complicated, perhaps branched mechanism, to quantitatively account for the observed pH jump kinetics to and from pH 8, but note that the results of these experiments are at least qualitatively consistent with the mechanism given in Schemes 1 and 2.
most likely associated with an amino acid residue located at or near the active site (11, 26).

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