Reaction of the C30A Mutant of Trimethylamine Dehydrogenase with Diethylmethylamine*

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The role played by the 6-S-cysteiny1-FMN bond of trimethylamine dehydrogenase in the reductive half-reaction of the enzyme has been studied by following the reaction of the slow substrate diethylmethylamine with a C30A mutant of the enzyme lacking the covalent flavin attachment to the polypeptide. Removal of the 6-S-cysteiny1-FMN bond diminishes the limiting rate for the first of the three observed kinetic phases of the reaction by a factor of 6, but has no effect on the rate constants for the two subsequent kinetic phases. The flavin in the C30A enzyme recovered from the reaction of the C30A enzyme with excess substrate is found to have been converted to the 6-hydroxy derivative, rendering the enzyme inactive. The noncovalently bound FMN of the C30A mutant enzyme is also converted to 6-hydroxy-FMN and rendered inactive upon reduction with excess trimethylamine, but not by reduction with dithionite, even at high pH or in the presence of the effector tetramethylammonium chloride. These results suggest that one significant role of the 6-S-cysteiny1-FMN bond is to prevent the inactivation of the enzyme during catalysis. A reaction mechanism is proposed whereby OH− attacks C-6 of a flavin-substrate covalent adduct in the course of steady-state turnover to form 6-hydroxy-FMN.

Trimethylamine dehydrogenase from the restricted facultative methylotroph Methylophilus methylotrophus is composed of two identical 83,000-Da subunits. Each subunit contains 1 eq of FMN and a ferredoxin-type 4Fe-4S cluster along with 1 eq of ADP of unknown function (1–6). The enzyme catalyzes the oxidation of FMN and a ferredoxin-type 4Fe-4S cluster with 1 eq of H2O. During the fast kinetic phase, the absorbance at 450 nm attributable to oxidized FMN is lost in a process that has been interpreted as giving rise to a covalent flavin-substrate adduct. The intermediate kinetic phase represents the breakdown of this covalent adduct and the subsequent rapid intramolecular electron transfer of one electron from the flavin hydroquinone thus formed to the iron-sulfur center. The slow kinetic phase has been interpreted as representing the dissociation of product followed by the binding of a second substrate molecule to the two-electron reduced enzyme, a process that coincides with the formation of the spin-interacting state (9). The oxidative half-reaction has also been studied using various forms of trimethylamine dehydrogenase and electron-transferring flavoprotein (10). It has been shown that electrons are transferred from trimethylamine dehydrogenase to electron-transferring flavoprotein via the iron-sulfur center of the former with a limiting rate constant of 172 s−1 (at pH 7.0 and 25 °C); this step is thus not rate-limiting in catalysis (10). As mentioned above, the FMN in trimethylamine dehydrogenase is covalently attached to Cys-30 via C-6 of the flavin ring by a 6-S-cysteiny1-FMN bond (1, 16, 17). When Cys-30 of the enzyme is replaced with alanine by site-directed mutagenesis, the mutant enzyme contains the full complement of 4Fe-4S and ADP, but only ~30% of the full complement of (now noncovalently bound) FMN (18, 19). To the extent that the C30A mutant possesses FMN, however, the mutant enzyme is catalytically active, albeit somewhat compromised. Steady-state kinetic experiments show that the removal of the 6-S-cysteiny1-FMN bond increases the apparent Km for trimethylamine by a factor of 100 and decreases the apparent kcat by a factor of 2 (18). The steady-state analysis, however, does not provide direct information about the role of the 6-S-cysteiny1-FMN bond in accelerating any of the specific steps of the reductive half-reaction. In this study, we have investigated the reaction of C30A mutant trimethylamine dehydrogenase with diethylmethylamine in order to examine in greater detail the role of the covalent flavin linkage in catalysis.

EXPERIMENTAL PROCEDURES

Enzyme Purification and Materials—Escherichia coli strain JM109 transformed with the plasmid pSV2mdvC30A was grown, and C30A mutant trimethylamine dehydrogenase was purified as described by Scrutton et al. (18). Enzyme concentrations were determined using an extinction coefficient of 197.1 M−1 cm−1 at 280 nm, which is calculated from native trimethylamine dehydrogenase. Routine enzyme assays were performed as described previously (9). Diethylmethylamine was purchased from Aldrich, and potassium phosphate, tetrasodium pyro
phosphate, trimethylamine chloride, FMN, and riboflavin were obtained from Sigma. Sodium dithionite was obtained from Virginia Chemicals. Ferricenium hexafluorophosphate was prepared as described by Lehman et al. (20, 21).

**Kinetic Experiments—**Pre-steady-state studies were carried out using a Kinetic Instruments stopped-flow apparatus equipped with an On-Line Instruments System Model 3020Z data collection system. Anaerobic solutions of oxidized C30A mutant trimethylamine dehydrogenase were prepared by alternately evacuating and flushing with O$_2$-free argon in a tonometer equipped with a side arm cuvette and a three-way stopcock valve possessing a male Luer connector. Anaerobic solutions of diethylmethylamine were prepared by bubbling buffer solution with O$_2$-free argon for 30 min in a 10-ml volume glass syringe and then injecting an appropriate volume of diethylmethylamine to give a final concentration of 20 mM. The glass syringe was equipped with a three-way valve to allow serial dilution of the diethylmethylamine stock solution thus prepared. The final concentrations of diethylmethylamine after mixing were between 1.25 and 10 mM, sufficient to ensure pseudo first-order conditions in the present stopped-flow experiments. Kinetic transients were obtained as transmittance voltage as a function of time, converted to absorbance change using On-Line Instruments System software, and fitted to the sum of exponentials using an iterative nonlinear least-squares Levenberg-Marquardt algorithm (22).

**RESULTS**

**Reductive Half-reaction Kinetics of C30A Trimethylamine Dehydrogenase with Diethylmethylamine—**To investigate the effect of removal of the covalent 6-S-cysteynl-FMN bond on the reductive half-reaction of trimethylamine dehydrogenase, the kinetics of the reaction of C30A mutant trimethylamine dehydrogenase with diethylmethylamine was examined. This slow substrate for native trimethylamine dehydrogenase has the advantage that several well resolved kinetic phases are observed in the course of its reaction with enzyme (9). As with native trimethylamine dehydrogenase, the reaction of the C30A mutant enzyme with diethylmethylamine at pH 8.0 exhibits three well resolved kinetic phases (Fig. 1). The transient observed at 362 nm consists of only the two slower kinetic phases (Fig. 1A), while that observed at 410 nm consists of the two faster kinetic phases (Fig. 1B); the transient observed at 450 nm contains all three kinetic phases (Fig. 1C). The observed rate constants for each kinetic phase, designated as $k_{fast}$, $k_{int}$, and $k_{slow}$, respectively, are each independent of observation wavelength. $k_{fast}$ exhibits hyperbolic dependence on the concentration of diethylmethylamine, and a fit of the data to the equation $k_{fast} = (k_{int}(S)/(K_d + S))$ (23) gives values for $k_{int}$ and $K_d$ of 80 s$^{-1}$ and 5.0 mM, respectively (Fig. 2). This $K_d$ (at pH 8.0) is within experimental error of the value seen with native trimethylamine dehydrogenase (4.3 mM) (9), whereas the value of $k_{int}$ exhibited by C30A at pH 8.0 is 6-fold smaller than that observed with the native enzyme. $k_{int}$ and $k_{slow}$ for the C30A mutant are both independent of diethylmethylamine concentration and have values of 6 and 0.2 s$^{-1}$, respectively, comparable to the values seen with the native enzyme (3.5 and 0.2 s$^{-1}$). At pH 6, $k_{int}/K_d$ for the fast kinetic phase is 0.05 mm$^{-1}$ s$^{-1}$, 40-fold smaller than that observed for the native enzyme at the same pH (2.1 mm$^{-1}$ s$^{-1}$), indicating that the removal of the 6-S-cysteynl-FMN bond has a comparatively larger effect as the pH is lowered.

**Spectra for Reaction Intermediates Seen in the Course of the Reaction of C30A Trimethylamine Dehydrogenase with Diethylmethylamine—**Spectra for intermediates seen in the course of the reaction of C30A trimethylamine dehydrogenase with diethylmethylamine (Fig. 3B) were calculated from the spectral changes associated with each of the three kinetic phases (Fig. 3A). The overall kinetic difference spectrum obtained from the stopped-flow experiment is consistent with the static difference spectrum observed upon the reduction of the C30A mutant enzyme with diethylmethylamine, indicating that there is no absorbance change in the dead time of the stopped-flow apparatus. The absorbance change associated with the fast kinetic phase is small below 380 nm, but is negative between 380 and 530 nm (Fig. 3A, closed circles). This spectral change seen with the C30A mutant is quite different from that associated with the fast phase seen with the native enzyme (9) and is typical of that observed upon reduction of normal FMN (as opposed to the 6-S-cysteynl-FMN found in the native enzyme). These results indicate that the fast phase represents bleaching of the enzyme FMN, presumably due to transient formation of a substrate-flavin covalent adduct as has been proposed with the native enzyme (9). The limiting rate constant for this process is 6-fold slower than that observed with the native enzyme, indicating

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1. The smaller limiting rate constant for the C30A mutant enzyme is not due to the 30% of the full complement of FMN because the deflavoenzyme is catalytically inert in these experiments.
that the formation of the covalent 6-S-cysteiny1-FMN bond facilitates this first step of the reaction, but this step is not rate-limiting.

The spectral change for the intermediate phase (Fig. 3A, open circles), which corresponds to intramolecular electron transfer from the flavin to the 4Fe-4S center, is also different from that seen with the native enzyme in the region of 390–550 nm, again due to changes in the absorbance spectrum of the flavin upon loss of the covalent linkage. The spectral deconvolution for the intermediate phase into components due to flavin hydroquinone → semiquinone and FeS red → FeS ox is possible because Δε for the latter process is well defined (10), and the result is shown in Fig. 3C. The difference spectrum for the 4Fe-4S center (Fig. 3C, closed triangles; taken from Ref. 10) is that observed upon reduction of phenylhydrazine-treated trimethylamine dehydrogenase, a modified enzyme that contains a redox-inert 4Fe-4S center (Fig. 3C, closed circles). The extinction change of the difference spectrum for the intermediate phase (Fig. 3C, closed circles) was obtained using a C30A mutant enzyme concentration of 9.6 μM and a 2-cm light path, taking into account the fact that only 30% of the C30A mutant enzyme contains flavin. The difference spectrum for one-electron oxidation of the reduced noncovalently bound FMNH2 (flavin semiquinone-flavin hydroquinone) (Fig. 3C, open circles) was obtained by subtracting the difference spectrum for the 4Fe-4S center from that for the intermediate phase. The difference spectrum thus obtained has absorbance maxima at 360 and 410 nm, analogous to the Δε seen for the semiquinone anion-hydroquinone of glucose oxidase (25), confirming that the flavin semiquinone formed with the C30A mutant enzyme is the anionic form, as is true of the native enzyme (26). The spectral change associated with the slow phase (Fig. 3A, closed triangles) has the same shape as that for the intermediate phase (open circles), again as true with the native enzyme. This has been interpreted as indicating a shift in internal oxidation-reduction equilibrium toward full reduction of the 4Fe-4S center concomitant with the formation of the spin-interacting state in this phase of the reaction (9).

Formation of 6-OH-FMN during Catalysis—Upon reaction of C30A mutant trimethylamine dehydrogenase with a 10-fold excess of trimethylamine or diethylmethylamine (as in the above experiments) and passage through a G-25 column to reoxidize the enzyme and to remove excess reagents, the absorption spectrum shown in Fig. 4 (dotted line) was obtained, which is seen to differ significantly from that for the as-isolated C30A mutant enzyme (solid line). The principal change is an absorption increase in the range of 300–450 nm and above 500 nm upon reaction with substrate. The substrate-treated C30A mutant enzyme cannot be reduced with substrate and exhibits negligible activity.

**Fig. 2.** Diethylmethylamine concentration dependence of the observed rate constants for the fast kinetic phase. The observed rate constants for the fast kinetic phase at pH 8.0 are plotted versus the concentration of diethylmethylamine after mixing. The closed circles represent kobs measured at 450 nm. The solid line represents the fit of the data to the hyperbolic function kobs = (k∞[diethylmethylamine])/(Kd + [diethylmethylamine]). The fitted values of k∞ and Kd are 80 s⁻¹ and 5.0 mM, respectively.

**Fig. 3.** Spectra for intermediates of the reaction of C30A mutant trimethylamine dehydrogenase with diethylmethylamine. Reaction conditions after mixing in a stopped-flow apparatus were as follows: [C30A mutant enzyme] = 9.6 μM, [diethylmethylamine] = 2.5 mM, 0.1 M sodium pyrophosphate, pH 8.0, at 25 °C. A, the kinetic difference spectra obtained from the absorbance changes associated with the fast kinetic phase (closed circles), the absorbance changes associated with the intermediate kinetic phase (open circles), and the absorbance changes associated with the slowest kinetic phase (closed triangles); B, the absorbance spectra of the oxidized C30A mutant enzyme (solid line), the intermediate formed by the fast kinetic phase (closed circles), the intermediate formed by the intermediate kinetic phase (open circles), and the final form at the end of the reaction (closed triangles); C, deconvolution of the difference spectrum for the intermediate kinetic phase. The difference spectrum for flavin (flavin semiquinone–flavin hydroquinone) (open circles) was calculated by subtracting the difference spectrum for the iron-sulfur center (reduced phenylhydrazine-treated, trimethylamine dehydrogenase-oxidized, phenylhydrazine-treated enzyme; taken from Ref. 12) (closed triangles) from the difference spectrum for the intermediate kinetic phase (closed circles).
Treatment of the C30A mutant enzyme with 0.5 M perchloric acid followed by neutralization and centrifugation results in the release of normal FMN (data not shown). The FMN content in the C30A mutant enzyme thus determined is ~30% of the full complement, consistent with the known flavin content determined by other means (18). When the same procedure was applied to the substrate-treated C30A mutant enzyme, however, a modified flavin was obtained. Fig. 5 shows the spectra for this modified FMN at pH 6 (solid line) and pH 10 (dotted line). The spectrum at pH 6 has an absorption maximum at 420 nm and a shoulder at 454 nm and is identical to that for the neutral form of 6-hydroxyflavin (27–29). Similarly, the spectrum at pH 10 has absorption maxima at 424 and 584 nm and is the same as the spectrum for the anionic form of 6-hydroxyflavin (27–29). The pH dependence of the absorption spectrum (Fig. 5, inset) corresponds to a pK<sub>a</sub> of ~7, within experimental error of that (7.1) for authentic 6-hydroxyflavin (27–29). Taken together, these results make it clear that the modified flavin released from the substrate-treated C30A mutant enzyme is 6-hydroxy-FMN. To make sure that 6-hydroxy-FMN is not the hydrolysis product of other flavin derivatives under the strongly acidic conditions of protein denaturation, the substrate-treated C30A mutant protein at pH 7.0 was heated in boiling water for 3 min. Heat treatment also resulted in the release of 6-hydroxy-FMN, indicating that 6-hydroxy-FMN is formed during the treatment of the C30A mutant with substrate and is not a product of hydrolysis in 0.5 M perchloric acid.

The most likely mechanism for the formation of 6-OH-FMN is via nucleophilic attack of OH<sup>-</sup> at flavin C-6. To determine more specifically how 6-hydroxy-FMN is formed in C30A trimethylamine dehydrogenase, the mutant enzyme was reduced with dithionite at pH 7.0 and 10.0, passed through a G-25 column, and reoxidized in air. In neither case was 6-hydroxy-FMN formed, indicating that OH<sup>-</sup> does not attack the flavin hydroquinone of the reduced enzyme. Similarly, reduction of the C30A mutant enzyme with dithionite in the presence of 3 mM tetramethylammonium chloride, which results in two-electron reduction of the enzyme and formation of the spin-interacting state, does not give 6-hydroxy-FMN, ruling out the possibility that OH<sup>-</sup> attacks the flavin semiquinone or the spin-interacting state of the two-electron reduced C30A mutant. The data suggest that a reaction intermediate form generated in the course of the reductive half-reaction is particularly susceptible to nucleophilic attack.

When C30A trimethylamine dehydrogenase was titrated with stoichiometric diethylmethylamine or trimethylamine at pH 7.0 and passed through a G-25 column, a spectrum similar to that for the as-isolated C30A mutant was obtained, indicating that the noncovalently bound FMN is not quantitatively modified during the first turnover. To determine how many times the C30A mutant of trimethylamine dehydrogenase does turn over prior to inactivation, enzyme assays utilizing excess ferricenium hexafluorophosphate as oxidant were carried out following the reaction at 300 nm. The amount of ferricenium hexafluorophosphate reduced subsequent to the addition of an excess of diethylmethylamine was calculated using its extinction coefficient of 4300 M<sup>-1</sup> cm<sup>-1</sup> at 300 nm. Because ferricenium hexafluorophosphate can accept only 1 reducing eq and substrate-reduced trimethylamine dehydrogenase possesses 2 reducing eq, the number of enzyme turnovers was obtained by dividing the amount of reduced ferricenium by 2 and by the amount of enzyme used. The results of these experiments (data not shown) indicate that the C30A mutant enzyme turns over approximately nine times before being inactivated.

When C30A trimethylamine dehydrogenase was treated with an excess of diethylmethylamine at pH 7.0, immediately passed through a G-25 column, and reoxidized in air, only part of the FMN was modified to 6-hydroxy-FMN. When the C30A mutant was incubated with an excess of diethylmethylamine overnight, however, all the enzyme-bound FMN was modified. On the other hand, when trimethylamine was used instead of diethylmethylamine and the mixture was immediately passed through a G-25 column, all the FMN was modified. These results indicate that the formation of 6-hydroxy-FMN is slow when the C30A mutant enzyme is treated with diethylmethylamine, but faster if trimethylamine is used.

It was of interest to see whether other nucleophiles could attack C-6 of the FMN in the C30A mutant enzyme to form other flavin derivatives. When oxidized C30A mutant trimethylamine dehydrogenase was treated with sodium sulfide, a spectrum resembling that for 6-mercaptoflavin (30, 31) was obtained, with absorption maxima at 428 and 632 nm (data not shown), suggesting that C-6 is also susceptible to the nucleophilic attack by S<sup>2-</sup>. On the other hand, treatment of oxidized trimethylamine dehydrogenase with sodium sulfide resulted in the formation of a spectrum resembling that for 6-mercaptoflavin (30, 31) was obtained, with absorption maxima at 428 and 632 nm (data not shown), suggesting that C-6 is also susceptible to the nucleophilic attack by S<sup>2-</sup>.
C30A trimethylamine dehydrogenase with potassium cyanide resulted in only partial bleaching of the spectrum between 370 and 520 nm. The spectrum of the oxidized C30A mutant was obtained upon reoxidation with air. Also, treatment of the C30A mutant protein with excess trimethylamine in the presence of potassium cyanide resulted in the formation of 6-hydroxy-FMN. These results indicate that CN− cannot attack C-6 of FMN to form 6-CN-FMN. Similarly, treatment of the oxidized C30A mutant with sodium sulfite and hydroxylamine hydrochloride in the absence or presence of trimethylamine did not result in the formation of flavin derivatives.

As is seen for native trimethylamine dehydrogenase, when the C30A mutant enzyme is reduced with dithionite in the presence of tetramethylammonium chloride, the unpaired electron spins of the flavin semiquinone and the reduced iron-sulfur center interact strongly to give rise to a spin-interacting state that exhibits an intense g = 4 EPR signal (18). To determine whether the unpaired electron spin of 6-hydroxy-FMN semiquinone can interact with that of the reduced iron-sulfur center, the substrate-treated C30A mutant enzyme was reduced with dithionite in the presence of tetramethylammonium chloride. No g = 4 EPR signal was observed, indicating that when FMN is modified to form 6-hydroxy-FMN, the unpaired electron spins of the two prosthetic groups cannot interact to give a spin-interacting state.

**DISCUSSION**

Similar to the reaction of native trimethylamine dehydrogenase with diethylmethylamine, the reaction of the C30A enzyme with this slow substrate is found to exhibit three kinetic phases, with only kfast dependent on the concentration of diethylmethylamine. The spectral change associated with the fast phase reflects reduction of the noncovalently bound FMN upon reaction with substrate. The spectral change associated with the intermediate phase represents intramolecular electron transfer from flavin hydroquinone to the iron-sulfur center, leaving flavin semiquinone anion. As with the native enzyme, the slow phase represents a small amount of additional electron transfer associated with the spin-interacting state. The mechanism of the C30A enzyme with diethylmethylamine thus appears to be fundamentally the same as with the native enzyme. The k fast for diethylmethylamine in the fast phase of the reaction has the same value for both the native and C30A mutant enzymes, indicating that the 6-S-cysteiny1–FMN bond has no effect on substrate binding. On the other hand, the value of kint,fast for the fast phase of the reaction of the C30A enzyme with diethylmethylamine is 6-fold smaller than that with the native enzyme, demonstrating that the removal of the 6-S-cystey1–FMN bond slows down the first step of the reductive half-reaction. The values of kint and kslow for the reaction of C30A trimethylamine dehydrogenase with diethylmethylamine are comparable to those with the native enzyme, indicating that the removal of the 6-S-cysteinyl–FMN bond has a minimal effect on the later steps in the catalytic sequence. It is to be emphasized that the first, mutation-sensitive step of the reductive half-reaction is not rate-limiting, and so the kinetic effect of loss of covalent flavin attachment is minimal.

This work demonstrates that the noncovalently bound FMN of the C30A enzyme is modified to form 6-hydroxy-FMN, and the enzyme becomes inactive after approximately nine turnovers. It is thus evident that one significant role for the 6-S-cystey1–FMN bond in native trimethylamine dehydrogenase is to prevent enzyme inactivation in the course of catalysis. The formation of 6-hydroxy-FMN seen during turnover with the C30A enzyme appears not to be due to an increase in solvent accessibility of the flavin as the active site is already quite accessible to solvent. Similarly, inactivation is not due to simple reduction of flavin in the course of catalysis as the C30A enzyme is not modified upon reduction with excess dithionite at either pH 7.0 or 10.0. Formation of the spin-interacting state does not result in formation of 6-hydroxy-FMN either since the C30A enzyme is unchanged upon reduction with dithionite in the presence of tetramethylammonium chloride.

The most likely mechanism whereby 6-hydroxy-FMN is formed is via nucleophilic attack of HO− at flavin C-6 in a manner similar to the cysteine thiolate attack that is thought to give rise to the covalent adduct of the native enzyme (18). With the above in mind, however, it must be a catalytic intermediate that is susceptible to nucleophilic attack, not simply oxidized or reduced flavin. We suggest that it is the covalent flavin-substrate adduct previously proposed (9) that is the form rendered susceptible to nucleophilic attack, as shown in Scheme 1. The covalent flavin-substrate adduct being the species susceptible to nucleophilic attack also provides a rationale as to why formation of 6-hydroxy-FMN occurs faster with trimethylamine than with diethylmethylamine. It is possible that formation of 6-hydroxy-FMN in the course of steady-state assays accounts for the large apparent value of kcat observed with C30A trimethylamine dehydrogenase (18).

The x-ray crystal structure of trimethylamine dehydrogenase shows that the flavin ring is distinctly nonplanar, bending 20° about the N-5/N-10 axis of the isoalloxazine ring (13, 14). It has been proposed that the nonplanarity of the flavin ring may facilitate the reductive half-reaction of the enzyme by raising the flavin reduction potential and may be due in part to the 6-S-cysteiny1 linkage (18). The results obtained in this study demonstrate that the removal of the 6-S-cysteiny1–FMN bond slows down the initial reaction of substrate with the enzyme-bound FMN, but not the overall reductive half-reaction because the rate of the rate-limiting step is unchanged. The modest effect seen for the C30A mutant enzyme on the limiting rate constant for the fast phase of the reaction with diethylmethylamine indicates that while the 6-S-cysteiny1–FMN bond may increase the FMN reduction potential, the effect is not profound from a kinetic standpoint. It appears instead that one major role of the covalent linkage is simply to prevent the formation of 6-hydroxy-FMN in the active site of trimethylamine dehydrogenase so that it does not become inactive under multiple turnover conditions. In this sense, it is possible that the covalent attachment found in the native enzyme is more appropriately thought of as an effect of other factors related to the flavin-binding site that renders (perhaps coincidentally) C-6 susceptible to nucleophilic attack, rather than a principal cause of the chemistry exhibited by the flavin that facilitates catalysis.

As is seen for native and recombinant wild-type trimethylamine dehydrogenases, the unpaired electron spins of the noncovalently bound flavin semiquinone and the reduced iron-sulfur center in the C30A mutant enzyme can interact to give rise to a spin-interacting state (18), demonstrating that the 6-S-cystey1–FMN bond does not play an important role in the formation of the spin-interacting state. When the ferredoxin-type 4Fe-4S center in the native enzyme is modified to an EPR-active iron-sulfur center by treatment of the native enzyme with ferricenium hexafluorophosphate at pH 10, the unpaired electron spin of the modified iron-sulfur center cannot
interact with that of the flavin semiquinone. Similarly, when the FMN in the C30A mutant enzyme is modified to 6-hydroxy-FMN, the unpaired electron spins of 6-hydroxyflavin semiquinone and the reduced iron-sulfur center no longer interact. All these results suggest that both the ferredoxin-type 4Fe-4S center and unmodified FMN are necessary for the formation of the spin-interacting state.

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