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Laser Flash Photolysis Study of Intermolecular and Intramolecular Electron Transfer in Trimethylamine Dehydrogenase†

James T. Hazzard,‡ William S. McIntire,§ and Gordon Tollin*,‡

Department of Biochemistry, University of Arizona, Tucson, Arizona 85721, Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121, and Department of Biochemistry and Biophysics and Department of Anesthesia, University of California, San Francisco, California 94143

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ABSTRACT: Laser flash photolysis has been used to investigate the kinetics of reduction of trimethylamine dehydrogenase by substoichiometric amounts of 5-deazariboflavin semiquinone, and the subsequent intramolecular electron transfer from the FMN cofactor to the Fe_4S_4 center. The initial reduction event followed second-order kinetics ($k = 1.0 \times 10^8$ M^{-1} s^{-1} at pH 7.0 and 6.4×10^7 M^{-1} s^{-1} at pH 8.5) and resulted in the formation of the neutral FMN semiquinone and the reduced iron–sulfur cluster (in a ratio of approximately 1:3). Following this, a slower, protein concentration independent (and thus intramolecular) electron transfer was observed corresponding to FMN semiquinone oxidation and iron–sulfur cluster reduction ($k = 62$ s^{-1} at pH 7.0 and 30 s^{-1} at pH 8.5). The addition of the inhibitor tetramethylammonium chloride to the reaction mixture had no effect on these kinetic properties, suggesting that this compound exerts its effect on the reduced form of the enzyme. Treatment of the enzyme with phenylhydrazine, which introduces a phenyl group at the 4a-position of the FMN cofactor, decreased both the rate constant for reduction of the protein and the extent of FMN semiquinone production, while increasing the amount of iron–sulfur center reduction, consistent with the results obtained with the native enzyme. Experiments in which the kinetics of reduction of the enzyme were determined during various stages of partial reduction were also consistent with these results, and further indicated that the FMN semiquinone form of the enzyme is more reactive toward the deazariboflavin reductant than is the oxidized FMN. The results of these experiments have been evaluated in terms of the X-ray structure of the enzyme and have been compared with previous results obtained with other multi-redox-center enzymes.

Trimethylamine dehydrogenase (TMAD)[1] is an iron–sulfur flavoprotein from a methylotrophic bacterium (W3A1) which catalyzes the conversion of trimethylamine to dimethylamine and formaldehyde, using as an acceptor an FAD-containing electron-transfer protein (Steenkamp & Mallinson, 1976; Steenkamp & Gallup, 1978). It is a tightly associated dimer

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* To whom correspondence should be addressed.
‡ University of Arizona.
§ Veterans Administration Medical Center and University of California, San Francisco.

[1] Abbreviations: dRf and dRfH•, oxidized and neutral semiquinone species of 5-deazariboflavin, respectively; FADH•, neutral semiquinone species of flavin adenine dinucleotide; FMN and FMNH•, oxidized and neutral semiquinone forms of flavin mononucleotide, respectively; Fe_4S_4, 4Fe–4S cluster; TMAC, tetramethylammonium chloride; TMAD, trimethylamine dehydrogenase.

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of M_r 166K which has two identical subunits each containing a 4Fe–4S (Fe_4S_4) cluster and a covalently bound FMN [via a (6*S*)-cysteinyl linkage; Steenkamp et al., 1978a]. A 2.4-Å X-ray structure determination has been published (Lim et al., 1986, 1988; Bellamy et al., 1989). Each subunit has distinct structural domains, the largest of which (approximately 40 kDa) contains the iron–sulfur cluster and the flavin. The closest contact distance between the two redox centers, which are both highly buried within the protein, is 5 Å (from the 8-methyl group of FMN to the sulfur atom of Cys-351 which is ligated to an iron atom of the Fe_4S_4 cluster). Microcoulometric analysis of TMAD at pH 7.0 (Barber et al., 1988) has determined the redox potentials of the enzyme to be as follows: +44 mV for FMN/FMNH•; +36 mV for FMNH•/$FMNH^-$; +102 mV for reduction of the iron–sulfur cluster. A recent report (Rohlfs and Hille, in press) demonstrates that it is the neutral flavin semiquinone which is generated upon dithionite reduction.

The enzyme has several unique and interesting features (Steenkamp et al., 1978b; Steenkamp & Beinert, 1982a,b). When reduced by substrate, it initially takes up two electrons at the FMN center and subsequently gives an ESR spectrum which indicates very strong coupling between the reduced iron–sulfur center and the flavin semiquinone (FMNH•) (Stevenson et al., 1986). In contrast, dithionite reduction, which can add three electrons per subunit, does not produce a spin–coupled species. Kinetic studies of substrate reduction using stopped flow indicate that the rate-limiting step in the overall catalytic cycle is electron transfer from fully reduced flavin to the iron–sulfur center (k = 8.6 s^{-1} at 20 °C and pH 7.7) (Steenkamp & Beinert, 1982b). The reduced flavin is generated with a limiting first-order rate constant at high substrate concentration of >500 s^{-1}. Full development of the spin-coupled ESR signal requires the presence of more than 1 mol of substrate per mole of enzyme, which has suggested the presence of an allosteric site and a substrate-induced conformational change (Steenkamp et al., 1978b), or, alternatively, excess substrate binding to the active site of reduced enzyme (Bellamy et al., 1989). Furthermore, it has been shown that tetramethylammonium chloride (TMAC), which is an inhibitor and not a substrate, will induce spin coupling between the flavin and iron–sulfur centers in the dithionite-reduced enzyme. Also, only two electrons can be added to the enzyme from dithionite when TMAC is bound, yielding reduced iron–sulfur and FMNH•, as opposed to three electrons in the absence of the inhibitor. Interestingly, recent X-ray studies (Bellamy et al., 1989) indicate that TMAC binds only at the active site and induces a local conformational change, without affecting the relative orientation or the distance between the FMN and the iron–sulfur cluster. Thus, there was no indication for the binding of this inhibitor to an allosteric site.

In the present study, we have used laser flash photolysis to investigate the accessibility of the two redox centers of the enzyme to an exogenous one-electron reductant, 5-deazariboflavin semiquinone (5-dRfH•), and to determine the rate constant for intramolecular electron transfer from the FMN semiquinone to the iron–sulfur cluster.

Materials and Methods

Bacterium W3A1 (NCIB 11348) was grown on trimethylamine as previously described (McIntire, 1990). The harvested cell paste was stored at −20 °C until used for the isolation of TMAD. The enzyme was purified as described by Steenkamp and Mallinson (1976) and was stored at a temperature of −20 °C in 25% v/v ethylene glycol in 50 mM phosphate buffer and 200 mM NaCl, pH 7.2.

In order to determine the concentration of oxidized redox centers during the course of the flash photolysis experiments, the enzyme (6.9 μM in 3 mL of 50 mM phosphate buffer, pH 7.0, ±1.0 mM tetramethylammonium ion) was reductively titrated under anaerobic conditions with sodium dithionite in a specially designed cuvette (Edmondson & Singer, 1973). To facilitate anaerobiosis, the enzyme solution contained 0.1 M D-glucose, 25 μg of glucose oxidase, and 4.8 μg of catalase. Air in the cuvette was replaced by oxygen-free argon by 7 vacuum pump/purge cycles. The dithionite solution was standardized by titration of a riboflavin solution of known concentration. An ϵ_{448} = 49.8 mM^{-1} cm^{-1} was obtained for oxidized TMAD dimer, which is within 10% of the published value of 55.8 mM^{-1} cm^{-1} (Kasprazak et al., 1983). The change in absorbance of TMAD at 448 nm was linear throughout the titration whether tetramethylammonium ion was present or not. Furthermore, during the flash photolysis studies, the absorbance due to the enzyme was corrected by subtracting the contribution resulting from the high concentration of 5-deazariboflavin (~80 μM).

The enzyme can be inactivated by incubation with phenylhydrazine (Nagy et al., 1979), which results in the introduction of a phenyl group at the 4a-position of the FMN cofactor, rendering it redox-inactive. A dithionite titration of the TMAD–phenylhydrazine adduct yielded an ϵ_{448} = 10.6 mM^{-1} cm^{-1} for each Fe_4S_4 cluster (from this, the ϵ_{448} for each FMN is calculated to be 14.3 mM^{-1} cm^{-1}). The approximate $\Delta\epsilon_{580}$ values given under Results are based on the changes one would expect to see for typical Fe_4S_{4ox} → Fe_4S_{4red} and FMN → FMNH• transitions (see below). Because of the unique properties of the prosthetic groups in TMAD, these values may not be very accurate and thus should be viewed only as rough estimates.

Laser flash photolysis experiments were carried out according to the procedures given in Hazzard et al. (1986, 1987). Excitation of dRf in the presence of EDTA as a sacrificial donor rapidly (<1 μs) generated the semiquinone (dRfH•) via triplet-state quenching, which either disproportionated or, in the presence of TMAD, reacted to generate reduced protein. All kinetic experiments were performed under pseudo-first-order conditions, in which the concentration of oxidized protein (>4 μM) was in large excess over the amount of dRfH• produced per flash (<0.7 μM). This ensures that each protein molecule reacts with no more than one dRfH• molecule, and simplifies the kinetic analysis. In such experiments, protein reduction is always in competition with disproportionation (k = 4.3 × 10^9 M^{-1} s^{-1} under comparable experimental conditions; Mark C. Walker, unpublished data). The extent of the contribution of the disproportionation reaction to the observed transient decay kinetics is determined by the magnitude of the second-order rate constant for protein reduction and the concentrations of the reacting species (i.e., protein vs dRfH•). In the present case, as will be documented below, the second-order rate constants for protein reduction are relatively small (≤10^8 M^{-1} s^{-1}), and, especially at the lower protein concentrations, it was necessary to deconvolute the kinetic transients in order to obtain an accurate value for the reduction rate constant. This was done by taking advantage of the fact that semiquinone disproportionation contributes predominantly to the initial portion of the decay curve (due to its dependence on the square of the semiquinone concentration). Thus, an adequate representation of the kinetics of protein reduction could be obtained by fitting a single-exponential curve to the longer time portion of the kinetic trace. The validity of this

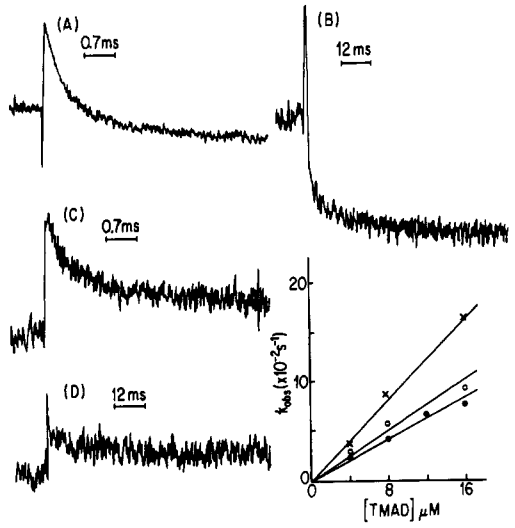

FIGURE 1: Transients observed upon reduction of TMAD (8 μM) by deazariboflavin semiquinone at pH 7.0. Signal intensities are not comparable. (A,B) 470 nm; (C,D) 580 nm. *Inset*: Plots of k_{obs} vs TMAD concentration (based on flavin content) for the fast phase of the 470-nm absorbance decay. (×) pH 7.0 ($k_2 = 1.0 \times 10^8$ M^{-1} s^{-1}); (○) pH 8.5 ($k_2 = 6.4 \times 10^7$ M^{-1} s^{-1}); (●) pH 7.0, phenylhydrazine treated ($k_2 = 5.0 \times 10^7$ M^{-1} s^{-1}).

procedure is attested to by the linear dependence of the first-order rate constants obtained in this way on the protein concentration. Although, in principle, it is possible to use computer methods to carry out this deconvolution, it has been our experience that this is less satisfactory than the above procedure because of the mixed first- and second-order kinetic behavior. We estimate that the uncertainty in the values of the rate constants determined here is ≤±10%.

All kinetic experiments were performed anaerobically and at room temperature in cuvettes which were deaerated by bubbling with water-saturated argon gas. Microliter volumes of a concentrated solution of protein were added via a syringe to the sealed cuvette containing 1–2 mL of deazaflavin-containing buffer subsequent to deoxygenation, and resulting traces of oxygen were removed by blowing argon gas across the surface of the solution. Laser photoexcitation was carried out with a nitrogen laser pumped dye solution (396-nm maximum wavelength).

Results

The reduction of TMAD by dRfH• generated by laser flash photolysis followed biphasic kinetics. This is shown in Figure 1 at two wavelengths, 470 (A,B) and 580 nm (C,D). In all of the traces, the initial rapid increase in absorbance is due to the formation of dRfH• by the laser flash. At 470 nm (cf. Figure 1A), the subsequent fast phase of the absorbance decrease decayed to a level below that of the preflash base line, consistent with reduction of the FMN and/or the iron–sulfur centers by dRfH•. At 580 nm (cf. Figure 1C), a rapid initial decay process also occurred subsequent to dRfH• formation, in this case leaving a small net positive absorbance, which indicates the net production of protein-bound neutral FMN semiquinone (FMNH•) based upon the spectral properties of the species involved [cf. Edmondson and Tollin (1983)]. However, if the *only* reaction proceeding during the initial electron transfer from deazariboflavin semiquinone was FMN reduction to the neutral semiquinone state, the 580-nm absorbance should have *increased* during this kinetic phase [$\Delta\epsilon_{580} \approx 4000$ M^{-1} cm^{-1}; cf. Mayhew and Ludwig (1975)]. On the other hand, if only the iron–sulfur center was reduced in the initial reaction, the signal should have decayed below the

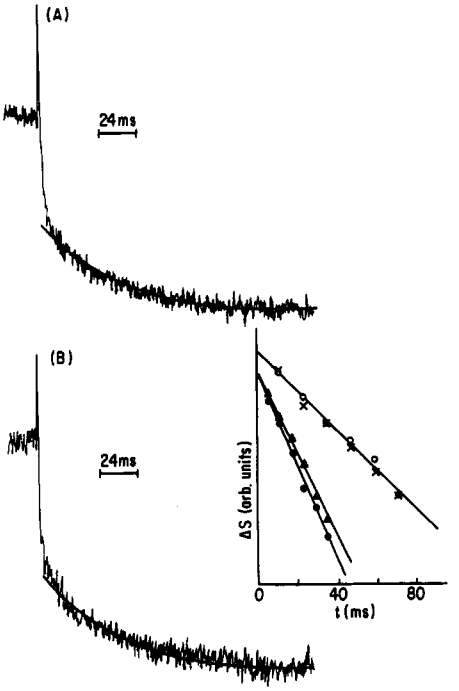

FIGURE 2: Transients observed at 470 nm upon reduction of TMAD by deazariboflavin semiquinone at pH 8.5. Solid lines are single-exponential fits to the slow phase of the decay. (A) 4 μM TMAD; (B) 8 μM TMAD. *Inset*: Semilog plots of the slow phase of absorbance decay monitored at 470 nm. (×) 4 μM TMAD, pH 8.5 ($k_1 = 30$ s^{-1}); (○) 8 μM TMAD, pH 8.5 ($k_1 = 30$ s^{-1}); (●) 4 μM TMAD, pH 7.0 ($k_1 = 62$ s^{-1}); (▲) 4 μM TMAD, pH 7.0, phenylhydrazine treated ($k_1 = 65$ s^{-1}).

preflash base line [$\Delta\epsilon_{580} \approx -1000$ M^{-1} cm^{-1}; cf. Palmer et al. (1967)]. The fact that the fast phase at 580 nm was manifested as a decrease in absorbance which remained above the preflash base line suggests that *both* FMN and iron–sulfur reduction occurred simultaneously. In view of the relative extinction coefficients and the residual positive absorbance at 580 nm for the initial reduction step (cf. 2-ms data points for the native enzyme in Figure 3), we estimate that the ratio of reduced Fe_4S_4 to FMNH• produced in this phase is approximately 3:1.

At both wavelengths, a slow absorbance decay followed these rapid changes and resulted in a further loss in absorbance (Figure 1B,D). However, the signal at 580 nm remained positive relative to the preflash base line, suggesting that there was still some residual FMNH• remaining at the end of the reaction. The decrease in absorbance at both wavelengths is, however, consistent with a decrease in the concentration of neutral FMN semiquinone and an increase in the extent of iron–sulfur center reduction (see below for further discussion).

The k_{obs} values, obtained from the slopes of semilog plots of the transient decay curves, for the initial rapid absorbance decay were protein concentration dependent. This is shown for the 470-nm data at two pH values in the inset to Figure 1. The same results were obtained at 580 nm (not shown). The calculated second-order rate constants (based on twice the molar concentration of redox centers and assuming equivalent FMN and iron–sulfur centers within the dimer) were 1.0×10^8 M^{-1} s^{-1} at pH 7.0 and 6.4×10^7 M^{-1} s^{-1} at pH 8.5. In contrast, the slow decay was concentration independent, i.e., first order, as is shown in Figure 2 at 470 nm. The observed first-order rate constants were 62 s^{-1} at pH 7.0 and 30 s^{-1} at pH 8.5.

Time-resolved difference spectra which support the above interpretations are shown in Figure 3. These are consistent with an initial electron transfer from dRfH• to TMAD which

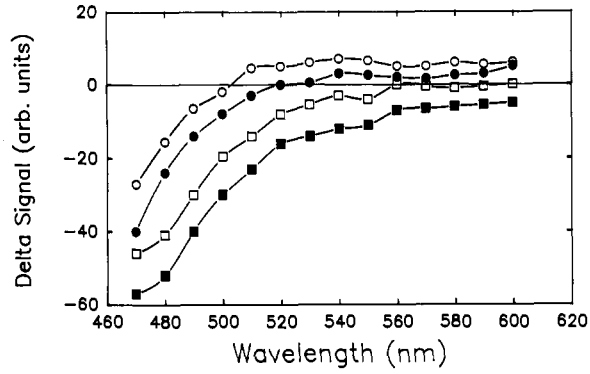

FIGURE 3: Time-resolved difference spectra for reduction of TMAD by deazariboflavin semiquinone. (○) 2 ms (after completion of the fast decay phase); (●) 30 ms (after completion of the slow decay phase); (□) 4 ms, phenylhydrazine treated (after completion of the fast decay phase); (■) 50 ms, phenylhydrazine treated (after completion of the slow decay phase).

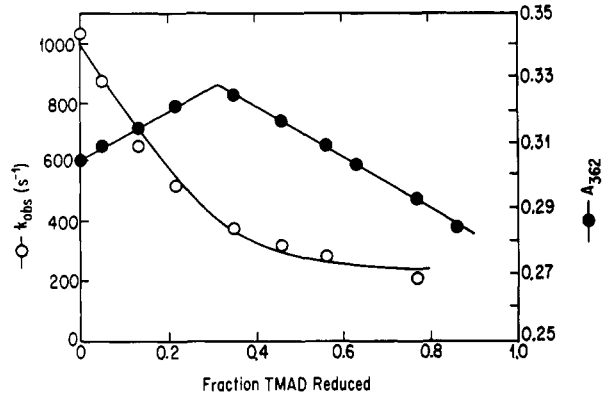

FIGURE 4: Observed rate constants for reduction of TMAD (fast kinetic phase) and absorbance changes at 362 nm during steady-state photititration. The solid curve through the rate constants is a nonlinear least-squares fit to the data according to the mechanism described in the text. (○) Observed rate constants; (●) 362-nm absorbance.

resulted in a mixture containing reduced iron–sulfur and the neutral FMN semiquinone. The subsequent slow decay process caused an overall decay in absorbance in the spectral region studied, which can most simply be attributed to a decrease in the FMN semiquinone concentration and a further increase in the reduced iron–sulfur concentration. Two other possible processes involving the loss of FMNH•, which might generate similar spectral behavior, can be argued against. Thus, the fact that the slow decay process was concentration independent eliminates any mechanism involving a bimolecular disproportionation of the FMN semiquinone species. Alternatively, the deprotonation of FMNH• to the anionic species, FMN^-•, should also result in a general decrease in absorbance within this spectral region [cf. Edmondson and Tollin (1983)]. If the deprotonation of FMNH• was the primary contributor to these spectral changes, the signal at 580 nm shown in Figure 1D and the difference spectrum for the native enzyme in Figure 3 should have returned to the preflash base line. In fact, these remained slightly positive relative to the preflash base line. Considering the magnitudes of the extinction coefficient changes for the flavin and iron–sulfur moieties, the data suggest that only a small amount of FMN semiquinone oxidation occurred during the second kinetic phase. Although the direction of electron transfer from FMN semiquinone to Fe_4S_4 is consistent with the reported redox potentials of the two centers (Barber et al., 1988), the relatively small magnitude of FMNH• oxidation is not consistent with a 58-mV difference in midpoint potentials for the two redox centers. The reason for this remains unclear. However, it must be noted that the flavin potential is determined in an enzyme species which has already undergone a one-electron reduction of the Fe_4S_4 cluster. In contrast to this, in the current studies, electron redistribution occurs in a one-electron-reduced protein. Furthermore, it is also possible that the relative redox potentials of the two centers under the nonequilibrium conditions of the flash experiments are not the same as those determined in the presence of dye mediators in an equilibrium experiment.

The addition of tetramethylammonium ion, to a final concentration of 1 mM, had no significant effect on the kinetic transients at either pH value (data not shown).

When the enzyme was treated with phenylhydrazine, the second-order rate constant for the initial phase of protein reduction decreased by approximately a factor of 2 (Figure 1). This decrease was most likely a consequence of the inactivation of FMN, and could also be a result of structural changes induced by the chemical modification (see below). The magnitude of the FMNH• contribution to the difference spectrum was appreciably decreased (cf. Figure 3), although some positive absorbance contribution was still evident, suggesting that some of the 4*a*-phenyl adduct had dissociated during the product workup [M. J. Barber (personal communication) has found that partial adduct dissociation occurs in experiments with this system)]. It can also be concluded from the flash-induced difference spectra in Figure 3 that the initial direct reduction process generated more reduced iron–sulfur in the phenylhydrazine-treated protein than was the case with the native enzyme. Biphasic kinetics were still observed with the phenylhydrazine-treated protein, and the rate constant for the slower concentration-independent process remained unchanged (Figure 2). At 470 nm, the percentage of the total signal change that could be attributed to the fast phase was much larger than seen with the native protein, which is consistent with a decrease in the amount of FMN reduction due to the phenylation process (cf. Figure 3). At the end of the slow decay phase, the difference spectrum was approximately that expected for a reduced iron–sulfur center. These results are consistent with the observations made with the untreated enzyme and provide strong support for the conclusion that during the initial reduction of native TMAD by deazariboflavin semiquinone a significant amount of FMNH• was produced (see below for further discussion).

Figure 4 shows the results of an experiment in which the reduction kinetics of TMAD by deazariboflavin semiquinone were monitored in a sample which was progressively reduced by steady-state irradiation with white light prior to laser photolysis. As is evident, the k_{obs} values (for the initial fast kinetic phase) decreased with the extent of photoreduction. Also plotted in Figure 4 are the absorbance values at 362 nm at the various stages of the photititration. These changes at 362 nm are taken to be primarily due to reduction of the iron–sulfur cluster. From a comparison of the data for both k_{obs} and A_{362}, we can conclude that there was a greater change in the rate constant (~77%) within the first 30% of the photititration (assuming a total of 3 reducing equiv per TMAD), which can be attributed primarily to the reduction of the Fe_4S_4 center. In the latter two-thirds of the photititration, k_{obs} can be attributed mainly to reduction of the FMN moiety to both the semiquinone and the fully reduced species.

If one assumes that the k_{obs} values during the photititration correspond to the sum of the rate constants for reduction of both the FMN and iron–sulfur centers of the enzyme, it is possible to analyze these data by using the published values for the reduction potentials (Barber et al., 1988). Thus, by means of the Nernst equation, one can calculate the concen-

trations of the oxidized and reduced forms of the two redox centers during the phototitration. By fitting the data to the following equation, values for the individual second-order rate constants can be obtained:

$$k_{obs} = k_{FMN}[FMN] + k_{FMNH}[FMNH\bullet] + k_{FeS}[FeS]$$

This analysis assumes that reduction of all of these species contributes to the observed kinetics and that both subunits behave independently. A nonlinear least-squares fit to the data points is shown by the solid curve in Figure 4, using the following values for the rate constants: $k_{FeS} = 5.2 \times 10^7$ M^{-1} s^{-1}; $k_{FMN} = 1.1 \times 10^7$ M^{-1} s^{-1}; $k_{FMNH} = 2.9 \times 10^7$ M^{-1} s^{-1}. It should be pointed out that it was not possible to fit the data well using only terms corresponding to the FMN and Fe_4S_4 contribution. It is evident from Figure 4 that the agreement between the observed data and the theoretical curve is satisfactory. It is important to note that the rate constant for reduction of the iron–sulfur center obtained from the data fit is approximately the same as that obtained from reduction of the phenylhydrazine-treated enzyme, in which the predominant site of reduction was clearly the iron–sulfur cluster. This is consistent with the microcoulometric results which indicate essentially no change in the redox potential of the iron–sulfur center upon phenylhydrazine treatment (Barber et al., 1988), and suggest that the phenylation does not result in significant structural changes which influence Fe_4S_4 reactivity. The conclusion that the rate constant for reduction of the iron–sulfur cluster is larger than that for the FMN center is consistent with the experiments using the fully oxidized enzyme. However, the sum of these two rate constants obtained from the data fit (6.3×10^7 M^{-1} s^{-1}) is somewhat smaller than the value obtained from the concentration dependence study with the oxidized native enzyme (1.0×10^8 M^{-1} s^{-1}; see above), and the rate constant ratio is somewhat larger than expected from the difference spectral measurements (Figure 3). It is not clear if this is due simply to the approximations inherent in the data analysis of Figure 4, according to the above equation, or to a small decrease in the rate constant for FMN reduction when the iron–sulfur center is reduced. This requires further study. The analysis also indicates that the semiquinone state of the FMN is more reactive toward deazariboflavin semiquinone than is the oxidized state.

Discussion

The results described above provide clear evidence for a second-order reaction between deazariboflavin semiquinone and TMAD which results in the reduction of both the FMN and iron–sulfur centers of the enzyme, although the latter is the predominant site. The observed second-order rate constants for this reaction (1.0×10^8 M^{-1} s^{-1} at pH 7.0 and 6.4×10^7 M^{-1} s^{-1} at pH 8.5) can be compared to values obtained previously for the reduction of flavin and iron–sulfur centers in other proteins. Thus, rate constants of 6.3×10^8 M^{-1} s^{-1} for the FAD center in spinach ferredoxin–$NADP^+$ reductase at pH 7.0 (Bhattacharyya et al., 1986), 3.7×10^8 M^{-1} s^{-1} for the FMN center of *Clostridium pasteurianum* flavodoxin at pH 7.5 (Simondsen & Tollin, 1983), and 6.7×10^8 M^{-1} s^{-1} for the iron–sulfur center of spinach ferredoxin at pH 7.0 (Bhattacharyya et al., 1986) have been reported. It is apparent that both redox centers in TMAD react appreciably more slowly with deazariboflavin semiquinone than these other proteins, which is probably at least partly a consequence of the highly buried nature of the redox centers in this enzyme (according to the X-ray structure, each center is approximately 20 Å from the surface of the protein; Lim et al., 1986). In fact, it is rather remarkable that protein redox center reduction can be achieved at all at rates which can compete favorably with deazaflavin semiquinone disproportionation.

Subsequent to the initial reduction step, an intramolecular electron-transfer process was observed, which was assigned to oxidation of FMNH• and reduction of the iron–sulfur center. The rate constant for this process (62 s^{-1}) can also be compared to values obtained for intramolecular transfer in other multicenter redox proteins. Thus, values of 120 s^{-1} for the FADH• to iron–sulfur transfer in milk xanthine oxidase at pH 7.2 (Edmondson et al., 1987), 220 s^{-1} for the FADH• to heme transfer in *p*-cresol methylhydroxylase at pH 7.6 (Bhattacharyya et al., 1985), 310 s^{-1} for the molybdenum to heme transfer in chicken liver sulfite oxidase at pH 7.0 (Kipke et al., 1988), and 70 s^{-1} for the FADH• to FMN transfer in rabbit liver NADPH–cytochrome P450 reductase at pH 7.0 (Bhattacharyya et al., 1991) have been measured. Again, TMAD is at the low end of this group of proteins, which is quite surprising inasmuch as the flavin and iron–sulfur centers are rather close in this enzyme (the distance from the 8-methyl of the FMN to the sulfur of Cys-351, which is one of the protein ligands of the Fe_4S_4 cluster, is 5 Å; Lim et al., 1986). However, it must be acknowledged that we know very little about the structural and energetic factors which control intramolecular electron-transfer rate constants in multicenter redox proteins, and thus comparisons of TMAD with other systems are probably not too meaningful at present. It is, however, important to compare this value to the rate constant determined for intramolecular electron transfer from fully reduced FMN to the iron–sulfur cluster in TMAD (8.6 s^{-1} at pH 7.7) from stopped-flow measurements of the reductive half-reaction (Steenkamp & Beinert, 1982b). The larger value for the semiquinone to iron–sulfur transfer is consistent with the fact that the enzyme does not stabilize the semiquinone forms of FMN.

The decrease in the intramolecular rate constant with an increase in pH from 7.0 to 8.5 (30 s^{-1}) is not consistent with the expected decrease in the FMN/FMNH• redox potential. Although it is not certain at present whether the iron–sulfur center potential is pH dependent, preliminary results indicate only a small effect at most (Barber et al., 1988). Thus, the decrease in the flavin potential would be in the direction of increasing the thermodynamic driving force for the electron-transfer reaction and, according to Marcus theory, increasing the rate constant. Apparently, this is not the controlling factor in determining the value of this rate constant, and thus structural parameters may be dominant. The fact that the calculated rate constant for reduction of the FMN semiquinone is larger than that for oxidized FMN is also inconsistent with the respective midpoint potentials. This may suggest an increase in the accessibility of the flavin in going to the semiquinone state.

After reaction of the enzyme with phenylhydrazine, we assumed that all of the enzyme-bound FMN was in the 4*a*-phenyl form, i.e., was redox-inactive, based upon the lack of indication of FMN_{ox} or FMNH• in the UV–visible spectrum, and the inactivity of the enzyme either before or after the flash photolysis experiments. Surprisingly, we observed a biphasic process following the formation of the 5-deazariboflavin semiquinone in the flash experiments, which is most simply explained if a small fraction of the FMN was still redox-active. Such a small amount of redox-active FMN could interfere with the accurate determination of the ϵ_{448} for the FeS cluster from the dithionite titration of the phenylhydrazine-inactivated enzyme. However, we assume that this had a minimal effect. We know of no way to determine this extinction coefficient

without assumptions. For example, we could assume that the ϵ values for enzyme-bound and free (6*S*)-cysteinyl-FMN are the same, i.e., $\approx$12 mM^{-1} cm^{-1}; under this assumption, an ϵ_{448} of $\approx$14 mM^{-1} cm^{-1} for each FeS cluster would be obtained. This latter value is the one derived by Kasprazak et al. (1983) from their dithionite titration of the phenylhydrazine-inactivated TMAD. These extinction coefficients, as well as the ones given herein, are all similar to corresponding values for these prosthetic groups in other proteins. Thus, we conclude that the ϵ values used in the present study could be in error by at most 30–40%. Uncertainties in the ϵ_{448} values for FeS_{ox} could affect our results in a quantitative way, but would not influence our analysis of the data, or the conclusions drawn from the values of the rate constants. We estimate that this extinction coefficient error would have to be >100% in order to alter our conclusions in any significant manner. In any event, the results of the experiments on the phenylhydrazine-treated enzyme should be viewed as only confirmatory, since they show that the spectral changes that occur in the fast phase for the unmodified TMAD are largely due to the direct reduction of the FeS_{ox} cluster by 5-deazariboflavin semiquinone, and the ensuing slow phase is a result of electron transfer from the smaller amount of FMNH•, formed in the fast phase, to FeS_{ox}.

It is of considerable interest that the presence of tetramethylammonium ion did not affect either the second-order rate constant for the initial reduction step or the first-order rate constant for the intramolecular reaction. This is consistent with the crystallography (Bellamy et al., 1989) and suggests that this inhibitor exerts its effect predominantly on the reduced form of the enzyme, thus stabilizing the spin-coupled species. Another possibility is that laser irradiation caused the rapid release of the inhibitor from the enzyme prior to the electron-transfer reaction, although we have no data to support such a proposal. This requires further study.

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