Evidence for a Tryptophan Tryptophylquinone Aminosemiquinone Intermediate in the Physiologic Reaction between Methylamine Dehydrogenase and Amicyanin[†]

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ABSTRACT: The tryptophan tryptophylquinone (TTQ) cofactor of methylamine dehydrogenase (MADH) is covalently modified by nitrogen during its two-electron reduction by methylamine to form an aminoquinol (N-quinol). It is possible, *in vitro,* to generate unmodified O-quinol and O-semiquinone forms of MADH with dithionite, as well as an N-semiquinone form which contains a substrate-derived nitrogen. Rapid-scanning stopped-flow spectroscopy and global kinetic analysis are used to demonstrate that N-semiquinone is a true physiologic reaction intermediate which accumulates during the two sequential one-electron oxidations of N-quinol MADH by amicyanin. In contrast, no detectable O-semiquinone accumulates during the two sequential one-electron oxidations of the O-quinol form of MADH by amicyanin. This is because the reaction of N-semiquinone with amicyanin is much slower (70 s^{-1} at 25 °C) than the reaction of O-semiquinone (>1000 s^{-1}). These rate constants obtained from global analysis of the overall reaction are the same as those obtained when each semiquinone form was made *in vitro* and then mixed with oxidized amicyanin. The presence of 200 mM NH_4Cl during the reaction of O-quinol MADH with amicyanin does not cause any detectable accumulation of a semiquinone species. Thus, the accumulation of the intermediate in the reactions of the N-quinol is not due to the influence of noncovalently bound ammonia at the active site of the O-semiquinone. These data indicate that the intermediate which accumulates during the complete oxidation of substrate-reduced N-quinol MADH is not the O-semiquinone, but the more slowly reacting N-semiquinone, and that the N-semiquinone is a physiologically relevant reaction intermediate. These results also provide good evidence in favor of an aminotransferase mechanism, as opposed to an imine elimination mechanism, for the reaction of MADH with substrate methylamine.

Enzyme-bound quinones and flavins are important cofactors in biological catalysis and electron transfer. The function of these prosthetic groups is to couple the two-electron oxidation of substrates to single-electron carrier proteins during metabolism and respiration. This requires the formation of a transient semiquinone intermediate. It is critical to understand the factors which influence the function, reactivity, and stability of such enzyme-bound semiquinone intermediates. This paper presents, for the first time, the detection of an enzyme-bound substrate-modified tryptophan tryptophylquinone (TTQ)[1] semiquinone intermediate during the physiologic reaction cycle of substrate-reduced methylamine dehydrogenase (MADH) with amicyanin. Rapid-scanning stopped-flow spectroscopy and global kinetic analysis are used to investigate the factors which influence the reactivity of this transient intermediate.

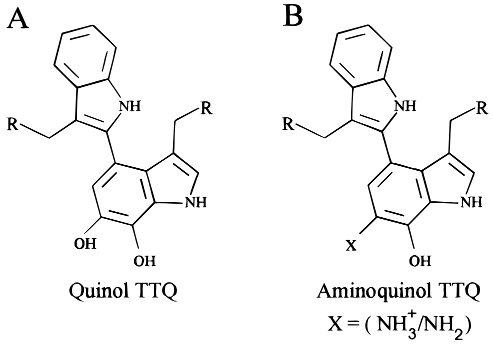

FIGURE 1: The two-electron-reduced forms of TTQ generated by reduction by dithionite (A, O-quinol) and the substrate methylamine (B, N-quinol). The protonation state of the amino group in (B) is unknown.

Paracoccus denitrificans is a facultative autotrophic bacterium which is capable of growth on primary amines as a sole source of carbon and energy. To do so, it utilizes MADH (Davidson, 1993), an inducible periplasmic enzyme which catalyzes the oxidative deamination of primary amines to their corresponding free aldehydes plus ammonia. MADH uses the covalently bound TTQ prosthetic group (McIntire *et al*., 1991) (Figure 1) to oxidize substrate and then donate the two substrate-derived electrons to a suitable single-electron acceptor. *In vivo,* MADH is reoxidized by two one-electron transfers from TTQ to a type-I blue copper protein, amicyanin, which mediates the transfer of electrons to a soluble *c*-type cytochrome (Husain & Davidson, 1985).

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[1] Abbreviations: TTQ, tryptophan tryptophylquinone; MADH, methylamine dehydrogenase; S/N, signal-to-noise; SVD, singular value decomposition; EV1, eigenvector 1; RSM, rapid scanning monochromator; O-quinol, unmodified fully reduced form of TTQ; O-semiquinone, unmodified semiquinone form of TTQ; N-quinol (i.e., aminoquinol), two electron reduced form of TTQ containing a substrate-derived nitrogen; N-semiquinone (i.e., aminosemiquinone), semiquinone modified by a substrate-derived nitrogen; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; ENDOR, electron nuclear double resonance.

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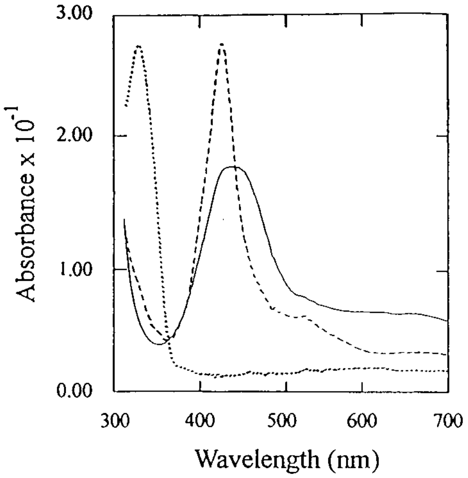

FIGURE 2: Spectra of the three redox forms of MADH generated by titration by dithionite. Spectra of the O-quinol (dotted line), O-semiquinone (dashed line), and quinone (solid line) redox forms are shown. These spectra were collected in 10 mM potassium phosphate buffer, pH 7.5 at 25 °C. The spectrum of the N-quinol is indistinguishable from that of the O-quinol in these reaction conditions (Davidson *et al.,* 1995). The N-semiquinone cannot be prepared in pure form, but its spectrum exhibits features which are similar to that of the O-semiquinone (discussed in text).

Amicyanin is an obligatory mediator in this physiologic electron transfer process, and the crystal structure of the ternary complex of MADH, amicyanin, and cytochrome *c*-551i has been described (Chen *et al.*, 1994).

TTQ in MADH can be chemically reduced by titration with dithionite to generate O-semiquinone and O-quinol forms of MADH (Husain *et al.*, 1987). These redox forms of MADH (Figure 1A) are stable and relatively inert toward oxygen. This has allowed us to characterize the static absorption spectra of these redox forms (Figure 2) (Husain *et al.*, 1987), and the electronic properties of the O-semiquinone have been characterized in detail by ESEEM spectroscopy (Warncke *et al.*, 1995). Alternatively, the two-electron reduction of TTQ by methylamine results in the replacement of the C6 quinone oxygen of TTQ with a substrate-derived amino group to form an aminoquinol (N-quinol) (Figure 1B) (Brooks *et al.*, 1993). Semiquinone TTQ is not formed during the reductive half-reaction of MADH (Brooks *et al.*, 1993) which involves the direct two-electron reduction of TTQ in the enzyme–substrate complex. An aminosemiquinone (N-semiquinone) can be generated *in vitro* by comproportionation of the fully oxidized quinone and substrate-reduced N-quinol forms of MADH at high pH (Davidson *et al.*, 1990). The presence of the substrate-derived nitrogen in this N-semiquinone has been confirmed by ENDOR and ESEEM spectroscopies (Warncke *et al.*, 1993).

The reoxidations of both O- and N-quinol MADH by amicyanin must proceed via two one-electron transfers through a TTQ semiquinone intermediate. The physiologic relevance of the N-semiquinone form of MADH has recently been disputed on the basis of transient kinetic and spectroscopic studies (Gorren & Duine, 1994; Gorren *et al.*, 1995a,b). We present, herein, results which establish that the N-semiquinone is the direct product of the one-electron oxidation of N-quinol MADH by amicyanin and is a true intermediate during the physiologic electron transfer reactions from methylamine-reduced MADH to amicyanin. It is also shown that the presence of the substrate-derived nitrogen on TTQ significantly affects the reactivity of the N-semiquinone with amicyanin relative to that of the O-semiquinone.

EXPERIMENTAL PROCEDURES

Purifications of MADH (Davidson, 1990) and amicyanin (Husain & Davidson, 1985) from *P. denitrificans* (ATCC 13543) were as previously described. MADH and amicyanin concentrations were calculated from known extinction coefficients (Husain & Davidson, 1985; Husain *et al.*, 1987) in 10 mM phosphate buffer, pH 7.5. The extinction coefficients of the different redox forms of MADH vary with pH (Davidson, *et al.*, 1990) and ionic composition of the buffer (Kuusk & McIntire, 1994). All reagents were purchased from Sigma Chemical Co.

O-Quinol and O-semiquinone MADH forms were prepared anaerobically at 25 °C in 10 mM potassium phosphate buffer (pH 7.5) with sodium hydrosulfite (dithionite) (Husain *et al.*, 1987). N-Quinol MADH was prepared anaerobically by reaction with the substrate methylamine hydrochloride. All reactions were monitored spectrophotometrically. Any excess dithionite was rendered inert and invisible by passing air through the system. Any excess methylamine was removed either by gel filtration using Sephadex G-50 or by dialysis. Both reduced forms were stable at room temperature in the presence of atmospheric oxygen.

Anaerobic experiments were performed, as previously described (Davidson *et al.*, 1992a), using a quartz cuvette fitted with an airtight rubber septum. The cuvette was connected to a Firestone rapid purge valve (Ace Glass, Vineland, NJ), a vacuum pump, and a source of oxygen-free argon. Using the valve, it was possible to alternate cycles of evacuations and flushing of the cuvette with argon. Such cycling was repeated immediately before each assay. Anaerobic solutions of dithionite and methylamine were prepared similarily in a separate reaction vessel and transferred to the reaction cuvette described above using a 25 μL gas-tight Hamilton syringe.

N-Semiquinone MADH was prepared by comproportionation of a 1:1 mixture of methlyamine-reduced (N-quinol) MADH and fully oxidized MADH (Davidson *et al.*, 1992b, 1995). This yields a mixture of O- and N-semiquinone (Warncke *et al.*, 1993). The comproportionation reaction is extremely sensitive to the reaction conditions. Optimum conditions for the generation of the N-semiquinone are 1–10 mM potassium phosphate, pH 9.5, 25 °C, with dilute concentrations (no more than 3 μM) of MADH in the presence of 0–0.2 M added KCl. In the presence of higher concentrations of KCl, anomalous results were obtained, which suggested that salt may affect either the comproportionation reaction or the stability of semiquinone products. The reaction takes from several minutes to hours and is dependent upon the ionic composition of the buffer, pH, and the enzyme preparation. The accumulating semiquinone is monitored spectrophotometrically. When the reaction is complete, the pH is adjusted to 7.5 by addition of monobasic potassium phosphate, and the solution is deoxygenated and stored under argon. The half-life of the equilibrium N-semiquinone mixture is somewhat sensitive to oxygen. As a result, N-semiquinone samples were either used immediately after preparation and pH adjustment, or frozen in liquid nitrogen and stored at −80 °C.

Rapid kinetic measurements were made using an On-Line Instrument Systems (OLIS) RSM1000 stopped-flow spectrophotometer. All reactions were monitored over a range between 300 and 500 nm where each of the three redox forms of TTQ exhibits a visible absorbance maximum. The visible absorbance maximum of amicyanin is at 595 nm, and its extinction coefficient is much smaller than that of MADH. Therefore, redox-linked changes in amicyanin do not interfere with the analysis of the absorbance changes of oxidized, semiquinone, or reduced MADH. Previous studies suggest that the spectra of the O-semiquinone and N-semiquinone are similar in that they exhibit their maximum absorbances at 330 and 425 nm (Davidson *et al.,* 1995). However, it is not clear whether or not they have identical extinction coefficients at these wavelengths. It has not been possible to generate pure N-semiquinone *in vitro*. N-Semiquinone is generated by comproportionation, and the reaction mixture is composed of O- and N-semiquinones. It is, therefore, difficult to obtain precise extinction coefficients for the N-semiquinone at these wavelengths. All kinetic data were collected at 25 °C in 10 mM potassium phosphate buffer, pH 7.5, in the presence or absence of added salts. In mixing experiments, MADH was the limiting reactant with its concentration fixed between 1 and 3 μM. All experiments were performed under pseudo-first-order conditions with the amicyanin concentration varied between 45 and 200 μM, which is at least 25-fold in excess of TTQ.

Kinetic data collected in rapid-scanning mode were reduced by factor analysis using Global Fit, the singular value decomposition (SVD) algorithm (Matheson, 1987) provided by OLIS. SVD reduced data were then globally fit by Matheson's robust version of the Levenberg and Marquardt nonlinear method of least squares using the fitting routines of the Global Fit software (Matheson, 1989, 1990). Following factor and global kinetic analysis, spectral data were reconstructed in a mechanism-dependent fashion using OLIS algorithms and software. Kinetic data were also collected in the single wavelength mode at 443 nm, which is isosbestic for the conversion between semiquinone and oxidized MADH, and at 425 nm where the semiquinone exhibits its maximum visible absorbance. In these experiments, observed rate constants were extracted from exponential fits of the single wavelength data by the method of successive integration using OLIS software.

RESULTS AND DISCUSSION

Global Kinetic Analysis of Reactions between Reduced MADH and Oxidized Amicyanin and Spectral Reconstructions of Reaction Intermediates. The two-step reoxidations of O-quinol and N-quinol MADH by oxidized amicyanin were studied by rapid-scanning stopped-flow spectroscopy. Kinetic data were fit by global kinetic analysis to distinguish the appropriate kinetic mechanism and determine rate constants for individual reaction steps. From the factor and global analyses it was possible to reconstruct spectra of intermediate absorbing species in the sequential one-electron oxidations of both dithionite- and methylamine-reduced (O- and N-quinol) TTQ by two amicyanin equivalents. Figure 3 presents the raw data, kinetic fits, and spectral reconstruction of intermediates of the reactions of O-quinol and N-quinol MADH with amicyanin in 10 mM buffer in the absence of added salt.

The reaction of the O-quinol MADH with a saturating concentration of excess amicyanin is depicted in Figure 3A. During the course of the reaction, no spectral evidence for the accumulation of an O-semiquinone intermediate is evident in the raw data, and the kinetics are monophasic. This monophasic process is globally fit best[2] by the equation for a single exponential with a correction for a visible background:

$$Y = a_0 \exp^{(-k_{\text{obs}}t)} + c \qquad (1)$$

The CALCULATED SPECTRA in Figure 3A are reconstructed from a one-step mechanism:

$$\mathrm{A} \rightarrow \mathrm{C} \qquad (2)$$

where A and C are the absorbing species: O-quinol MADH and fully oxidized quinone MADH, respectively. The observed rate constant for this process is 10.2 ± 0.7 s^{-1}. The absence of an intermediate B in this simplified mechanism does not indicate the absence of B during the reoxidation, but rather exemplifies the point that the decay of B into C (O-semiquinone to quinone) is so fast, relative to the formation of O-semiquinone, that it does not accumulate during the reaction.

In striking contrast to the results obtained for the O-quinol MADH, when N-quinol MADH is mixed with oxidized amicyanin under identical conditions, there is an accumulation of an intermediate species centered at approximately 425 nm (Figure 3B), which is characteristic of semiquinone (Figure 2). The global kinetic analysis of the data obtained for the two-step oxidation of N-quinol by amicyanin is described best by the equation for a two exponential process with a correction for a visible background:[3]

$$Y = a_0 \exp^{(-k_{\text{obs1}}t)} + b_0 \exp^{(-k_{\text{obs2}}t)} + c \qquad (3)$$

The raw spectral data shown in Figure 3B are best deconvoluted by the equation for a two-step sequential reaction:[3]

$$\mathrm{A} \rightarrow \mathrm{B} \rightarrow \mathrm{C} \qquad (4)$$

where A is fully reduced N-quinol MADH, B is the N-semiquinone intermediate, and C is the quinone. The observed rate constants for these two steps are 42.0 ± 2 s^{-1} and 72.0 ± 8 s^{-1}. In the O-quinol system (Figure 3A), the rate of O-semiquinone disappearance was very rapid relative to its formation, thus preventing the accumulation of that intermediate. This is not the case for the intermediate in the reaction of the N-quinol (Figure 3B). Because the absorption spectra of O- and N-semiquinone are difficult to distinguish, one cannot, from the features of the spectra, judge which one is accumulating. The identity of this intermediate can, however, be deduced from knowledge of

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[2] Residual plots from the global kinetic analysis of the data contain a periodic noise background with a frequency of 16 ms. Since the noise is periodic, its effect on the uncertainty of the fits shown in Figures 3 and 4 is negligible.

[3] The number of rate constants that can be accurately determined by any fitting routine is a function of the signal-to-noise ratio (S/N) that is inherent in the data. For example, if two rate constants are to be accurately solved, then a S/N ratio of ~10% is adequate. Any more, and the solved parameters become less reliable (I. B. C. Matheson, personal communication). Thus, the S/N ratio limits the complexity of models that can be used to describe the decay processes. The S/N ratio of our data is between 5% and 10%. As a result, the two parameters obtained from two exponential fits of the data are quite reliable as are the resulting spectral reconstructions.

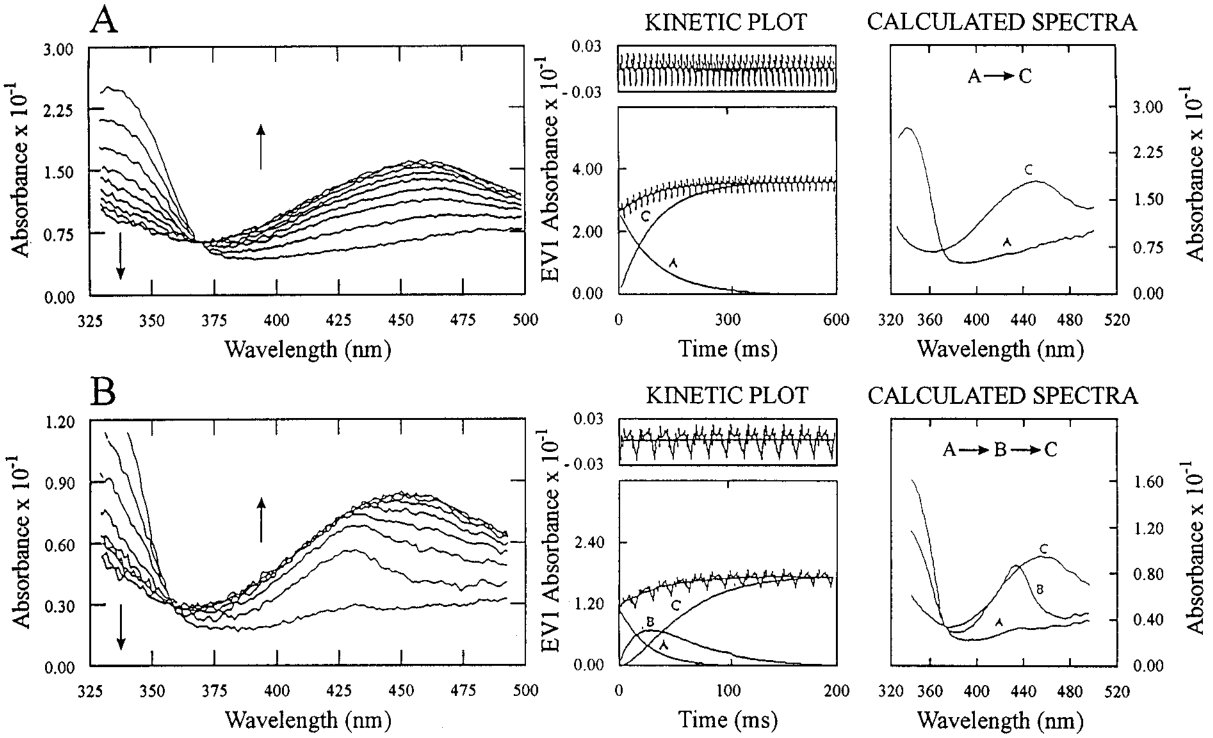

FIGURE 3: Complete oxidation of reduced MADH by oxidized amicyanin in low salt. (Panel A) The reaction of 2 μM dithionite-reduced (O-quinol) MADH with 50 μM amicyanin at 25 °C in 10 mM potassium phosphate buffer, pH 7.5, with no added KCl. The left-most plot shows the raw data collected during this experiment. The arrows indicate the directions of the spectral changes. Spectra were collected every millisecond. For clarity, only every 30th scan is shown. The KINETIC PLOT depicts global fits of the most statistically significant eigenvector (EV1) of the SVD reduced three-dimensional data. Progress curves for the quinol and quinone species are labeled as A and C, respectively. The plot labeled CALCULATED SPECTRA is the reconstruction of the initial and final absorbing species (A and C, respectively) for the A → C mechanism which best describes the data. (Panel B) The reaction of 2 μM substrate-reduced N-quinol MADH with 50 μM amicyanin. Reaction conditions were identical to those in panel A, and spectra were recorded every millisecond. For clarity, only every 20th scan is shown. Progress curves for the species A (N-quinol), B (N-semiquinone), and C (quinone) are shown in the KINETIC PLOT. CALCULATED SPECTRA is the reconstruction of the initial, intermediate, and final absorbing species for the A → B → C mechanism which best describes the data.

Table 1: Rates of Oxidation by Amicyanin of Reduced and Semiquinone Forms of MADH

reaction[a]	added salt[b]	wavelength(s) analyzed (nm)	k_{obs1} (s^{-1})[c,d]	k_{obs2} (s^{-1})[c,d]
O-quinol to quinone		global (300−500)	10.2 ± 0.7	
O-quinol to O-semiquinone		443	8.6 ± 0.5	
O-semiquinone to quinone		425		≥1000[e]
N-quinol to quinone		global (300−500)	42 ± 2	72 ± 8
N-quinol to N-semiquinone		443	38.2 ± 2	
N-semiquinone to quinone		425		60 ± 20
O-quinol to quinone	0.2 M KCl	global (300−500)	11.2 ± 0.5	
O-quinol to O-semiquinone	0.2 M KCl	443	9.3 ± 1.1	
O-semiquinone to quinone	0.2 M KCl	425		≥1000[e]
N-quinol to quinone	0.2 M KCl	global (300−500)	148 ± 10	69 ± 5
N-quinol to N-semiquinone	0.2 M KCl	443	162 ± 8	
N-semiquinone to quinone	0.2 M KCl	425		75 ± 10
O-quinol to quinone	0.2 M NH_4Cl	global (300−500)	8.8 ± 0.8	

[a] O-Quinol and O-semiquinone contain oxygen bound to the C6 carbon of TTQ. N-Quinol and N-semiquinone contain a substrate-derived nitrogen bound to the C6 carbon in TTQ. Methods for generating these forms are described in the text. [b] All reactions are performed in 10 mM potassium phosphate, pH 7.5 at 25 °C, in the presence or absence of added salts, except for the reactions performed in the presence of 200 mM ammonium chloride which were buffered with ammonium phosphate, pH 7.5. [c] The rate constants k_{obs1} and k_{obs2} represent the rates of the first and second one-electron oxidation steps in the conversion of the quinol to quinone forms that occur via semiquinone intermediates. [d] All parameters are obtained from fits of the data from multiple experiments by either eq 1 or eq 3 and are presented ± one standard deviation within a 95% confidence interval. [e] The reaction was complete within the dead time for mixing in the stopped-flow and assumed, therefore, to be at least 1000 s^{-1}.

the kinetics of the reactions of the different redox forms of MADH with amicyanin. The intermediate in Figure 3B cannot be O-semiquinone because the rate of its decay is much too slow (i.e., $\ll$1000 s^{-1}).

The experiments which are presented in Figure 3 were repeated in 10 mM potassium phosphate buffer, pH 7.5, in the presence of added 200 mM KCl (referred to as high salt conditions). Under these conditions, the rate of the first one-electron oxidation step of the O-quinol is unchanged but the rate of this step for the N-quinol is much faster (Table 1). The global fit of the kinetic data for the reaction of O-quinol MADH with amicyanin is again best described by a single exponential (eq 1), and the spectral data are again best deconvoluted by the single-step mechanism shown in eq 2

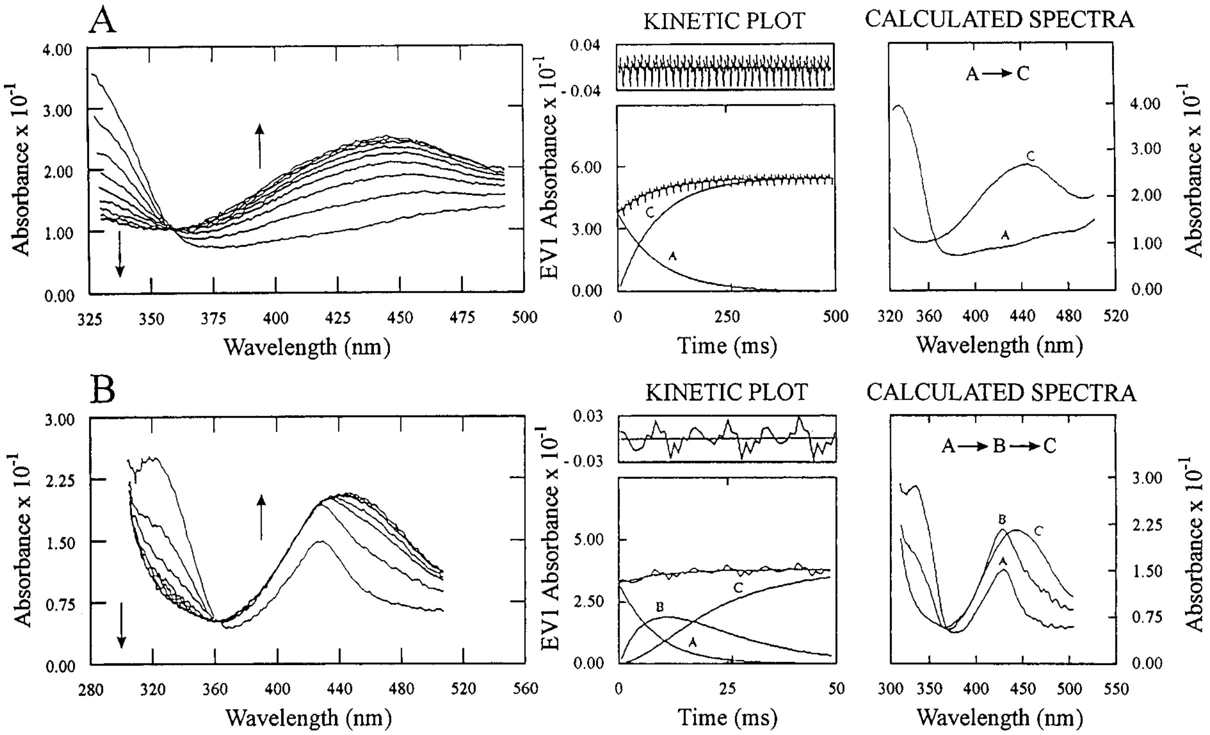

FIGURE 4: Complete oxidation of reduced MADH by oxidized amicyanin in high salt. (Panel A) The reaction of 2 μM dithionite-reduced (O-quinol) MADH with amicyanin (65 μM) at 25 °C in 10 mM potassium phosphate buffer, pH 7.5, with 200 mM potassium chloride. The left-most plot shows the raw data collected during this experiment. The arrows indicate the directions of the spectral changes. Spectra were collected every millisecond. For clarity, only every 30th scan is shown. The KINETIC PLOT depicts global fits of the statistically most significant eigenvector (EV1) of the SVD reduced three-dimensional data. Progress curves for the O-quinol and quinone species are labeled as A and C, respectively. The plot labeled CALCULATED SPECTRA is the reconstruction of the initial and final absorbing species (A and C, respectively) for the A → C mechanism which best describes the data. (Panel B) The reaction of 2 μM substrate-reduced N-quinol MADH with 45 μM amicyanin. Reaction conditions were identical to panel A, and spectra were recorded every millisecond. For clarity, only every 5th scan is shown. Progress curves for the species A (aminoquinol), B (N-semiquinone), and C (quinone) are shown in the KINETIC PLOT. CALCULATED SPECTRA is the reconstruction of the initial, intermediate, and final absorbing species for the A → B → C mechanism which best describes the data.

(Figure 4A). In contrast, Figure 4B shows the progression of the N-quinol reaction with amicyanin in the presence of 200 mM KCl. As in the low salt conditions, there is an accumulation of an intermediate species centered at 425 nm. The kinetics are again globally fit best by eq 3, and the spectra for the biphasic process are reconstructed best by eq 4. It should be noted that species A in the CALCULATED SPECTRA panel of Figure 4B is not the spectrum of the starting N-quinol species. This is because the conversion of species A into B is too fast to observe the complete reaction in the rapid-scanning mode. Prior to mixing, the spectrum of the N-quinol was as in Figures 2 and 3B. It should also be noted that the reconstructed spectra (Figure 3 and 4) of the reduced forms of MADH slope upward at higher wavelengths. This is simply due to the absorbance of excess amicyanin in this region.

The spectra of the accumulating semiquinones, which are reconstructed from the low and high salt data and presented in the far right panels of Figure 3B and 4B, exhibit a smaller extinction coefficient at 425 nm than the spectrum of the O-semiquinone generated by dithionite which is presented in Figure 2. This may be due to inherent differences in the O- and N-semiquinone spectra. It is also possible that the semiquinone spectrum is perturbed after amicyanin binds to MADH. It is known that the spectrum of oxidized MADH is perturbed on binding of amicyanin (Gray *et al.*, 1988). Alternatively, the differences in the spectra pictured in Figure 2 and those in Figure 3B and 4B could arise from kinetic considerations, since the rate of N-semiquinone decay is fast enough that it may not fully accumulate. For example, the presence of 200 mM KCl increased not only the rate of appearance of the intermediate in the overall reaction of N-quinol, but also the extent of N-semiquinone accumulation relative to low salt conditions. This is because the rate of the first one-electron oxidation of N-quinol is increased relative to low salt conditions, while the rate of oxidation of the N-semiquinone remains unchanged (Table 1). Thus, the rate of the one-electron oxidation of the N-quinol is salt dependent. In contrast, the rate of the second oxidation step, where the N-semiquinone is converted to the oxidized quinone form of TTQ, is independent of the salt concentration over this range.[4]

Reactions of Preformed O-Semiquinone and N-Semiquinone MADH with Oxidized Amicyanin. To verify the results of the global kinetic analysis of the overall two-step oxidation reactions described above, efforts were made to directly measure each of the rate constants for the individual one-electron oxidations by mixing each reduced and semiquinone form of MADH with amicyanin and analyzing the kinetic data at single wavelengths which exhibit changes in absorbance that are known to describe each reaction step of interest. These results are summarized in Table 1. As noted

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[4] When these same experiments are performed in the presence of ≥400 mM KCl, anomalous behavior was observed. Thus, the observation that the rate of N-semiquinone oxidation to the quinone by amicyanin is independent of the KCl may be valid only over a limited range of specific cation concentrations. Further studies are in progress to elucidate the basis for this phenomenon.

previously (Brooks & Davidson, 1994a), the rate of oxidation of the O-semiquinone by amicyanin is at least 2 orders of magnitude faster than the rate of the oxidation of the O-quinol. In both low and high salt, the rate of oxidation of the O-semiquinone by amicyanin is faster than can be detected by rapid-mixing in the stopped-flow. The lower limit for the rate of O-semiquinone oxidation by amicyanin is obtained from the collection rate and mixing time of the instrument (∼2 ms) while using the single wavelength mode. The progression of a reaction with an observed rate constant $\geq 1000\ s^{-1}$ will not be detected even when high enzyme concentrations are used to maximize the S/N ratio. This is the case for the reaction of the O-semiquinone at 25 °C.

Analysis of the rate of the reaction of the N-semiquinone that is generated *in vitro* by comproportionation of a 1:1 solution of the N-quinol and quinone forms of MADH is complicated because after comproportionation one obtains a mixture of O- and N-semiquinone that cannot be separated. Depending upon salt concentration, some N-quinol may also be present in the mixture. Since the reaction of the O-semiquinone is too rapid to detect with our instrumentation, any reaction of a semiquinone species that is observed in this experiment will be the slower and detectable reaction of the N-semiquinone decay into the quinone. With our knowledge of the kinetics of the reactions of the N-quinol with amicyanin, it is also possible to deconvolute the kinetic data to identify the rate constant which describes the reaction of N-semiquinone with amicyanin. Global analysis of kinetic data obtained for the reaction of the mixture containing preformed N-semiquinone with amicyanin in the rapid-scanning mode yields a similar rate constant; however, spectral reconstructions are complicated by the presence of multiple species. This rate constant is essentially the same as that obtained from the global kinetic analysis described earlier (Table 1). These comparative studies allow us to conclude that the intermediate which accumulates during the two-step reaction between N-quinol MADH and amicyanin is kinetically identical to the N-semiquinone which has been generated *in vitro*, by comproportionation, and which was previously shown (Warncke *et al.*, 1993) to possess a substrate-derived nitrogen.

Insensitivity of MADH Oxidation by Amicyanin to Ammonium Chloride. It has been suggested that the N-semiquinone does not exist as a reaction intermediate and that properties attributed to the N-semiquinone which was generated *in vitro* by comproportionation may alternatively be explained by the influence of ammonia which is noncovalently bound in the active site in close proximity to the C6 oxygen of the O-semiquinone species (Gorren *et al.*, 1995a). This seems unlikely since the reported K_d for this putative ammonia binding site is at least in the millimolar range (McIntire, 1987; Davidson & Jones, 1992; Gorren & Duine, 1994), and during *in vitro* preparation of the N-semiquinone, any ammonia generated from the reaction of stoichiometric concentrations of methylamine with MADH would be present in a concentration orders of magnitude less than this. However, to test this hypothesis, the reaction of O-quinol MADH with amicyanin was repeated in the presence of 200 mM ammonium chloride in 10 mM ammonium phosphate buffer, pH 7.5, under pseudo-first-order reaction conditions. The results (data not shown) were exactly as shown in Figures 3A and 4A in the absence of ammonia, with no detectable accumulation of a semiquinone intermediate. Furthermore, all kinetics were best described by a single exponential decay (eq 1), and all spectra were best reconstructed by eq 2. The presence of excess ammonia, therefore, does not mimic the N-quinol results of Figures 3B and 4B. Thus, the kinetic behavior, which we have attributed earlier to being that of the N-semiquinone, cannot be due instead to the effect of noncovalently bound ammonia on the O-semiquinone.

Physiologic Relevance of the N-Semiquinone. These data provide strong evidence against alternative mechanisms for the amicyanin-mediated oxidation of MADH where ammonia is released from TTQ before the first or second one-electron oxidation steps. The fact that an intermediate is detectable (Figures 3B and 4B) in the reaction of N-quinol MADH demonstrates two points. First, the product of the one-electron reduction of the N-quinol is not O-semiquinone and, therefore, must still retain the amino group (i.e., N-semiquinone). Second, the presence of the substrate-derived amino group on TTQ dramatically decreases the reactivity of the N-semiquinone relative to that of O-semiquinone.

It was recently suggested (Gorren *et al*., 1995a) that the N-quinol has not been definitively shown to be the product of the reduction of MADH by substrate. These authors suggest that the oxidation of methylamine may proceed via an imine elimination mechanism without formation of a stable covalent adduct between methylamine and TTQ. This mechanism contradicts a large body of evidence which has established that the reductive half-reaction of MADH involves a covalent substrate−TTQ adduct and an aminotransferase mechanism involving a carbanion intermediate (Davidson *et al*., 1992b, 1995; Brooks *et al*., 1993; Davidson & Jones, 1992, 1995). Furthermore, we have provided strong data in this, and previous, reports that clearly demonstrate that the N-quinol is a stable and discrete species with different reactivity toward amicyanin than the O-quinol. This was supported by kinetic, thermodynamic, and deuterium kinetic solvent isotope effect studies which showed that the rate-limiting step for the first electron transfer from N-quinol MADH to amicyanin is very different than that from the O-quinol MADH, the former reaction being gated by proton transfer (Bishop & Davidson, 1995). In a subsequent study, Gorren *et al.* (1995b) make no distinction between substrate- and dithionite-generated MADH semiquinones. The results described herein clearly confirm structural differences in the two semiquinone forms of MADH and prove that they cannot be explained merely by the influence of noncovalently bound ammonia on O-semiquinone TTQ.

It has been reported (Gorren & Duine, 1994) that monovalent cations stimulated the rate of oxidation of methylamine-reduced *Thiobacillus versutus* MADH and that the semiquinone intermediate, which was proposed to be O-semiquinone, only accumulated in the presence of added salt (200 mM in that study). Our results confirm that the rate of the first oxidation step of N-quinol MADH is dependent upon the salt concentration. However, important distinctions must be clarified. Only the N-quinol rate exhibited the observed salt dependence. The rate of the first electron transfer from the dithionite-generated O-quinol MADH, and the rate of the electron transfer from the N-semiquinone, were not affected by salt concentration in the range of 0−200 mM. If O-semiquinone were the intermediate for the reactions with the N-quinol, it would not accumulate under these conditions since its rate is $>1000\ s^{-1}$, which is beyond the limits of detection by stopped-flow spectroscopy. Therefore, the N-quinol must be the reacting species during the physiologic reaction cycle. We do observe that the N-semiquinone accumulates to a greater extent in high salt, but in contrast to the results obtained with *T. versutus* MADH (Gorren & Duine, 1994), we also observe the N-semiquinone in the absence of added salt. This discrepancy may be due to differences in the source of the enzyme, but it also may be due to differences in the techniques used to monitor the reactions. Their kinetic data were collected by monitoring absorbance changes at single wavelengths. For the data shown in Figure 3B of this paper, if only changes in absorbance at selected single wavelengths were monitored, it would have been very difficult, if not impossible, to make conclusions about the accumulation of a semiquinone intermediate. Global kinetic analysis of absorbance data collected over a wide range of wavelengths versus time provides a more complete and detailed analysis of the spectral data concerning the accumulation of spectral intermediates, as well as the exponential equations which describe the decay processes. These points reveal the value of the rapid-scanning technology and global kinetic analysis for identifying reaction intermediates.

CONCLUSIONS

These results and previous work in our laboratory (Bishop & Davidson, 1995) show that the first one-electron oxidations of N-quinol and O-quinol MADH to their respective semiquinone intermediates by amicyanin are mechanistically distinct. Relative to the reaction of the O-quinol, the rate of reaction of the N-quinol form of MADH with amicyanin is rapid and dependent upon salt concentration (Table 1), and exhibits a large primary deuterium solvent kinetic isotope effect which is consistent with a proton-linked mechanism of electron transfer (Bishop & Davidson, 1995). Conversely, the rate of the first oxidation step of the O-quinol form of TTQ to amicyanin is slower and independent of salt concentration (Table 1), and is rate-limited by nonadiabatic electron transfer as determined by the temperature (Brooks & Davidson, 1994b; Bishop & Davidson, 1995) and free energy dependencies of the reaction rate (Brooks & Davidson, 1994a). These findings establish critical differences in reactivity and structure between the O-quinol and N-quinol forms of MADH. The present work also characterizes differences between the O- and N-semiquinone forms of MADH and establishes that the latter is stable enough to serve as an intermediate during the physiologic reactions between MADH and amicyanin. Our data, as that obtained with *T. versutus* MADH (Gorren & Duine, 1994; Gorren *et al.*, 1995a,b), also suggest that monovalent cations selectively affect the rate of reaction of the N-quinol. How this is mechanistically done and how this relates to the stability of the N-semiquinone are fascinating questions. Further studies are in progress in our laboratory to precisely define these phenomena.

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