Semiquinone Radicals of Methylamine Dehydrogenase, Methoxatin, and Related *o*-Quinones: A Pulse Radiolysis Study†

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ABSTRACT: Methoxatin is a novel *o*-quinone coenzyme found in a variety of dehydrogenases and oxidases. In this paper we present the visible absorption spectra, apparent pK_a, and midpoint potentials of the methoxatin and two analogous phenanthrolinedione semiquinones. We have also obtained absorption spectra for the semiquinone forms of the methoxatin-containing holoenzyme methylamine dehydrogenase and of its resolved methoxatin-containing subunit. The two protein and the methoxatin semiquinone spectra all differ from one another.

Methoxatin, also called pyrroloquinoline quinone (PQQ,[1] Figure 1), is an *o*-quinone found in oxidases and dehydrogenases from a variety of bacteria [e.g., Westerling et al. (1979), Duine et al. (1980, 1981), deBeer et al. (1983), and Kawai et al. (1985)], plants (Glatz et al., 1987), and mammals [e.g., Ameyama et al. (1984, 1985), Lobenstein-Verbeek et al. (1984), van der Meer and Duine (1986), and Williamson et al. (1986)]. The crystal structure of methoxatin (Salisbury et al., 1979) has been verified by the many de novo syntheses (Gainor & Weinreb, 1981; Corey & Tramontano, 1981; Hendrickson & deVries, 1982; MacKenzie et al., 1983). The methoxatoenzymes are a new class of proteins that from their ubiquity appear to be important. PQQ itself may be a vitamin (Killgore et al., 1989). Thus, we have chosen to study the chemistry of this *o*-quinone, and since methoxatoenzymes stabilize the PQQ semiquinone ($PQQ^{\bullet-}$), we have begun with the physical properties of this radical, both in aqueous solution and when attached to the enzyme methylamine dehydrogenase (MADH).

MADH, an $\alpha_2\beta_2$ complex with one PQQ bound covalently to each β-subunit, catalyzes electron transfer from two-electron primary amine donors to one-electron acceptors (Tobari & Harada, 1981; Husain et al., 1986; Chandrasekar & Klapper, 1986). Since MADH stabilizes a spectral species believed to be a one-electron-reduced semiquinone $PQQ^{\bullet-}$ (Kenney & McIntire, 1983), MADH catalysis may involve one-electron shuttling through this radical. Although spectral and redox properties of various *o*- and *p*-semiquinones are known [e.g., Swallow (1982)], only preliminary data are available for the semiquinones of PQQ (Eckert et al., 1982; Duine et al., 1984; Faraggi et al., 1986) and its close relative 9-decarboxymethoxatin (Rodriguez et al., 1987). The *o*- and *p*-semiquinone radicals are difficult to study at neutral pH in aqueous solution since they decay rapidly by disproportionation and/or radical–radical recombination. Still, with the technique of pulse radiolysis (Matheson & Dorfman, 1969) we can study such semiquinones in general [e.g., Swallow (1982)] and $PQQ^{\bullet-}$ in particular (Faraggi et al., 1986).

Despite their instability in aqueous solution, semiquinones can be studied with the rapid kinetic technique of pulse radiolysis. Short pulses (<1 μs) of high-energy electrons (ca. 3.5 MeV from the Ohio State Chemistry Department Linear accelerator) interact with water to produce the three primary radicals: the hydrated electron ($e_{aq}^{\bullet-}$), the hydroxyl radical ($OH^{\bullet}$), and the hydrogen atom ($H^{\bullet}$). In the presence of formate and saturating N_2O these radicals are converted within 1 μs to the formate radical ($CO_2^{\bullet-}$) in the following series of reactions:

$$e_{aq}^{\bullet-} + N_2O \xrightarrow{H_2O} N_2 + OH^{\bullet} \quad (1a)$$

$$OH^{\bullet} + HCOO^- \rightarrow CO_2^{\bullet-} + H_2O \quad (1b)$$

$$H^{\bullet} + HCOO^- \rightarrow CO_2^{\bullet-} + H_2 \quad (1c)$$

With a redox potential more negative than −1 V, $CO_2^{\bullet-}$ effects the one-electron reduction of many molecules including quinones and oxygen:

$$CO_2^{\bullet-} + Q \rightarrow Q^{\bullet-} + CO_2 \quad (2a)$$

$$CO_2^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + CO_2 \quad (2b)$$

These reactions are readily monitored with the UV/visible absorbances of the various radicals involved. The preparation of superoxide ($O_2^{\bullet-}$) from oxygen does not require N_2O, for the $CO_2^{\bullet-}$ made in reactions 1b and 1c reduces O_2 in reactions 2b and the pulse-generated $e_{aq}^{\bullet-}$ reduces oxygen in reaction 3.

$$e_{aq}^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} \quad (3)$$

Semiquinone radicals can be effectively stable over the short time of the pulse radiolysis experiment. Thus, it has been possible to measure semiquinone absorption spectra, redox potentials, and the pK_a for the equilibrium

$$QH^{\bullet} \rightleftharpoons Q^{\bullet-} + H^+ \quad (4)$$

We have used the radical chemistry outlined briefly to (i) extend our earlier work on the PQQ semiquinone, (ii) discuss the properties of the related semiquinones formed from 1,10-phenanthroline-5,6-dione (1,10-PD) and 1,7-phenanthroline-5,6-dione (1,7-PD) (Figure 1), and (iii) de-

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[1] Abbreviations: $CO_2^{\bullet-}$, formate radical; DCPIP, dichlorophenolindophenol; $e_{aq}^{\bullet-}$, hydrated electron; E_m', midpoint potential at pH 7.0 relative to NHE; $H^{\bullet}$, hydrogen atom; $OH^{\bullet}$, hydroxyl radical; MADH, methylamine dehydrogenase; $MV^{\bullet+}$, methylviologen cation radical; $O_2^{\bullet-}$, superoxide anion radical; 1,10-PD, 1,10-phenanthroline-5,6-dione; 1,10-$PD^{\bullet-}$, 1,10-PD anionic radical semiquinone; 1,10-$PDH^{\bullet}$, 1,10-PD protonated radical semiquinone; 1,7-PD, 1,7-phenanthroline-5,6-dione; 1,7-$PD^{\bullet-}$, 1,7-PD anionic radical semiquinone; 1,7-$PDH^{\bullet}$, 1,7-PD protonated radical semiquinone; PQQ, methoxatin or pyrroloquinoline quinone; $PQQ^{\bullet-}$, PQQ anionic radical semiquinone; $PQQH^{\bullet}$, PQQ neutral radical semiquinone; Q, generic quinone; $Q^{\bullet-}$, anionic radical semiquinone; $QH^{\bullet}$, neutral radical semiquinone.

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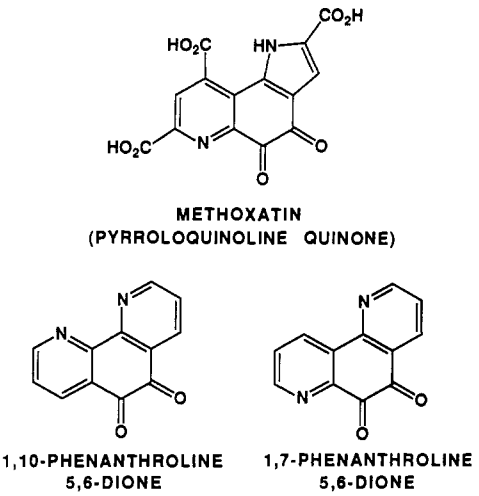

FIGURE 1: Structures of methoxatin and phenanthrolinediones.

scribe the preparation of the purported PQQ semiquinone species of MADH by one-electron reduction with the methylviologen cation radical ($MV^{\bullet+}$).

Materials and Methods

We have already described our linear accelerator and pulse radiolysis methods (Felix et al., 1967; Faraggi et al., 1985). We obtained semiquinone − quinone difference spectra, reconstructed from separate kinetic experiments at individual wavelengths, with oxygen-free (Meites & Meites, 1948) N_2O-saturated solutions that contained 0.1 M formate, 5 mM phosphate, millimolar quantities of the quinone under study, and micromolar quantities of $CO_2^{\bullet-}$. Reaction conditions for $O_2^{\bullet-}$ reduction experiments were the same except for the replacement of N_2O by O_2. N_2O-saturated solutions still contained enough contaminating oxygen to oxidize the methylviologen cation radical, $MV^{\bullet+}$. To avoid this side reaction, we added an enzymatic O_2 scavenging system: 5 mM D-glucose with catalytic amounts of glucose oxidase and catalase (Englander et al., 1987). The added O_2 scavenging system did not compete effectively for the reactant radicals in control experiments.

We isolated W3A1 methylamine dehydrogenase with a procedure modified slightly from that of Chandrasekar and Klapper (1986). W3A1 cultures were stored in glass ampules at −70 °C. The bacteria were grown at room temperature with aeration. Into 16 L of sterilized minimal liquid medium (Owens & Keddie, 1969) we added unsterilized methylamine to a final concentration of 0.3% (w/v) and then immediately innoculated with 1 L of a 2-day-old W3A1 culture grown in the same liquid medium. When the suspension had an absorbance reading of about 1 at 600 nm (ca. 1–2 days), we collected the bacteria by centrifugation at 15 000 rpm in a Sorvall SS-4 centrifuge equipped with an SS-34 rotor and a Szent–Gyorgyi continuous-flow system (Ivan Sorvall, Norwalk, CN). The resultant pink pellets were then wet slightly with 20 mM phosphate buffer, pH 7, and stored for up to 2 weeks at −20 °C. The thawed cell paste was suspended (ca. 200 g of wet cell paste to 500 mL) in 20 mM phosphate, pH 7. With the beaker immersed in ice–water, this suspension was sonicated (Fisher Sonic Dismembrator Model 33) for 4 min at maximum intensity. After centrifugation of the sonicated suspension at 11 000 rpm for 20 min, we saved the supernatant and gently washed the pink pellet with deionized water to remove a viscous top layer. The washed pellet was resuspended in 20 mM phosphate, pH 7, to a volume of 500 mL for a second sonication. We adjusted the pH of the two supernatants back to pH 7 with NaOH and then heated the combined solution for 20 min at 70 °C. After cooling, the suspensions were centrifuged for 45 min at 11 000 rpm, and we either processed the final supernatant immediately or stored it at −20 °C for later use.

After a 1:1 dilution with cold water, the preparation was passed through a Sephadex CM-50 (Pharmacia Inc., Piscataway, NJ) 15 × 5 cm column previously equilibrated with 20 mM phosphate buffer, pH 7. The protein on the column was washed first with 1 L of the same buffer and then with 500 mL of 35 mM, pH 7, phosphate. This 35 mM buffer wash eluted no protein but did establish proper separation in the subsequent elution of MADH with 60 mM phosphate buffer, pH 7. The green protein fractions were combined, diluted to 20 mM phosphate with cold water, and passed through a 3 × 1 cm column of the same ion exchange resin. After being washed with 0.5–1.0 L of the 20 mM, pH 7 buffer, the concentrated protein was eluted with 0.4 M, pH 7.5, phosphate buffer. Since this otherwise pure enzyme was always partially reduced, we incubated the protein with 5–10 mM $K_3Fe(CN)_6$ at 5 °C for 15–60 min in the dark. The oxidized preparation was then passed through a 8 × 1 cm 1-X8 (J. T. Baker Chemical Co., Phillipsberg, NJ) column to remove ferri- and ferrocyanide (Klapper & Klotz, 1968). The final preparation was stored either after exchanging the buffer to 0.1 M, pH 7.5, phosphate and freezing or after exchanging into 0.2 mM, pH 7.2, phosphate and lyophilizing. Both preparations were stable for months.

MADH is an $\alpha_2\beta_2$ protein. We resolved the smaller β-subunit with its covalently attached quinone from the α-subunit in two ways. In the first, we started by rapidly stirring cold glacial acetic acid into ice-cold MADH in 0.2 M phosphate buffer, pH 7.0. The resulting brown solution at 35–40% (v/v) acetic acid was passed through a Sephadex G-75 column previously equilibrated with 25% acetic acid. The brown β-subunit containing fractions were combined, dialyzed against 5 L of water for approximately 30 min, and lyophilized to remove remaining acetic acid. After resuspension in water, the protein solution was centrifuged to remove insoluble material and then lyophilized for storage. In this procedure the colorless larger α-subunit denatures. In the second method, we added guanidine hydrochloride to 5.5 M while maintaining the solution pH above 6.5 and then separated the two subunits on a Sephadex G-75 column equilibrated with 0.5 M guanidine hydrochloride. The two sets of fractions containing the resolved subunits were pooled separately, dialyzed against six 1-L changes of 0.1 M, pH 7.5, phosphate, and stored frozen at −20 °C. We measured the concentrations of the tetrameric MADH and the PQQ-containing β-subunit from their visible absorbances with respective extinction coefficients of ϵ_{444} = 8340 ± 230 cm^{-1} M^{-1} and ϵ_{425} = 8340, determined as described below.

To reconstitute MADH from its subunits, we added varying amounts of the α-subunit to 1 mL of the stock β-subunit, obtained either by glacial acetic acid or guanidine hydrochloride resolution. Each reconstitution mixture was diluted to 3 mL and a final phosphate concentration of 0.2 M, pH 7.5. After incubation at 5 °C for 60 h, we determined specific activity (moles of DCPIP reduced per mole of β-subunit per second) by the method of Eady and Large (1968). We measured the α-subunit concentration with the Bradford (1976) assay, in which bovine serum albumin was the reference.

Methoxatin was synthesized by the method of MacKenzie et al. (1983). 1,7-Phenanthroline-5,6-dione, synthesized by

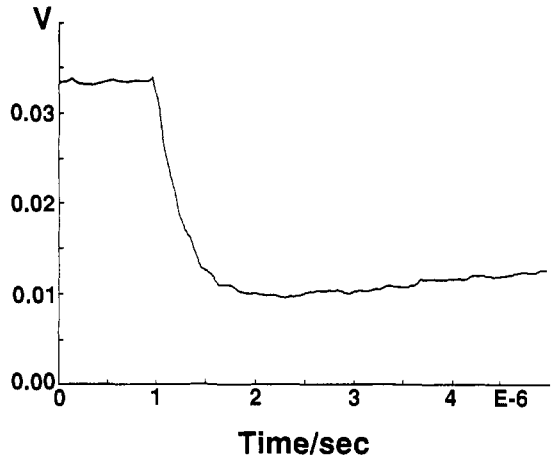

FIGURE 2: $CO_2^{\bullet-}$ reduction of 1,7-phenanthroline-5,6-dione: transmittance–time profile. The initial plateau is the base line. After the pulse, the transmittance decrease (absorbance increase) at 630 nm is due to the reduction of 190 μM 1,7-PD by 3 μM $CO_2^{\bullet-}$ at pH 3.91, 25 °C. The subsequent slow transmittance increase (absorbance decrease) is the start of the second-order 1,7-PDH$^{\bullet}$ radical decay.

Table I: Summary of Semiquinone Physical Properties[a]

semi-quinone	λ_{max} (nm)[a]	ϵ (cm^{-1} M^{-1})[a]	pK_a	$k_{(CO_2^{\bullet-}+Q)}$ (M^{-1} s^{-1})[b]	E_m' (mV)[c]
PQQ	460 (7.3) 470 (2.0)	3200 (7.3) 1600 (2.0)	4.7, 6.8	0.28×10^9	−122 (3)
1,7-PD	490 (8.0) 500 (3.0)	4800 (8.0) 3700 (3.0)	6.2	1.8×10^9	4.5 (4)
1,10-PD	490 (8.0) 510 (3.0)	4500 (8.0) 3400 (3.0)	5.1	1.8×10^9	−3 (3)

[a] Figure given in parentheses is the pH at which the value was determined. [b] Measured at pH 7.2. [c] Estimated standard deviations for E_m' values are within parentheses.

the method of Eckert et al. (1982), was recrystallized twice from chloroform/hexane (1:1). Methylviologen dichloride (Sigma Chemical Co., St. Louis) was recrystallized three times from cold ethanol. 2,6-Dimethyl-1,4-benzoquinone (Aldrich Chemical Co, Milwaukee, WI) was sublimed from watchglass to watchglass three times. 1,10-Phenanthroline-5,6-dione was purchased from Alfa Products (Danvers, MA) and used without further purification. Remaining reagents came from common commercial sources.

All nonlinear least-squares data analyses were done with the software package MINSQ (Micromath, Salt Lake City, Utah) running on an IBM PC compatible.

Results

Spectral and Acid/Base Properties of o-Semiquinones. $CO_2^{\bullet-}$, generated by pulse radiolysis, reduces quinones to their semiquinone forms, eq 2a, as seen in the Figure 2 representative transmittance–time profile for formation and decay of the semiquinone 1,7-PDH$^{\bullet}$ from 1,7-phenanthroline-5,6-dione (1,7-PD). The initial transmittance decrease (absorbance increase) at 630 nm reflects $CO_2^{\bullet-}$ reduction of excess 1,7-PD. The observed dependence of the pseudo-first-order rate constant on quinone concentration gives the second-order one-electron-reduction rate constant (Table I). The slower transmittance increase in Figure 2 is the initial portion of a second-order radical decay, since 1,7-PDH$^{\bullet}$ is unstable in aqueous solution, as are most semiquinones.[2] Because radical decay is second order and radical formation is pseudo first order, we effectively separate semiquinone formation from its decay by raising the quinone concentration and lowering the amount of generated $CO_2^{\bullet-}$. In this way semiquinone loss was kept at ≤10% of its maximum concentration for a time of ca. $20t_{1/2}$ (growth); i.e., the semiquinone was effectively stable during our experiments. We repeated the experiment of Figure 2 with fresh solutions at a number of wavelengths in order to construct time-dependent difference (semiquinone − quinone) spectra. Under our experimental conditions, the quinone reacts with more than 95% of the generated $CO_2^{\bullet-}$ so that the maximum absorbance excursion yields a good estimate at each wavelength of $\Delta\epsilon$ for the conversion of quinone to semiquinone. The difference spectra for the PQQ, 1,7-PD, and 1,10-PD semiquinones are in Figure 3; their measured λ_{max} and ϵ_{max} are in Table I. With difference spectra and the independently measured spectra of the fully oxidized quinones available, we calculated absolute semiquinone spectra (Figure 4). These differ for all three *o*-quinones at neutral and acidic pH, a pH

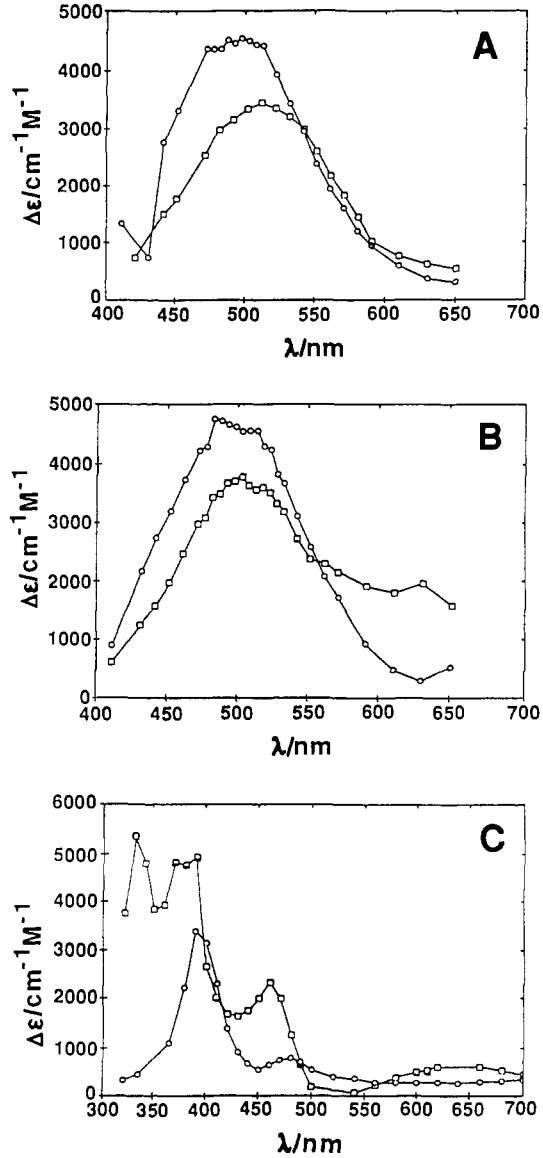

FIGURE 3: Semiquinone difference spectra. The semiquinone minus quinone difference spectra were obtained as described in the text. The lines were drawn in arbitrarily. (A) 1,10-Phenanthroline-5,6-dione at pH 3.1 (□) and 8.4 (○), determined with a solution containing 1.0 μM $CO_2^{\bullet-}$ and 1 mM 1,10-PD. (B) 1,7-Phenanthroline-5,6-dione at pH 3.0 (□) and 8.0 (○), determined with a solution containing 1.0 μM $CO_2^{\bullet-}$ and 1 mM 1,7-PD. (C) Methoxatin at pH 2.0 (○) and 7.3 (□), determined with a solution containing 2.5 μM $CO_2^{\bullet-}$ and 0.2 mM methoxatin.

[2] The approximate second-order decay rate constants at pH 7.3 for the two phenanthroline semiquinones are 1×10^9 M^{-1} s^{-1} and for the PQQ semiquinone 1×10^8 M^{-1} s^{-1}.

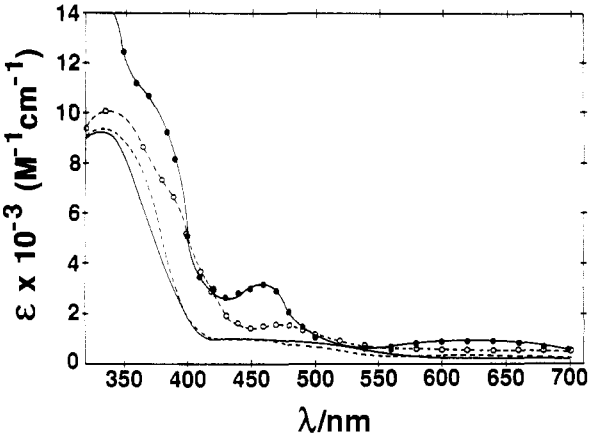

FIGURE 4: Absolute spectra of methoxatin and the methoxatin semiquinone: (—) methoxatin at pH 7.3; (●) methoxatin semiquinone at pH 7.3; (---) methoxatin at pH 2.0; (○) methoxatin semiquinone at pH 2.0. The conditions for the production of the semiquinone species were those described in the legend for Figure 3C. The dashed lines connecting the points were drawn in arbitrarily. The methoxatin spectra were obtained with a Uvikon 820, Kontron spectrophotometer.

dependence due to the weak acidity of semiquinones, eq 4 (Swallow, 1982). From the observed pH dependence of the absorbance changes we extracted the apparent pK_a (Table I) by nonlinear fitting. *Single* acid/base equilibria with pK_a of 5.1 (±0.2) and 6.2 (±0.1) adequately describe the pH dependence for both phenanthroline semiquinones, 1,10-PDH$^•$ and 1,7-PDH$^•$, respectively. The PQQ semiquinone data, however, require an assumption of *two* acid/base groups, and the pK_a we extracted from this set of data are 4.6 (±0.1) and 6.8 (±0.1).

Redox Potentials of o-Semiquinones. One can obtain redox potentials for the three semiquinone radical couples (Q/Q$^{•-}$) by comparison against an appropriate reference (R/R$^{•-}$) in the equilibrium

$$Q + R^{•-} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} Q^{•-} + R \qquad (5)$$

The primary requirement to such a measurement is that the equilibrium be established more rapidly than the decay of either R$^{•-}$ or Q$^{•-}$. Pulse radiolysis, a fast kinetic technique, permits us to meet this requirement, and as described elsewhere (DeFelippis et al., 1989), the equilibrium constant, K_{eq}, is measurable by both a kinetic and an equilibrium protocol. The pseudo-first-order equilibration rate constant, k_{app}, is given by eq 6 if (i) reaction 5 is elementary with $K_{eq} = k_f/k_b$, (ii) the reference couple is $O_2/O_2^{•-}$ (Ilan et al., 1976; Land et al., 1983; Faraggi et al., 1986), and (iii) the initial concentrations Q_o and $O_{2,o}$ are in large excess over those of $O_2^{•-}$ and Q$^{•-}$:

$$k_{app} = k_f Q_o + k_b O_{2,o} \qquad (6)$$

From the linear correlations of k_{app} with Q_o at different $O_{2,o}$, we obtain the individual rate constants, k_f and k_b, and so K_{eq}. This is the kinetic protocol.

The equilibrium protocol, based entirely on measured equilibrium absorbances (A_{eq}), is independent on the reaction scheme. The appropriate expression for the analysis of the equilibrium data (DeFelippis et al., 1989) obtained with the reference radical $O_2^{•-}$ is

$$A_{eq} - A_o = \frac{K_{eq}(\epsilon_{Q•^-} - \epsilon_Q)}{K_{eq} + O_{2,o}/Q_o} D \qquad (7)$$

with A_o the absorbance at the reaction time zero and D, $O_{2,o}$ and Q_o the total radical, initial oxygen, and initial quinone concentrations, respectively. We obtained K_{eq} by the nonlinear fit of eq 7 to the measured dependence of $A_{eq} - A_o$ on the concentration ratio $O_{2,o}/Q_o$. The K_{eq} obtained by the kinetic and equilibrium protocols for the quinones PQQ, 1,10-PD, 1,7-PD, and 2,6-dimethylbenzoquinone (DMBQ) were always similar within experimental error—consistent with a single-step reaction (eq 5). The values of K_{eq}, averaged between the two protocols, are collected in Table II.

Table II: Midpoint Potentials of Semiquinones[a]

quinone	ref[b]	K_{eq}[c]	ΔE (mV)[d]	E_m' (mV vs NHE)
1,10-PD	$O_2/O_2^{•-}$	174 (11.9)	144 (5)	−11
1,7-PD	$O_2/O_2^{•-}$	170 (24.4)	144 (8)	−11
DMBQ	$O_2/O_2^{•-}$	28.3 (5.8)	98 (12)	−70 (2)
PQQ	$O_2/O_2^{•-}$	3.11 (0.7)	41 (13)	−122 (3)
1,10-PD	DMBQ/DMBQ$^{•-}$	14.9 (5.5)	99 (22)	−3 (3)
1,7-PD	DMBQ/DMBQ$^{•-}$	17.2 (3.3)	130 (11)	4.5 (4)

[a] Estimated standard deviations are given in parentheses. [b] The reference potentials used for $O_2/O_2^{•-}$ and DMBQ/DMBQ$^{•-}$ were −0.155 V (Ilan et al., 1976) and −0.70 V (McWhirter, 1989), respectively. [c] Experiments with the $O_2/O_2^{•-}$ reference were done at pH 7.2 and those with the DMBQ/DMBQ$^{•-}$ at pH 7.5. The constants presented are averages taken from the two sets of data obtained by the kinetic and the equilibrium protocols. [d] Corrected to pH 7 on the assumption of the Nernst equation and a single proton acid/base equilibrium. The correction is negligible for the 1,10-PD/PD$^{•-}$ couple, 2.5 mV for the 1,7-PD/PD$^{•-}$ couple and 4 mV for the PQQ/PQQ$^{•-}$ couple.

With the known redox potential for the reference couple $O_2/O_2^{•-}$, −155 mV vs NHE (Ilan et al., 1976), it is possible to compute the unknown quinone redox potential from the measured K_{eq} since

$$\Delta E = \frac{RT}{nF} \ln K_{eq} \qquad (8)$$

$$E_{Q/Q•^-} = \Delta E + E_{O_2/O_2•^-} \qquad (9)$$

where R, T, and F are the gas constant, the absolute temperature, and Faraday's constant, respectively, $n = 1$, ΔE is the redox potential difference between quinone and the oxygen reference, and E is an absolute midpoint potential. The computed difference and absolute potentials are collected in Table II. We determined the DMBQ potential for two reasons. The first was to check our procedures by comparison with earlier measurements on the same compound. Our measured DMBQ E_m' of −70 ± 2 mV is comparable to the −80 mV quoted to Swallow (1982). Second, the two phenanthrolinedione redox potentials are closer to that of the DMBQ than to that of the superoxide potential. In our experience, the chance for error is greater when the ΔE between unknown and reference is larger than ca. 150 mV (DeFelippis et al., 1989; McWhirter, 1989). For this reason, we repeated the phenanthrolinedione measurements with DMBQ/DMBQ$^{•-}$ as reference. This second set of potentials (Table II) are the same, within experimental error, as those obtained against $O_2/O_2^{•-}$, and are also reported in Table I.

Spectral Properties of the Methylamine Dehydrogenase Semiquinone. We determined the extinction coefficient of the visible MADH absorption band with a titration between oxidized protein and the two tight binding inhibitors semicarbazide and phenylhydrazine (Kenney & McIntire, 1983). Adding either results in a spectral change that depends linearly on the inhibitor concentration up to a titration break (e.g., Figure 5, inset) at 1.09 (semicarbazide) and 1.04 (phenylhydrazine) mol/mol of β-subunit. From the average of these two and assuming a true equivalence of 1 mol/mol, we calculated an extinction coefficient for the visible band of oxidized MADH: $\epsilon_{444} = 8340 \pm 230$ cm^{-1} M^{-1}. This value served for all remaining concentration measurements. Since MADH as isolated was always a mixture of oxidized, semiquinone, and

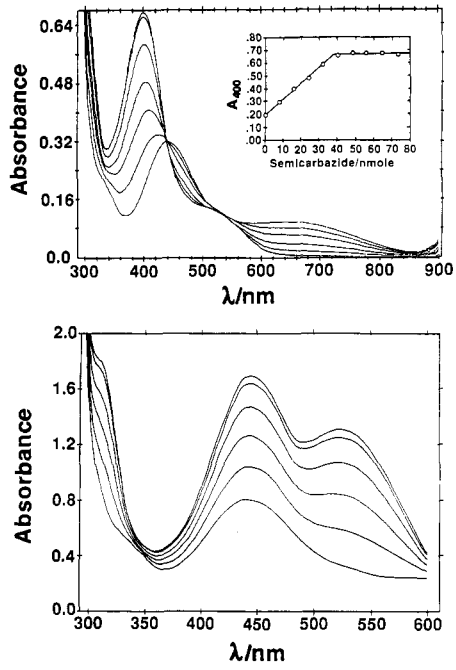

FIGURE 5: Reaction of MADH with semicarbazide and phenylhydrazine. Incremental addition of semicarbazide to oxidized MADH (42 and 92 nmol of active site, respectively, in semicarbazide and phenylhydrazine titrations) in 1.0 mL of 0.1 M phosphate, pH 7.5, and incubated for 3 h. The inset tracts A_{400} as a function of semicarbazide added. The active site concentration is based on an MADH absorbance at 278 nm of 0.92 cm^{-1} mL/mg^{-1} and a protein molecular weight of 125000 (Kenney & McIntire, 1983). (Top) Semicarbazide titration. (Bottom) Phenylhydrazine titration; absorbance increases with increasing titrant.

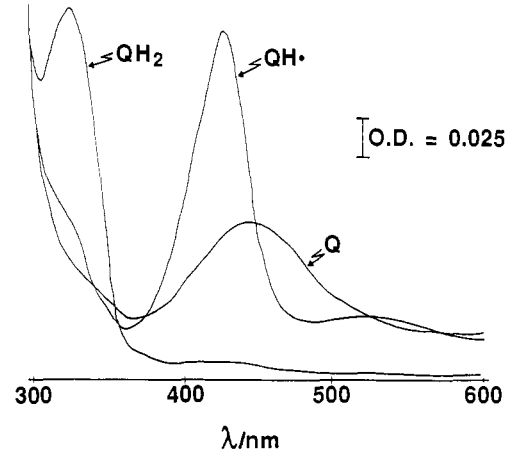

FIGURE 6: Redox states of W3A1 methylamine dehydrogenase. All spectra were obtained as described in the text at room temperature, by use of 11 μM active site in 0.1 M phosphate buffer, pH 7.5. Q, QH•, and QH_2 are the oxidized, half-reduced, and fully reduced coenzyme forms of the enzyme. The semiquinone spectrum was produced by addition of excess alkylamine to oxidized enzyme under aerobic conditions. This semiquinone spectrum is identical with that one obtained by addition of dithionite at a stoichiometry of 1 dithionite to 1 MADH.

reduced forms, we routinely oxidized the protein with ferricyanide before using the visible absorption band as a measure of concentration.

MADH is an $\alpha_2\beta_2$ protein. The two subunits are separable by addition either of 35–40% acetic acid or of 5.5 M guanidine hydrochloride at pH 7, resolution with Sephadex G-75, and dialysis to remove the denaturant. While we could recover the smaller β-subunit after either treatment, we recovered the larger α-subunit after the guanidine but not the acetic acid treatment. The β-subunit contains the coenzyme and has a spectrum closely similar to those published for other β-subunit preparations (Kenney & McIntire, 1983; Matsumoto et al., 1980). The spectrum of the tetrameric protein when diluted into 20% acetic acid is identical with that of the β-subunit dissolved in water. Thus, in dilute acetic acid the protein appears to dissociate into a stable β-subunit and a denatured α-subunit. So simply by measuring and comparing protein spectra before and after addition of acetic acid, we obtained the extinction coefficient of the β-subunit at its visible absorption maximum, ϵ_{425} = 8340 M^{-1} cm^{-1}.

We were also able to reconstitute tetrameric $\alpha_2\beta_2$ from the separated subunits. Various amounts of the α-subunit, isolated with guanidine hydrochloride, were incubated at 5 °C with a fixed amount of the β-subunit, prepared with either acetic acid or guanidine treatment, and the specific activity of the reconstituted enzyme then measured. Both acid- and guanidine-prepared β-subunit yielded titrations with equivalence points of 1.1 (±0.1) and 1.3 (±0.3) mol of β/mol of α, respectively, and approximately 100% recovered activity. Similar reconstitutions have been done previously (Matsumoto & Tobari, 1978; Matsumoto et al., 1980) but not with the acetic acid resolved β-subunit.

Titration of MADH with dithionite results in an intermediate species with an intense absorbance band centered at 428 nm (Kenney & McIntire, 1983; Husain et al., 1987). Because of the observed stoichiometry in these dithionite titrations, both laboratories assigned the 428-nm spectrum to the MADH PQQ semiquinone, a one-electron-reduced species. We have obtained this same spectrum with both dithionite (not shown) and excess methylamine (Figure 6). Kenney and McIntire (1984) also reported a qualitative correlation between the 428-nm band and a measured ESR signal. Altogether, these observations do not establish unequivocally that the 428-nm species is the semiquinone radical. Such an assignment is especially problematic since the spectrum of the 428-nm species is strikingly unlike the spectrum of the free PQQ semiquinone, at either acid or neutral pH (Figure 4). In order to prove that the 428-nm species is a one-electron-reduced, semiquinone, we turned to pulse radiolysis. Our strategy was to reduce the protein so rapidly with a well-defined one-electron reductant that there was no possibility of kinetic intermediates. The observation of only the 428-nm species under these experimental conditions would establish it as the semiquinone form of the enzyme.

We designed this series of experiments around the methylviologen cation radical ($MV^{\bullet+}$), a more specific reductant than either $e_{aq}^{\bullet-}$ or $CO_2^{\bullet-}$ because it has a more positive redox potential (−449 mV vs NHE; Szentrimay et al., 1977) and it resembles phenazine methosulfate, a known electron acceptor in the MADH-catalyzed oxidation of amines. In fact, $MV^{\bullet+}$ does react rapidly with excess MADH in a single pseudo-first-order process observed throughout the visible region. From the linear dependence of the apparent first-order reduction rate constant on the MADH concentration, we obtained a second-order rate constant of 6.1 (±0.1) × 10^8 M^{-1} s^{-1}. This reduction product has a spectrum closely resembling that of the 428-nm species (Figure 7). Because the $MV^{\bullet+}$ reduction is so rapid, we shall argue later that this product, and so also the 428-nm species, must be the MADH semiquinone.

We have also looked at the reaction of $MV^{\bullet+}$ with the isolated β-subunit. When protein is in excess over $MV^{\bullet+}$, this reduction is pseudo first order and the measured second-order rate constant is 2.4 × 10^8 M^{-1} s^{-1}. The spectrum of the $MV^{\bullet+}$-reduced subunit differs from the spectra of both the PQQ semiquinone (Figure 4) and the $MV^{\bullet+}$-reduced MADH,

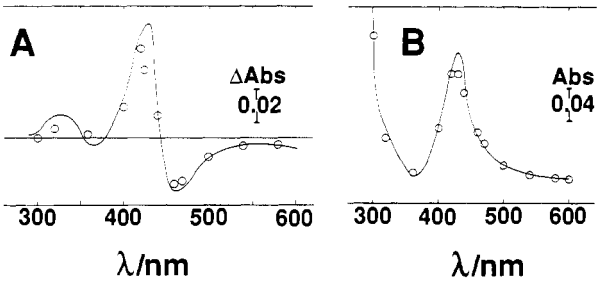

FIGURE 7: Spectrum of MADH reduced by the one-electron donor $MV^{\bullet+}$. Spectrum obtained at pH 7.5 with initial concentrations of 200 μM methylviologen and 19 μM tetrameric MADH. The $MV^{\bullet+}$ concentration formed by the pulse was 4.2 μM. Remaining reaction conditions are given in the text. The solid lines have been computed from the spectra of Figure 6. (O) Measured absorbances 0.2 ms after the pulse. (A) Difference spectrum of MADH minus $MV^{\bullet+}$-reduced MADH; (B) absolute spectrum of $MV^{\bullet+}$-reduced MADH.

seen by comparison of Figures 7 and 8. This latter difference is not surprising, since the spectra of oxidized subunit and native tetramer are also not identical.

Discussion

In spite of their instability in aqueous solution, the physical properties of several *o*- and *p*-semiquinones have been measured (Swallow, 1982). Those of benzo-, naptho-, and anthraquinones have absorption bands in the region of 370–450 nm, both in the neutral and in the anion radical forms with extinction coefficients ranging from 4 to 12 mM^{-1} cm^{-1}. In comparison, the visible bands of the three semiquinones studied here appear slightly more to the red and are less intense (Table I).

Semiquinone pK_a reported in the literature range from approximately 4 to 5.5, much lower than the pK_a associated with their hydroquinones. Thus, the pK_a of 5.1 and 6.2 that we observed for 1,10-PDH$^\bullet$ and 1,7-PDH$^\bullet$, respectively, are consistent with proton dissociation from the semiquinone. On the other hand, two pK_a are required to describe the titration of the PQQ semiquinone, and it is presently unclear which of the various potential sites on the PQQ radical are involved.

The observed midpoint potentials of the three radicals at pH 7 fall in a range, ca. −0.12 to 0 V, comparable to potentials of other simple semiquinones, ca. −0.4 to 0.2 V. Since the reported two-electron reduction potential of PQQ is 0.095 V (Duine et al., 1981; Eckert et al., 1982), the estimated potential for the one-electron reduction of $PQQ^{\bullet-}$ to PQQH2 is ca. 220 mV. Thus, in terms of the pH 7 standard potentials, the two-electron oxidation of fully reduced PQQ by O_2 is energetically favorable; the one-electron oxidation is not:

$$PQQH_2 + O_2 \rightarrow PQQ^{\bullet-} + O_2^{\bullet-} + 2H^+ \qquad \Delta E = -0.37 \text{ V}$$

$$PQQH_2 + O_2 \rightarrow PQQ + H_2O_2 \qquad \Delta E = 0.29 \text{ V}$$

On the other hand, the one-electron acceptors that participate in the MADH catalysis (Chandrasekar & Klapper, 1986; Gray et al., 1986) would be energetically favorable oxidants of both $PQQH_2$ and $PQQ^{\bullet-}$. Therefore, were the redox potentials of the enzyme-bound PQQ not greatly altered, then the quinone would be poised at formation of the semiquinone during one-electron reoxidations. But the occurrence of the semiquinone during MADH reoxidation is presently only a conjecture. It is also interesting that known methoxatoenzymes are dehydrogenases and not oxidases unless the protein also contains copper.

Overall, the properties of the PQQ, 1,7-PD, and 1,10-PD semiquinones are unexceptional. Hence, it is unsettling that

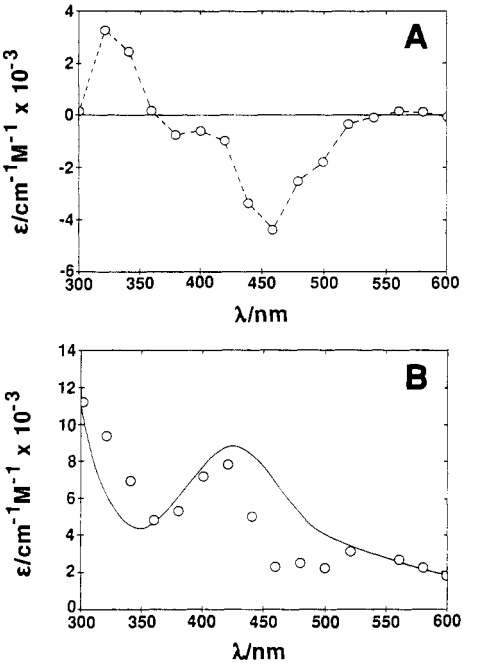

FIGURE 8: Spectrum of the β-subunit reduced by the one-electron-donor $MV^{\bullet+}$. The reaction conditions were those of Figure 7 except for 9.8 μM $MV^{\bullet+}$ produced by the pulse and 45 μM β-subunit. (A) Difference spectrum of β-subunit minus one-electron-reduced species obtained at 0.6 ms after the pulse. The dashed line has been drawn in arbitrarily. (B) Absolute spectrum of one-electron-reduced β-subunit. (O) Absolute spectrum after $MV^{\bullet+}$ reaction calculated from the results of the data in panel A. (—) Spectrum of oxidized β-subunit. There is a clear spectral change on one-electron reduction.

the spectra of oxidized MADH and PQQ are dissimilar and that the spectrum of the purported MADH semiquinone species does not resemble that of the PQQ semiquinone at either acid or neutral pH. This lack of resemblance raises questions about the identity of the MADH cofactor and of the purported enzyme semiquinone. Also troubling are the tentative partial structures for the MADH cofactor proposed by McIntire and Stults (1986) from MS and NMR analyses and by Vellieux et al. (1989) from an initial crystal structure of an MADH. If either proposal is correct, then the MADH cofactor could be a PQQ analogue but not PQQ itself. On the other hand, the observed differences between MADH and PQQ spectra could be due to a coenzyme–protein interaction. For example, a PQQ–protein charge-transfer complex could cause the very large visible absorption band so characteristic of MADH but absent from the spectra of many other methoxatoenzymes. The observation of an intense absorbance band at 400 nm in the spectrum of a methanol dehydrogenase with a noncovalently attached PQQ (Frank et al., 1988) supports this possibility.

Although we cannot comment presently on the precise identity of the MADH cofactor, we can argue from the rapid [$k = (6.1 \pm 0.1) \times 10^8$ M^{-1} s^{-1}] $MV^{\bullet+}$ reduction of MADH that the 428-nm MADH species is a one-electron-reduced species of the enzyme. Since the $MV^{\bullet+}$ reaction is close to diffusion controlled, it is improbable that two $MV^{\bullet+}$ will react with one protein molecule when protein is present in large excess. With this premise and the following example, it will become clear that the 428-nm species must be a semiquinone. In the reaction of ca. 80 μM MADH (160 μM β-subunit quinone) with 4.2 μM $MV^{\bullet+}$ there is only one observed kinetic process, and it has an apparent first-order rate constant of 4.5×10^4 s^{-1}. The product spectrum is that of the MADH 428-nm species (Figure 7). There are, thus, three possibilities for the stable 428-nm MADH species formed in the dithionite or

aerobic alkylamine reaction. (i) This species is identical with that formed in the $MV^{\bullet+}$ reduction. (ii) This species is not the $MV^{\bullet+}$ reduction product, even though their spectra are closely similar. (iii) The 428-nm species is in reality a two-electron product since the $MV^{\bullet+}$ one-electron-formed semiquinone is further reduced so rapidly that we were unable to see the one-electron intermediate. The second suggestion is too improbable for serious consideration. The third is unlikely, for under our reaction conditions that second electron could have come only with the second-order collision of another protein molecule. (Reduction by two $MV^{\bullet+}$ is unlikely.) Since the $t_{1/2}$ of the reaction we do observe is 15 μs, an appropriate bimolecular protein collision would require a half-life less than 15 μs. Hence, $15 \times 10^{-6} > 1/(4.2 \times 10^{-6})$ $(1/k_2)$, the first half-life of a second-order collision at a radical concentration of 4.2 μM; this limit implies that the second-order protein collision rate constant k_2 would have to be $>1.5 \times 10^{10}$ M^{-1} s^{-1} or greater than the diffusion-controlled limit for a reaction between two proteins. We conclude, therefore, that the MADH species with a major absorbance band centered around 428 nm is the one-electron-reduced semiquinone form of MADH. The reason(s) for the spectral differences between the MADH and free PQQ semiquinones remain(s) to be established.

We have also seen the $MV^{\bullet+}$ reduction of resolved β-subunit (Figure 8). With an argument similar to that just presented, we conclude that the one-electron-reduced semiquinone is the product of this reaction also. Since the three semiquinones of free PQQ, holoenzyme, and β-subunit have different visible spectra, the protein structure must affect the spectrum of the semiquinone, as it also affects the spectrum of the quinone.

Added in Proof

Janes et al. (1990) have just proposed that the covalently attached cofactor of the bovine serum amine oxidase is not PQQ as previously believed. Rather, they conclude that the cofactor is 2,4,5-trihydroxyphenylalanine (6-hydroxyDOPA). This conclusion together with the data we have reported here suggests that the identity of the MADH cofactor should be reviewed with care.

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Registry No. MADH, 60496-14-2; PQQ$^{\bullet}$, 92714-14-2; 1,10-PD$^{\bullet}$, 78803-63-1; 1,7-PD$^{\bullet}$, 127492-64-2; $CO_2^{\bullet}$, 2564-86-5; DMBQ, 527-61-7.

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