Oxidation–Reduction Potentials of Flavin and Mo–Pterin Centers in Assimilatory Nitrate Reductase: Variation with pH†

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ABSTRACT: Potentiometric titrations of assimilatory nitrate reductase from *Chlorella vulgaris* were performed within the pH range 6.0–9.0. Mo(V) was measured by room temperature EPR spectroscopy while the reduction state of FAD was monitored by CD spectroscopy. Between pH 6 and 8.5, the line shape of the Mo(V) EPR signal was constant, exhibiting superhyperfine coupling to a single, exchangeable proton. Potentiometric titrations indicated the E_m values for the Mo(VI)/Mo(V) (+61 mV, pH 6) and Mo(V)/Mo(IV) (+35 mV, pH 6) couples decreased with increasing pH by approximately −59 mV/pH unit, consistent with the uptake of a single proton upon reduction of Mo(VI) to Mo(V) and Mo(V) to Mo(IV). The pK_a values for the dissociation of these redox-coupled protons appeared to lie outside the pH range studied: p$K_{o(MoVI)}$, p$K_{o(MoV)}$ <5.5; p$K_{r(MoV)}$, p$K_{r(MoIV)}$ >9. The E_m (n = 2) for FAD (−250 mV, pH 7) varied by approximately −30 mV/pH unit within the pH range 6.0–9.0. Low-temperature EPR potentiometry at the extreme pH values indicated less than 0.5% conversion of FAD to the semiquinone form at the midpoint of the titrations. In contrast, NADH-reduced enzyme exhibited approximately 3–5% of the FAD in the semiquinone form, present as the anionic ($FAD^{\bullet -}$) species, the spectrum characterized by a line width of 1.3 mT at both pH 6.0 and 9.0. These results were consistent with the following reduction scheme: $FAD + e^- \rightarrow FAD^{\bullet -} + e^- + H^+ \rightarrow FADH^-$, with p$K_{r(FADH^-)}$ of >9.5, a pH-independent value for E_m ($FAD/FAD^{\bullet -}$) of <−458 mV, and a pH dependence of the E_m for the $FAD^{\bullet -}/FADH^-$ redox couple of −59 mV/pH unit. Combining these results with data previously published for the heme center [Kay, C. J., Solomonson, L. P., & Barber, M. J. (1986) *J. Biol. Chem. 261*, 5799–5802], the uptake of four protons appears to be coupled to the oxidation–reduction reactions of *Chlorella* nitrate reductase at room temperature: one stoichiometrically coupled to $FAD^{\bullet -}$ reduction, one weakly coupled to heme reduction, and two independent coupled to reduction of Mo(VI) and Mo(V). At all pH's examined, nitrate reductase activity remained thermodynamically favorable.

Nitrate reductase (NR)[1] catalyzes the initial and rate-limiting step in the assimilation of inorganic nitrogen, the NAD(P)H-dependent reduction of nitrate to nitrite (Solomonson & Barber, 1990). The enzyme isolated from the unicellular green alga *Chlorella vulgaris* is a homotetramer, each subunit (M_r 96 000) containing FAD, cytochrome b_{557}, and Mo–pterin in a 1:1:1 stoichiometry (Howard & Solomonson, 1982). Limited proteolysis of NR using a specific corn inactivator protease, or *Staphylococcus aureus* V8 protease, liberates a 30-kDa FAD-containing fragment which catalyzes NADH oxidation with ferricyanide as acceptor and a 70-kDa fragment which catalyzes nitrate reduction at the Mo–pterin center with artificial electron donors such as reduced methyl viologen (Solomonson et al., 1986). The function of the b_5-type cytochrome appears to be to shuttle reducing equivalents from FAD to Mo–pterin in a linear pathway (Kay & Barber, 1986). At pH 7.0, this hypothesis is consistent with the thermodynamic properties of the three prosthetic groups in NR: the midpoint potentials become more oxidizing in the sequence from FAD ($E^{\circ\prime}$ = −272 mV, n = 2) to heme ($E^{\circ\prime}$ = −164 mV, n = 1) to Mo–pterin ($E^{\circ\prime}$ = −5 mV, n = 2) (Kay et al., 1986, 1988).

Examination of the pH dependence of the oxidation–reduction midpoint potential of the b_5-type cytochrome of *Chlorella* NR detected a single protonatable group, with pK_o = 5.8 and pK_r = 6.1 (Kay et al., 1986). Crystallographic studies of microsomal cytochrome b_5, which exhibits significant sequence similarity with the heme domain of NR, have revealed the cause of the pH dependence of the heme midpoint potential to be electrostatic interaction between one heme propionate group and the heme–Fe (Reid et al., 1982).

We have examined the effects of pH on the flavin and Mo–pterin midpoint potentials of *Chlorella* NR to establish the sequence of electron and proton transfers for the various redox centers over a wide pH range, the relative protonation states of the prosthetic groups, and whether electron transfer in a linear pathway is thermodynamically favorable under all conditions. Potentiometric titrations were performed at room temperature, since comparative studies of both NR (Kay et al., 1988) and other complex metalloflavoproteins (Porras & Palmer, 1982) have shown that artifacts introduced during the freezing of samples for low-temperature EPR analysis may invalidate thermodynamic measurements owing to the redistribution of reducing equivalents among the various redox-active prosthetic groups.

Experimental Procedures

Enzyme Purification. Nitrate reductase was isolated from *C. vulgaris* according to the affinity purification procedure of Howard and Solomonson (1981) and assayed as previously described (Kay & Barber, 1986). The enzyme exhibited a

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[1] Abbreviations: NR, nitrate reductase; EPR, electron paramagnetic resonance; SHE, standard hydrogen electrode; EXAFS, X-ray absorption extended fine structure; NADH:NR, NADH-dependent nitrate reductase activity; NADH:CR, NADH-dependent cytochrome *c* reductase activity; MV:NR, reduced methyl viologen dependent nitrate reductase activity.

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NADH:NR activity of greater than 80 units/mg of protein and an A_{280}/A_{413} ratio of less than 1.8. The NR heme concentration was determined by using an extinction coefficient of 117 mM^{-1} cm^{-1} at 413 nm (Solomonson et al., 1984). All enzyme samples were prepared in an appropriate zwitterionic buffer by using "Ultrol"-grade reagents (Calbiochem, San Diego, CA) and extensively dialyzed by ultrafiltration. Contamination with Cl^- was determined colorimetrically by using mercury thiocyanate and ferric ion (Iwasaki et al., 1952). Chloride contamination of buffers was below 0.4 mM. The Mo content of NR samples was determined colorimetrically as previously described (Solomonson et al., 1975). Buffer concentrations required to maintain constant ionic strength were calculated as described by Ellis and Morrison (1982). All buffers contained 0.1 mM EDTA.

EPR Spectroscopy. Spectra were recorded by using a Varian E109 Century Series EPR spectrometer (Varian Associates, Palo Alto, CA) operating at 9 GHz with 100-kHz modulation and equipped with a variable-temperature accessory. Room temperature spectra were generally recorded with an incident microwave power of 50 mW and a modulation amplitude of 0.32 mT. Low-temperature spectra were recorded at 173 K, with a microwave power of 5 mW and a modulation amplitude of 0.32 mT. Double integration of EPR spectra was performed as described by Wyard (1965).

EPR Spectral Simulations. Simulations of low-temperature Mo(V) EPR spectra were performed according to a modified version of the program described by Lowe (1978) using the published spectral parameters for *Chlorella* NR (Kay & Barber, 1989).

Potentiometry. Room temperature EPR potentiometric titrations were performed as previously described (Kay et al., 1988; Kay & Barber, 1989) in the presence of dye mediators. Spin concentrations in the flat cell were calibrated with quinhydrone (Eastman Kodak Co., Rochester, NY) as standard. Oxidation–reduction midpoint potentials, expressed relative to the standard hydrogen electrode, were derived by least-squares fitting of the appearance and disappearance of Mo(V) to the Nernst equation for the proportion of intermediate formed by two consecutive one-electron reduction processes.

Potentiometric CD titrations were performed as described by Kay et al. (1988). The proportions of oxidized FAD and flavin hydroquinone produced during potentiometric titrations in the presence of dye mediators were determined at 460 nm. Midpoint potentials were obtained by fitting the change in CD at 460 nm to the Nernst equation for a concerted $n = 2$ reductive process by using a linear least-squares procedure.

Results

EPR Spectra of Mo(V). Three distinct Mo(V) EPR species have been detected for NR from either *Chlorella* (Kay & Barber, 1989) or spinach (Gutteridge et al., 1983), depending on the pH and anion and buffer composition of the medium. For *Chlorella* NR, two EPR species, designated "signal A" ($g_1 = 1.996$, $g_2 = 1.969$, $g_3 = 1.967$, $g_{av} = 1.977$, $A^H_1 = 1.25$ mT, $A^H_2 = 1.18$ mT, $A^H_3 = 1.63$ mT, $A^H_{av} = 1.35$ mT) and "signal B" ($g_1 = 1.996$, $g_2 = 1.969$, $g_3 = 1.967$, $g_{av} = 1.977$), are observed at low pH. These have identical g values and exhibit near-axial symmetry but differ in that "signal A" exhibits superhyperfine coupling to a single exchangeable proton, while "signal B" is devoid of superhyperfine splittings. A third signal with different g values ($g_1 = 1.984$, $g_2 = 1.951$, $g_3 = 1.947$, $g_{av} = 1.961$) and near-rhombic symmetry has been reported at high pH (Solomonson et al., 1984). The proportion of the "high-pH" signal generated is strongly buffer-dependent

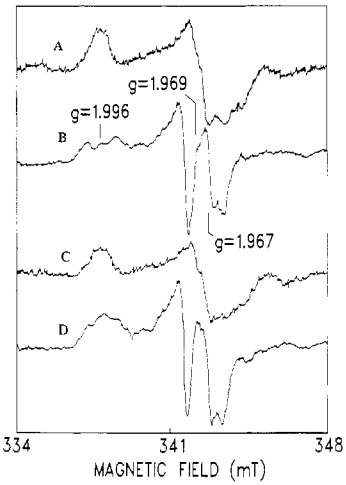


FIGURE 1: Comparison of room temperature and low-temperature Mo(V) EPR spectra obtained at pH 6.0 and 8.5. NR (12 μM heme), in the appropriate buffer, was poised close to the appropriate $n = 2$ midpoint potentials given in Table I in the presence of dye mediators. Spectra A and B: 50 mM MES and 0.1 mM EDTA, pH 6.0. Spectra C and D: 50 mM Bicine and 0.1 mM EDTA, pH 8.5. Spectra A and C were recorded at 25 °C in a flat cell while spectra B and D were recorded at 173 K, following anaerobic transfer from the titration vessel and freezing in an EPR tube. The field scale corresponds to a frequency of 9.427 GHz.

(unpublished data). To determine the nature of the Mo(V) EPR species present during potentiometric titrations, samples of *Chlorella* NR were poised at controlled potentials in the presence of dye mediators at pH 6.0 and 8.5, transferred anaerobically to EPR tubes, and rapidly frozen in liquid N_2 for subsequent EPR analysis. These spectra are presented in Figure 1B,D, together with spectra obtained during potentiometric EPR titrations performed at 25 °C in the same buffers (Figure 1A,C). At 173 K, the Mo(V) spectra obtained at pH 6.0 (Figure 1B) and pH 8.5 (Figure 1D) were similar and showed superhyperfine interaction, although with improved resolution, compared to the corresponding spectra obtained at room temperature. At low temperature, the Mo(V) spectra obtained at pH 6.0 and 8.5 appeared to be exclusively of the "low-pH" forms, and both "signal A" and "signal B" were present, the former exhibiting superhyperfine splitting to a single, exchangeable proton. Computer additions of simulated spectra corresponding to the paramagnetic species obtained at 173 K indicated that, at pH 6.0 (Figure 1B), the observed Mo(V) EPR spectrum comprised approximately 90% "signal A" and 10% "signal B" whereas at pH 8.5 (Figure 1D), the composition had changed to approximately equivalent proportions of "signal A" and "signal B". The "high-pH" form of Mo(V), readily detectable in the presence of the "low-pH" forms owing to their higher g values, was not observed, even at pH 8.5. However, the "high-pH" Mo(V) species was detected at pH 9. Thus, the range of pH values at which potentiometric determinations of the Mo midpoint potentials could be performed was bounded at pH 9 by the appearance of the "high-pH" Mo(V) species, and at low pH values by precipitation of NR, which occurred below pH 6.0. Within the pH range 6.0–8.5 NADH:NR activity and MV:NR activities were stable, indicating functional integrity of FAD, heme, and Mo–pterin prosthetic groups.

Mo(V) Potentiometric Titrations. The results of room temperature EPR potentiometric titrations of Mo(V), performed in the presence of dye mediators within the pH range 6.0–8.5, are shown in Figure 2. Titrations were performed in both oxidative and reductive directions by the addition of $K_3Fe(CN)_6$ and reduced methyl viologen, respectively. At

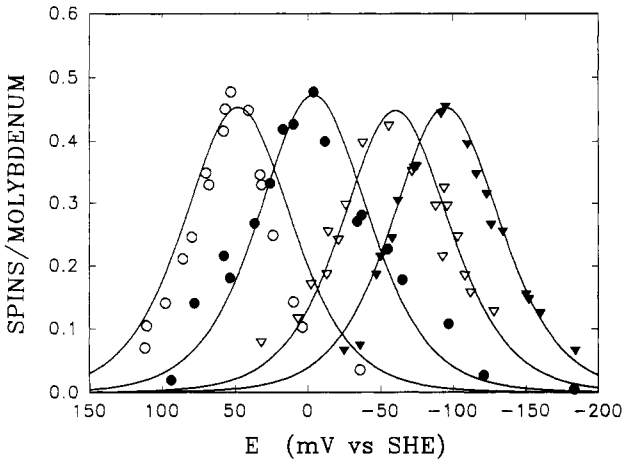


FIGURE 2: pH dependence of the behavior of the Mo(V) EPR signal during room temperature potentiometric titrations. NR (12–16 μM heme) was poised in an anaerobic EPR flat cell at controlled potentials in the presence of dye mediators by addition of reduced methyl viologen (10 mM) or $K_3Fe(CN)_6$ (20 mM). Mo(V) EPR spectra were recorded following attainment of redox equilibrium. (○) 50 mM MES, pH 6; (●) 50 mM MOPS, pH 7; (▽) 50 mM Tricine, pH 8; (▼) 50 mM Bicine, pH 8.5. Data are fitted to Nernst curves representing two sequential one-electron processes, with Mo(V) as intermediate.

Table I: Midpoint Reduction Potentials for the FAD and Mo–Pterin Centers of *Chlorella* Nitrate Reductase as a Function of pH[a]

		E_m (mV vs SHE)		
pH	buffer	Mo(VI)/Mo(V)	Mo(V)/Mo(IV)	FAD/FADH⁻
6	MES	61	35	−250
6.5	MES	nd	nd	−263
7[b]	MOPS	11	−19	−272
8	Tricine	−48	−73	−310
8.5	Bicine	−82	−108	nd
9	Bicine	nd	nd	−342

[a] All titrations were performed at 25 °C in the presence of dye mediators in buffers containing 0.1 mM EDTA and at an ionic strength of 0.1. Values given represent computer best fits to the experimental data. [b] Data from Kay et al. (1988).

each pH value, the Mo(V) integrated signal intensity initially increased as the potential was lowered, reached a maximum, and then decreased as the potential was further decreased. This behavior was consistent with two consecutive one-electron (n = 1) reduction steps forming Mo(V) as the intermediate, corresponding to reduction of Mo(VI) to Mo(V), and subsequent reduction of Mo(V) to Mo(IV). At pH 6,[2] the integrated intensity of the Mo(V) EPR signal reached a maximum at approximately +50 mV and corresponded to conversion of 48% of the total enzyme-bound Mo to Mo(V), yielding midpoint potentials of +72 and +28 mV for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples, respectively. As the pH was increased, at constant ionic strength, the potential corresponding to the maximum Mo(V) signal intensity became more negative, corresponding to potentials of −4 (pH 7), −61 (pH 8), and −95 mV (pH 8.5). However, the maximum proportion of Mo(V) obtained during the titrations remained

[2] The narrower width of the Mo(V) vs potential curve than would be expected from ideal n = 1 behavior, seen in the data of Figure 2 at pH 6.0, was quite reproducible compared to the nearly ideal Nernstian behavior shown at pH's ≥ 7.0. Since deviations from ideality have been frequently observed in titrations of other Mo-containing enzymes, such as xanthine oxidase (Barber & Siegel, 1982), and since the shape of the theoretical titration curve does not change appreciably with minor changes in the integrated Mo(V) intensity at approximately 45–50% conversion, we have weighted the curve-fitting process at pH 6 to fit the maximum level of Mo(V) formed during the titration.

Table II: Limiting pK_a Values and Nernst Equations for Redox-Linked Protonations of FAD, Heme, and Mo–Pterin Prosthetic Groups

redox couple	$E^{\circ\prime}$ (mV)	p$K_{a(o)}$	p$K_{a(r)}$	Nernst equation[a]
Mo(VI)/Mo(V)	+11	<5.5	>9.0	$E_m = E^{\circ\prime} + 59\ (7 - pH)$
Mo(V)/Mo(IV)	−19	<5.5	>9.0	$E_m = E^{\circ\prime} + 59\ (7 - pH)$
heme$_o$/heme$_r$[b]	−162	5.8	6.1	$E_m = E^{\circ\prime} + 59 \log (1 + 10^{pK_r - pH}) - 59 \log (1 + 10^{pK_r - pH})$
FAD/FADH⁻	−272	<6.0	>9.0	$E_m = E^{\circ\prime} + 30\ (7 - pH)$

[a] The equation gives the effective midpoint potential at any pH within the range pH 6.0–8.5 for Mo and pH 6.0–9.0 for flavin. [b] $E^{\circ\prime}$ represents the midpoint potential of the unprotonated species and equals −162 mV. Heme data are taken from Kay et al. (1986).

effectively constant. Midpoint potentials for the two couples, calculated from the experimental data, are listed in Table I. The pH dependence of the midpoint potentials for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples is shown in Figure 5. The pH dependencies of both redox couples were linear, yielding a pH dependence of approximately −59 mV/pH unit. No evidence was found for pH independence of either of the two Mo couples over the pH range examined. Behavior of this type can be analyzed (Clarke, 1960) in terms of both one-electron reduction steps, within the accessible pH range, being coupled to uptake of a single proton according to the scheme:[3]

$$\text{Mo(VI)}\cdot m\text{H}^+ \overset{1e^-,\,1H^+}{\leftrightarrow} \text{Mo(V)}\cdot(m+1)\text{H}^+ \overset{1e^-,\,1H^+}{\leftrightarrow} \text{Mo(IV)}\cdot(m+2)\text{H}^+$$

where m represents the total number of protons associated with Mo(VI), which cannot be determined from these data. The close approximation of the pH dependencies of the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples to the theoretical −59 mV/pH unit value suggested that the pK_a values for the ionizations of any of these protons must be outside the pH range 6–8.5. Specifically, the pK_a's for protonation of each form of Mo shown above are below 6.0, while the pK_a values for deprotonation of each Mo species shown are greater than 8.5. Theoretical calculations of the pH dependencies of the two Mo couples yielded limiting values for the pK_a's of these possible ionizations. Thus p$K_{o(MoVI)}$ and p$K_{o(MoV)}$ must be less than or equal to 5.5 while p$K_{r(MoV)}$ and p$K_{r(MolV)}$ must be greater than or equal to 9.0. The estimates for these pK_a's and the equations used to calculate the pH dependencies of the two Mo couples are given in Table II.

CD Potentiometry of FAD. The degree of reduction of the flavin chromophore of NR was determined by CD potentiometry in the presence of dye mediators, as previously described (Kay et al., 1988). Flavin reduction was monitored at 460 nm, and titrations were performed in media of constant ionic strength (μ = 0.1) within the pH range 6.0–9.0. The NADH:CR partial enzyme activity was stable within this pH range, indicating the presence of a functional FAD-binding domain. Titrations of the 460-nm CD band, which is lost upon reduction of FAD, were consistent with a concerted (n = 2)

[3] All protonated species in this paper are designated relative to the least protonated form of the most oxidized species found in the pH range 6–9. Thus the actual number of protons associated with the species termed Mo(VI)·H⁺ is not known, but this species does in fact contain one more proton than the species termed Mo(VI). This convention has also been used for the FAD center, which may cause some confusion with respect to the flavin dissociation states since the absolute numbers of protons associated with the various oxidized and reduced free flavins are known (Muller et al., 1970). Thus, the species we refer to as "FAD" does contain one potentially dissociable proton (whose dissociation pK in nitrate reductase lies outside the pH range examined in this work); this species would be designated as FADH in the flavin literature. Similarly, according to the same convention, the anionic semiquinone and hydroquinone would thus be referred to as $FADH^{\bullet-}$ and $FADH_2^-$, respectively.

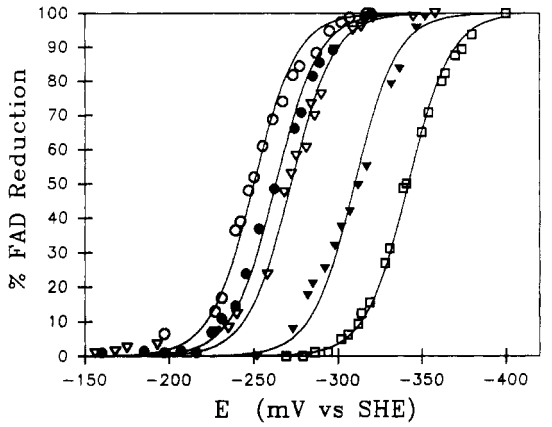


FIGURE 3: CD potentiometric titrations of the FAD prosthetic group at various pH values. NR (15 μM heme), in the appropriate buffer, was reduced or oxidized in the presence of dye mediators by addition of methyl viologen (10 mM) or $K_3Fe(CN)_6$ (20 mM). The decrease in CD at 460 nm was used to monitor the extent of FAD reduction. (○) 50 mM MES, pH 6.0; (●) 50 mM MES, pH 6.5; (▽) 50 mM MOPS, pH 7.0; (▼) 50 mM Tricine, pH 8.0; (□) 50 mM Bicine, pH 9.0.

reduction process. At pH 6.0, the midpoint potential for FAD reduction was −248 mV, which decreased by approximately 30 mV/pH unit up to pH 9.0 (Table I). The observed pH dependence of the FAD midpoint potential is shown in Figure 3. The behavior of the flavin was consistent with the uptake of a single proton upon two-electron reduction, according to the equation:

$$E_{m,pH} = E^{\circ\prime} + 30(7 - pH)$$

In principle, the proportion of flavin semiquinone formed during the titration may be determined from the slope of the titration at the (n = 2) midpoint potential, according to the method of Elema (1933). However, since the proportion of flavin semiquinone formed during methyl viologen reduction of *Chlorella* NR is low (Kay et al., 1988), application of the method of Elema could have resulted in significant errors in the estimation of the levels of flavin semiquinone formed at each pH. Therefore, flavin semiquinone levels were determined directly by using low-temperature EPR samples poised at the appropriate (n = 2) midpoint potentials at pH 6.0 and 9.0. Examination of the EPR spectra of these potentiometric samples showed no detectable flavin semiquinone, indicating a maximum conversion of FAD to the semiquinone form below the detection limits of the method, estimated to be 0.5% (Kay et al., 1989). However, in contrast, flavin semiquinone was readily observed in NR samples reduced with NADH and rapidly frozen in the presence of cyanide. Spectra obtained at 173 K at pH 6.0 and 9.0 are shown in Figure 4. At both pH values a g = 2.004 flavin semiquinone EPR spectrum was observed, representing approximately 3–5% of the total FAD. At pH 6 and 9, identical line widths (1.3 mT) were observed (Figure 4), typical of a red, anionic flavin semiquinone, indicating that the anionic flavin semiquinone of NADH-reduced NR was not protonated at pH 6.0 or above.

Since uptake of a single proton appeared to be coupled to complete (n = 2) reduction of FAD, the formation of an anionic flavin semiquinone is most simply explained by the following scheme in which proton uptake is coupled to flavin semiquinone reduction:

$$FAD \overset{1e^-}{\leftrightarrow} FAD^{\bullet -} \overset{1e^-,\,1H^+}{\leftrightarrow} FADH^-$$

The absence of any detectable levels of $FAD^{\bullet -}$ in the poised samples suggested a minimum separation between the $FAD/FAD^{\bullet -}$ and $FAD^{\bullet -}/FADH^-$ redox couples of greater

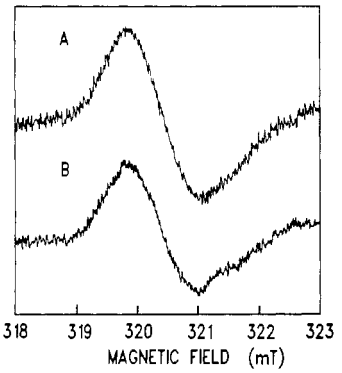


FIGURE 4: EPR spectra of FAD semiquinone obtained following NADH reduction at pH 6 and 9. Samples of NR (10 μM heme) in the presence of KCN (50 μM) were rapidly mixed with NADH (1 mM) in a quartz EPR tube and frozen in liquid N_2. Spectrum A: 50 mM MES, pH 6.0. Spectrum B: 50 mM Bicine, pH 9.0. Spectra were recorded at 173 K by using 5-mW microwave power and 0.2-mT modulation. The field scale corresponds to a frequency of 8.986 GHz.

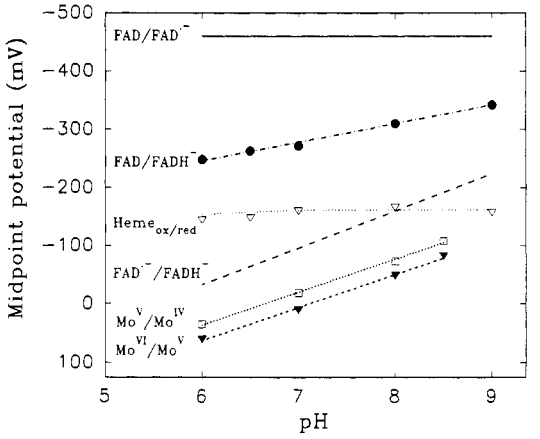


FIGURE 5: Variation with pH of the oxidation–reduction midpoint potentials for nitrate reductase. Calculated pH dependencies for the midpoint potentials for the Mo(VI)/Mo(V), Mo(V)/Mo(IV), $heme_o/heme_r$, $FAD/FAD^{\bullet -}$, $FAD^{\bullet -}/FADH^-$, and $FAD/FADH^-$ couples, derived by using the equations given in Table II, are shown together with the experimentally determined values obtained from the room temperature EPR and CD potentiometric titrations (Table I). Data for the heme potential were taken from Kay and Barber (1984).

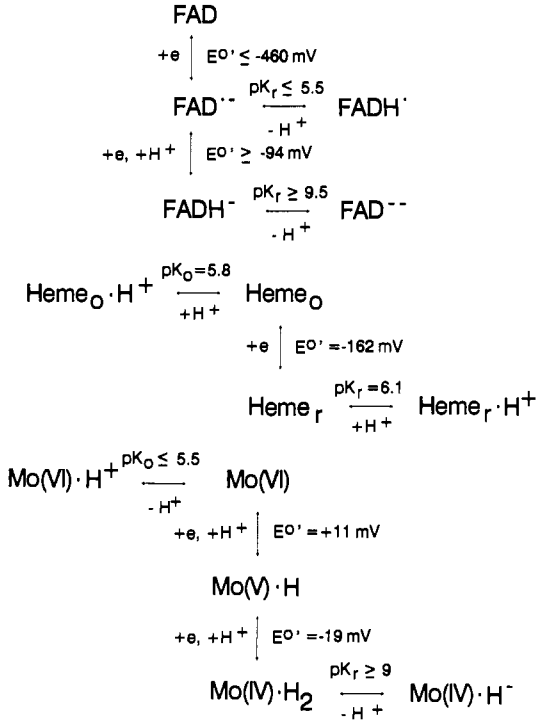
than 240 mV. Since formation of $FAD^{\bullet -}$ from FAD was not accompanied by proton uptake, the E_m value would be expected to be pH-independent. An upper limit of −460 mV for the midpoint potential of the $FAD/FAD^{\bullet -}$ couple could therefore be estimated from the apparent absence (<0.5%) of $FAD^{\bullet -}$ in the samples poised at pH 9. The pH dependencies of the $FAD/FAD^{\bullet -}$ and $FAD^{\bullet -}/FADH^-$ couples, by use of these estimates for the minimal separations, are shown in Figure 5.

Discussion

The preceding results, when combined with our previous work on the pH dependence of the *Chlorella* NR heme midpoint potential (Kay et al., 1986), provide the first complete description of the pH dependence of the redox properties of the flavin, cytochrome b_{557}, and Mo–pterin prosthetic groups of an assimilatory NR.

Within the pH range examined, four protonation reactions appear to contribute to the pH dependence of the redox properties of the enzyme. Between pH 6 and pH 9, the oxidation–reduction midpoint potentials of the FAD, heme, and Mo–pterin centers vary significantly with pH and can be summarized as shown in Figure 5 and Scheme I. With the

Scheme I: Summary of the Oxidation–Reduction Properties of the FAD, Heme, and Mo–Pterin Prosthetic Groups of Nitrate Reductase



exception of the $heme_o$/$heme_r$ and $FAD^{\bullet -}$/$FADH^-$ couples above pH 8, none of the midpoint potentials were reversed within the accessible pH range, indicating that the majority of the electron-transfer reactions occurring in the linear pathway from FAD to heme to Mo–pterin remain thermodynamically favorable. However, above pH 8, where the $heme_o$/$heme_r$ and $FAD^{\bullet -}$/$FADH^-$ midpoint potentials cross (Figure 5), electron transfer from $FADH^-$ to oxidized heme would become thermodynamically unfavorable. This would not necessarily result in inactivation of NR, since the subsequent reoxidation of reduced heme by Mo–pterin and nitrate ($E^{\circ\prime}$ = +420 mV) is thermodynamically favorable. In addition, it is unlikely that the flavin and heme midpoint potentials cross under physiological conditions, since the flavin semiquinone appears to be stabilized by the presence of NADH/NAD^+ and is readily detected in NR when NADH, but not methyl viologen or dithionite, is used as the reductant (Solomonson & Barber, 1984). Previous work on the pH profile of the NADH:NR activity of the *Chlorella* enzyme has shown a pH optimum of 8, with the enzyme retaining >50% of its maximal activity at pH 9 (Kay & Barber, 1986).

Mo–Pterin. At present, xanthine oxidase is the only other Mo–pterin-containing enzyme for which detailed studies of the pH dependence of the Mo potentials have been performed (Barber & Siegel, 1982; Porras & Palmer, 1982). Two xanthine oxidase Mo(V) species, distinguished by their characteristic EPR spectra, were examined and found to differ in their midpoint potentials and protonation behavior. Both "rapid" and "slow" species exhibited pH-dependent midpoint potentials for their Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples. The studies of xanthine oxidase were performed over a wider pH range than the results presented here for *Chlorella* NR, which were limited by both enzyme precipitation at low pH and a change in the nature of the Mo(V) species ("high pH") at alkaline values. Within the pH range 6–8.5, the pH depeendence of the Mo(VI)/Mo(V) couple for *Chlorella* NR closely resembled the behavior of the "slow" signal of xanthine oxidase, corresponding to an approximate −60 mV/pH unit dependence for both enzymes. This behavior suggests that the reduction of Mo(VI) to Mo(IV) in *Chlorella* NR involves two successive cycles of reduction and protonation, as shown in Scheme I. While pK_a values for the redox-associated protonation/deprotonation reactions of the Mo species of xanthine oxidase were obtained, these protonation/deprotonation steps occurred outside the pH range accessible to our current examination of NR. Consequently, only limiting values can be provided concerning the pK_a's of any protonation/deprotonation steps for the Mo–pterin center of *Chlorella* NR.

FAD. CD potentiometric titrations were performed between pH 6 and 9, using the spectral change at 460 nm to monitor the amount of oxidized NR flavin. In each case, the titration curves were consistent with a concerted $n = 2$ reduction process. The CD spectrum of the flavin semiquinone form of NR has not been determined; however, the monotonic behavior of the CD spectral change and the n value of 2 indicated the virtual absence of this species during the titrations. The absence of any significant formation of the flavin semiquinone species was confirmed by low-temperature EPR analysis of *Chlorella* NR samples poised at the midpoints of the titrations, which failed to elicit detectable flavin semiquinone. In contrast, a flavin semiquinone form of NR has been readily obtained under nonequilibrium conditions, by freezing samples promptly following addition of NADH (Solomonson et al., 1984). Flavin semiquinone was detected in NADH-reduced samples at both pH 6 and 9 (Figure 4) and exhibited a g value of 2.004 as previously reported (Solomonson et al., 1984). The line width of 1.3 mT, which is characteristic of a red, anionic flavin semiquinone (Massey & Palmer, 1966), did not change within the accessible pH range, suggesting the pK_a for protonation (producing the blue, neutral form) is equal to or lower than 5.5. Formation of flavin semiquinone in the presence of NADH is accompanied by appearance of a broad charge-transfer band in the visible spectrum at 600 nm and a positive shift in the E_m for the FAD/$FADH^-$ couple (unpublished work). The altered properties of the FAD in the presence of NADH/NAD^+ suggested that the anionic flavin semiquinone is not the intermediate formed in the absence of NADH/NAD^+. However, the pH dependence of the midpoint potential of the FAD/$FADH^-$ couple reported in this study is fully consistent with this hypothesis. The pH dependence of −30 mV/pH unit observed within the pH range 6–9 indicated that a single proton was bound upon complete ($n = 2$) reduction of FAD. This behavior strongly suggests that $FADH^-$ is the fully reduced form, with the anionic flavin semiquinone being protonated upon the second one-electron reduction. Our results suggest that conversion of FAD to $FADH^-$ requires two reduction steps followed by a single protonation (Scheme I).

Sequence analysis of cDNA of higher plant NR's has shown significant sequence similarity between the C-terminal flavin-containing domain of NR and the flavoprotein cytochrome b_5 reductase (Crawford et al., 1988). Potentiometric titrations of cytochrome b_5 reductase (Iyanagi et al., 1984) have yielded a midpoint potential for the FAD/$FADH^-$ couple of −258 mV (pH 7), similar to the corresponding value of NR. In addition, the flavin midpoint potential of cytochrome b_5 reductase exhibited a −30 mV/pH unit dependence over the pH range 6.0–9.0 while significant levels of the anionic semiquinone were only detected for the NADH-reduced enzyme, suggesting similar properties for the flavin prosthetic group in both enzymes.

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Registry No. NR, 9013-03-0; FAD, 146-14-5; Mo, 7439-98-7.

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A γ-Carboxyglutamic Acid (γ) Variant (γ^6D, γ^7D) of Human Activated Protein C Displays Greatly Reduced Activity as an Anticoagulant†

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ABSTRACT: Site-specific mutagenesis has been employed to alter the cDNA of human protein C (PC), such that the γ-carboxyglutamic acid (γ) pair at positions 6 and 7 of the recombinant (r) protein would be changed to aspartic acid residues. This variant, [γ^6D, γ^7D]r-PC, and its wild-type (wt) counterpart have been expressed in human kidney 293 cells. After purification, forms of wtr-PC that were fully γ-carboxylated and β-hydroxylated and of [γ^6D, γ^7D]r-PC that lacked only the two altered γ-residues at amino acid sequence positions 6 and 7 were obtained. Subsequent to its conversion to activated PC (APC), [γ^6D, γ^7D]r-APC displayed a greatly reduced activity in the activated partial thromboplastin time of PC-deficient plasma, as compared to wtr-APC and human plasma APC. In addition, the activity of [γ^6D, γ^7D]r-APC toward inactivation of purified human factor VIII was reduced to less than 5% of that of wtr-APC and human plasma APC. These results, with the first reported mutations at γ-residues of PC produced by recombinant DNA technology, indicate that the paired γ-residues at positions 6 and 7, which are highly conserved in all vitamin K dependent coagulation proteins, are very important to generation of fully functional APC. Additional results demonstrate further that lack of γ-carboxylation at positions 6 and 7 of PC does not substantially affect this same processing reaction at other relevant glutamic acid residues.

Protein C (PC)[1] is a plasma protein that has sequence homology with other vitamin K dependent serine protease zymogens. PC functions as an anticoagulant subsequent to its conversion to activated protein C (APC) by virtue of its limited proteolytic inactivation of cofactors necessary for clot formation, viz., factor V (f-V) and factor Va (f-Va) (Kisiel et al., 1977), as well as factor VIII (f-VIII) and factor VIIIa (f-VIIIa) (Vehar & Davie, 1980), in a reaction that is stimulated by Ca^{2+}, phospholipid (Kisiel et al., 1977), and a cofactor, protein S (Walker, 1980). Maximal activation of PC occurs at the endothelial cell surface as a result of a limited proteolytic event, catalyzed by thrombin, along with the

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[1] Abbreviations: PC, human protein C; APC, activated human protein C; [γ^6D, γ^7D]r-PC(APC), a recombinant protein C (or activated protein C) containing aspartic acid residues substituted for γ-carboxyglutamic acid residues at positions 6 and 7 of the protein C amino acid sequence; γ, γ-carboxyglutamic acid; βOH-D, β-hydroxyaspartic acid; r, recombinant; wt, wild type; DodSO$_4$/PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fmoc, [(9-fluorenylmethyl)oxy]carbonyl.

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