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EPR and Kinetic Analysis of the Interaction of Halides and Phosphate with Nitrate Reductase†

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ABSTRACT: Electron paramagnetic resonance spectra obtained during turnover of the Mo center of NADH:nitrate reductase at pH 8 were comprised of two Mo(V) species, signal A (g_1 = 1.996, g_2 = 1.969, g_3 = 1.967, A_1^H = 1.25 mT, A_2^H = 1.18 mT, and A_3^H = 1.63 mT) and signal B (g_1 = 1.996, g_2 = 1.969, and g_3 = 1.967), the former exhibiting superhyperfine interaction due to strong coupling with a single, exchangeable proton. Binding of halides and nitrite to the Mo center increased the proportion of signal A whereas phosphate had no effect on the EPR line shape. Halides decreased and phosphate increased the rates of enzyme activities involving the Mo center (NADH:nitrate reductase and reduced methyl viologen:nitrate reductase), but neither had any effect on activities involving FAD (NADH:ferricyanide reductase) or heme (NADH:cytochrome *c* reductase), indicating specific binding of halides to the Mo center. Halides were found to be weak, mixed competitive–noncompetitive inhibitors (Cl^- K_I = 39 mM, μ = 0.2 M, pH 8) of nitrate reductase forming a catalytically inactive ternary halide–nitrate–enzyme complex. Inhibition patterns changed from nearly noncompetitive (F^-) to nearly competitive (I^-). The weakening of nitrate binding due to halide binding correlated with increased halide electronegativity rather than ionic radius. In contrast, phosphate (K_d = 7.4 mM, μ = 0.2 M, pH 8) and arsenate were determined to be nonessential activators, characterized by a constant value of $(V_{max}/K_m)_{app}$, increasing nitrate reductase activity by weakening nitrate binding without affecting the stability of the transition state. Phosphate had no effect on product inhibition by nitrite (K_I = 0.33 mM) or the oxidation–reduction midpoint potentials of the Mo center.

Assimilatory nitrate reductase (NR)[1] from *Chlorella vulgaris* is a homotetrameric enzyme of molecular weight 375K, containing FAD, heme, and Mo–pterin prosthetic groups in a ratio of 1:1:1 per subunit (Giri & Ramadoss, 1979; Howard & Solomonson, 1982). The enzyme catalyzes the initial and regulated step in the assimilation of nitrate, the NADH-dependent reduction of nitrate to nitrite (Guerrero et al., 1981; Dunn-Coleman et al., 1984). The Mo prosthetic group of NR is the site of nitrate reduction and is complexed to a sulfur-containing pterin derivative (molybdopterin) (Johnson et al.,

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[1] Abbreviations: NR, nitrate reductase; EPR, electron paramagnetic resonance; EXAFS, X-ray absorption fine structure; NADH:NR, NADH-dependent nitrate reductase; NADH:FR, NADH-dependent ferricyanide reductase; NADH-CR, NADH-dependent cytochrome *c* reductase; FH_2:NR, reduced FMN-dependent nitrate reductase; MV:NR, reduced methyl viologen dependent nitrate reductase.

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1984). The Mo–pterin complex cycles between Mo(VI) and Mo(IV) redox states while catalyzing the reduction of nitrate (Solomonson et al., 1984).

The environment of the catalytic Mo center has been studied primarily by EXAFS (Cramer et al., 1984) and EPR spectroscopy (Solomonson et al., 1984). The EXAFS study indicated that the Mo center in *Chlorella* NR closely resembled that of the related Mo-containing hydroxylase sulfite oxidase (Cramer et al., 1981) in that the Mo was complexed to at least two sulfur ligands, together with one oxo group ligand in the dithionite-reduced enzyme and two oxo ligands in the oxidized state (Cramer et al., 1984). EPR studies of Mo(V) in partially reduced *Chlorella* NR (Solomonson et al., 1984) have indicated that the Mo center of NR may exist in either a "low-pH" or a "high-pH" form, each with distinct g values. In addition, limited studies with the closely related enzyme from spinach have indicated that the "low-pH" species exists in two forms, the interconversion of which is influenced by the presence of various anions, particularly chloride (Gutteridge et al., 1983). In the presence of chloride, the EPR spectrum exhibited superhyperfine structure due to strong coupling to a single exchangeable proton, resulting in an EPR signal designated signal A, while in the absence of chloride the superhyperfine coupling was substantially decreased (signal B). A similar duality of low-pH EPR spectra has been reported for sulfite oxidase (Bray et al., 1983). For this enzyme, a 10-fold increase in the concentration of chloride raised the pK_a for interconversion between the two EPR species by 1 pH unit, suggesting that chloride and proton ligation may be stoichiometrically coupled (Bray et al., 1983). This behavior has been interpreted as a ligand exchange reaction in which the binding of chloride to Mo is accompanied by conversion of an existing oxo ligand of Mo into the hydroxyl form (Bray et al., 1983; Bray, 1986). Direct ligation of chloride to the Mo of sulfite oxidase has been inferred from $^{19}F^-$ superhyperfine splittings apparent in the Mo(V) EPR signal (Bray et al., 1983).

In addition to NADH-dependent nitrate reduction, NR catalyzes a number of partial enzyme activities involving one or more of the prosthetic groups: ferricyanide and cytochrome c may accept electrons from NADH-reduced enzyme, whereas reduced FMN and reduced methyl viologen can act as alternative electron donors to the enzyme (Guerrero et al., 1981; Dunn-Coleman et al., 1984). Several anions containing oxo groups (phosphate, arsenate, pyrophosphate, selenate, tellurate, and α-glycerophosphate) stimulate NR activity (Nicholas & Scawin, 1956; Howard & Solomonson, 1981). Activation is specific for partial enzymatic activities catalyzing nitrate reduction, implicating Mo as the site of action (Kay & Barber, 1986). Phosphate is the most effective activator and has been suggested as a possible physiological regulator of NR activity in vivo (Nicholas & Scawin, 1956). Stimulation by phosphate was distinct from a strong dependence of NADH:NR activity on ionic strength, since stimulation by increasing ionic strength was not dependent on the nature of the ions present (Kay & Barber, 1986). No consensus currently exists concerning the kinetic mechanism of phosphate stimulation, possibly because of inadequate control of assay conditions in some previous studies. The need to maintain constant ionic strength and avoid the presence of halides has not previously been recognized.

We have examined the effects of halides and phosphate on both the EPR and steady-state kinetic properties of *Chlorella* NR to differentiate these effects. Halide ligation is shown to fully inactivate NR and is of consequence to earlier kinetic studies in which chloride was present in the assay media. Chloride, fluoride, and nitrite facilitated conversion of Mo(V) to a species exhibiting superhyperfine interaction with a single exchangeable proton. In contrast, phosphate did not significantly perturb either the Mo(V) EPR spectrum or the midpoint potentials of the Mo center, providing no evidence for formation of a Mo–phosphate complex. Under controlled assay conditions, kinetic analysis indicated that activation by phosphate and arsenate may be explained as anion-induced weakening of nitrate binding to NR, and we propose a kinetic mechanism for this process.

Materials and Methods

Enzyme Preparation. Nitrate reductase was isolated from *C. vulgaris* as previously described (Howard & Solomonson, 1981), with the addition of a final step involving preparative HPLC on a TSK4000 column. The enzyme exhibited an 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). ^2H-Substituted NR was prepared by extensive dialysis of native enzyme against buffers using ^2H_2O as solvent.

All enzyme samples were prepared in zwitterionic buffers using "Ultrol" grade reagents (Calbiochem, San Diego, CA) and were analyzed colorimetrically for Cl^- contamination by using mercury thiocyanate and ferric ion (Iwasaki et al., 1952). Under these conditions, endogenous Cl^- concentrations were generally in the range 0.05–0.4 mM.

^{17}O-Substituted phosphate was prepared as described by Cohn and Drysdale (1955).

EPR Spectroscopy. EPR spectra were recorded by using a Varian E109 Century Series spectrometer (Varian Associates, Palo Alto, CA) operating at 9 GHz with 100-kHz modulation and equipped with a variable-temperature accessory. Mo(V) spectra were recorded at 173 K using a modulation amplitude of 0.3 mT and an incident microwave power of 5 mW. Samples of NR (10–20 μM heme) were poised at the appropriate potentials in an anaerobic potentiometric titration vessel in the presence of dye mediators as previously described (Solomonson et al., 1984).

To avoid contamination of samples by Cl^- efflux from the Ag/AgCl micro reference electrode in the titration cell, some Mo(V) samples were prepared by quick-freezing enzyme during turnover using a coupled NADH-generating system comprising NAD^+ (0.1 mM), D-glucose 6-phosphate (2 mM), KNO_3 (2 mM), and sufficient glucose-6-phosphate dehydrogenase (Sigma type XXIV from *Leuconostoc mesenteroides*) to enable NR to reduce nitrate at approximately 20% of the maximum activity. The amount of glucose-6-phosphate dehydrogenase required was determined for each assay medium used. Reagents, at the final concentrations indicated, were rapidly mixed into a quartz EPR tube and immediately frozen in an isopentane/liquid N_2 mixture.

Room temperature EPR potentiometric titrations of Mo(V) were performed as previously described (Kay et al., 1988). Simulated EPR spectra were calculated as described by Lowe (1978). For simulations of EPR spectra in ^2H_2O, ^2H splittings were taken to be 6.51 times smaller than ^1H splittings (Lamy et al., 1978).

Enzyme Assays. Full and partial enzyme activities were determined at 25 °C as previously described (Kay & Barber, 1986). Enzyme activities, measured as initial rates, were expressed in units of micromoles of substrate consumed or product generated per minute per nanomole of NR heme. Unless otherwise stated, assays were performed at pH 8 in "Ultrol" grade buffers in the presence of 0.1 mM EDTA at

Table I: EPR Parameters for the "Low pH" Mo(V) Spectra of *Chlorella* Nitrate Reductase[a]

species	g_1	g_2	g_3	g_{av}	A_1^H	A_2^H	A_3^H	A^H_{av}	Δ_1[b]	Δ_2[b]	Δ_3[b]
signal A	1.996	1.969	1.967	1.977	1.25	1.18	1.63	1.35	0.2	0.15	0.15
signal B	1.996	1.969	1.967	1.977					0.2	0.15	0.15

[a] g values and A values were obtained by comparison of experimental and simulated spectra. g values were determined to an accuracy of ±0.0005. [b] Δ_1, Δ_2, and Δ_3 represent the half line widths used in the simulations.

a total ionic strength of 0.2 M. The concentrations of "Good" buffers required to maintain a given pH and ionic strength were calculated as previously described (Kay & Barber, 1986). Phosphate assay medium (μ = 0.2 M) was prepared by mixing solutions of 0.2 M KH_2PO_4 and 66.7 mM K_2HPO_4, each containing 0.1 mM EDTA, to the correct pH. Kinetic parameters were derived from the experimental initial rate data by least-squares fitting to the original hyperbolic rate equations using the software "Enzfitter" (Elsevier Biosoft).

Results

EPR Spectra of Mo(V) and Mo(V)–Anion Complexes. EPR spectra of Mo(V), in the absence and presence of various anions, are shown in Figure 1. To yield the species corresponding to partially reduced native enzyme under conditions of minimal chloride contamination and in the absence of exogenous phosphate (Figure 1A), NR was frozen during turnover by using an NADH-generating system. Under these conditions, the spectrum, which corresponded to approximately 20% conversion of Mo to Mo(V), exhibited a complex line shape, suggesting the presence of two paramagnetic species, one exhibiting superhyperfine interaction due to strong coupling with a single $s = {}^1/_2$ nucleus and one in which this superhyperfine interaction was either substantially reduced or abolished. This is most clearly seen in the low-field g_1 feature, centered at 321 mT, where three partially resolved peaks are discernible. Previous work with the spinach enzyme (Gutteridge et al., 1983) has referred to these split and unsplit Mo(V) species as signal A and signal B, respectively.

In addition to Mo(V), low levels of flavosemiquinone radical were also observed in spectra of enzyme samples frozen during turnover. Exhibiting a g value of approximately 2.004 and a line width of 0.15 mT, these values are characteristic of the anionic flavosemiquinone (Solomonson et al., 1984). Double integration of the spectra indicated that only 1–2% of the FAD was present as the flavosemiquinone.

Substitution of MOPS with phosphate buffer of equivalent ionic strength had little effect on the line shape of the Mo(V) species (Figure 1B), indicating only minor alteration in the relative proportions of signal A and signal B. Identical spectra were obtained for samples poised at controlled potentials in either regular phosphate buffer or buffer prepared using ^{17}O-substituted phosphate, the latter samples showing no detectable broadening or splitting of the Mo(V) line shape attributable to hyperfine interaction of Mo(V) with ^{17}O ($I = {}^5/_2$).

In contrast, Mo(V) spectra of NR poised at controlled potentials in the presence of 100 mM Cl^- (Figure 1C) corresponded to predominantly signal A while samples frozen during turnover in chloride-containing buffer also showed significantly more signal A than those obtained in the absence of Cl^-, although conversion to signal A was incomplete. This is presumably due to competition between Cl^- and nitrate binding during turnover. The line shapes of spectra obtained in the presence of F^- (50 mM) were indistinguishable from those obtained in the presence of Cl^-.

Addition of nitrite also increased the proportion of signal A to signal B in samples prepared by quick-freezing during turnover (Figure 1D).

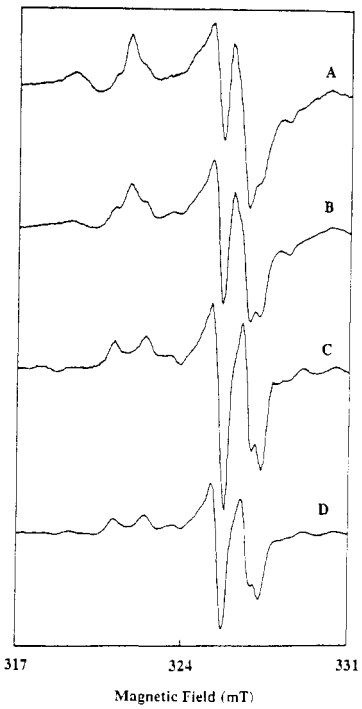


FIGURE 1: Influence of anions on *Chlorella* nitrate reductase Mo(V) EPR spectra. Spectra A, B, and C were obtained from enzyme samples (10–20 μM heme) quick-frozen during turnover using the NADH-generating system. Spectrum C was obtained from a sample of enzyme (15 μM heme) poised at −10 mV in the presence of dye mediators. (A) MOPS buffer, μ = 0.2 M, pH 8.0. (B) Phosphate buffer, μ = 0.2 M, pH 8.0. (C and D) MOPS buffer, pH 8.0, containing either 50 mM KCl or 50 mM KNO_2, respectively, with a final μ = 0.2 M. The field scale corresponds to a microwave frequency of 8.984 GHz.

To determine the EPR parameters for signal A and signal B, we utilized comparisons of the spectra obtained in the presence and absence of chloride. The hypothesis that the Mo(V) spectrum obtained in the presence of excess Cl^- corresponded to a single species (signal A) was confirmed by computer simulations of the EPR signals obtained in both ^1H_2O and ^2H_2O using the g and A values shown in Table I. The experimental spectrum in H_2O (Figure 2A) could be adequately simulated (Figure 2B) by using a single set of g values and assuming superhyperfine interaction with a single strongly coupled proton while the spectrum obtained in ^2H_2O (Figure 2C) could be stimulated by using the same g values but with A values decreased to reflect substitution of ^2H for ^1H (Figure 2D).

To determine the EPR parameters for signal B, we initially used the spectra obtained from enzyme samples during turnover in the absence of Cl^- (Figure 3A). Under these conditions, the line shape corresponded to approximately equal proportions of signals A and B as judged from computer-generated spectra. In ^2H_2O, this complex line shape was reduced to a nearly axial signal (Figure 3C) which could be adequately simulated (Figure 3D) by using the same parameters obtained for signal A in ^2H_2O, indicating that signals A and B were the same species except that signal A showed strong superhyperfine coupling to a single proton. To confirm this analysis, we generated the experimental spectrum con-

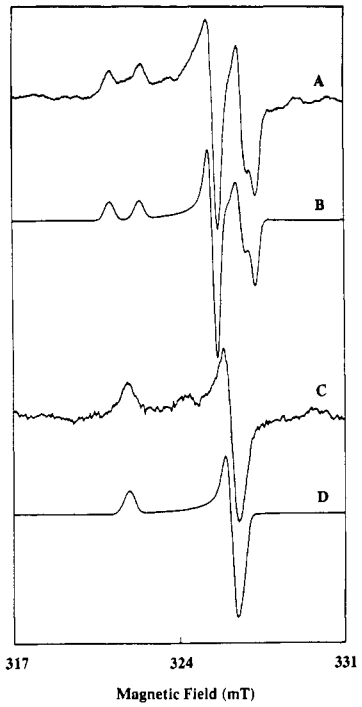


FIGURE 2: EPR spectra of nitrate reductase Mo(V) signal A in ^1H_2O and ^2H_2O. Samples of nitrate reductase (10–20 μM heme) were poised at −10 mV in the presence of dye mediators. (A) MOPS buffer (^1H_2O), pH 8.0, containing 50 mM KCl (μ = 0.2 M). (B) Computer simulation of spectrum A. (C) MOPS buffer (^2H_2O), pD 8.0, containing 50 mM KCl (μ = 0.2 M). (D) Computer simulation of spectrum C. The parameters used to simulate the experimental spectra are given in Table I. The microwave frequency was the same as Figure 1.

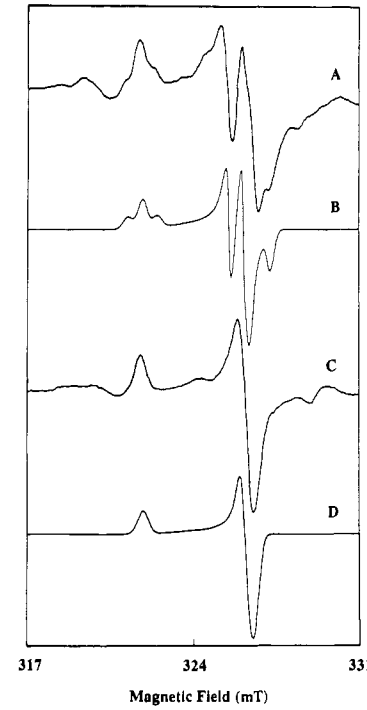


FIGURE 3: EPR spectra of nitrate reductase Mo(V) signal B in ^1H_2O and ^2H_2O. Samples of nitrate reductase (10–20 μM heme) were quick-frozen during turnover using the NADH-generating system. (A) MOPS buffer (^1H_2O, μ = 0.2 M), pH 8.0. (B) Computer simulation of spectrum A obtained by adding equal proportions of the simulated spectra shown in Figure 2B and Figure 3D. (C) MOPS buffer (^2H_2O, μ = 0.2 M), pD = 8.0. (D) Computer simulation of spectrum C. The parameters used to simulate the experimental spectra are given in Table I. The microwave frequency was the same as Figure 1.

sisting of a mixture of signals A and B (Figure 3A) by adding together, in equal proportions, the computer simulations of signals A and B (Figure 3B).

Halide Inhibition. In the course of this study, it was noted that chloride exerted a weak inhibition on NADH:NR activity, noticeably increasing the K_m for nitrate. Chloride has previously been shown to inhibit the related molybdoenzyme sulfite oxidase (Bray et al., 1983) but not assimilatory nitrate reductase. In addition, chloride inhibition is relevant to the interpretation of several previous studies where chloride was included in assay media.

Inhibition of NR by chloride was specific for nitrate-reducing partial enzymatic activities, as shown in Figure 4. The diaphorase partial enzymatic activities (NADH:FR and NADH:CR) were unaffected by chloride concentrations up to 150 mM. In contrast, activities involving the Mo–pterin center and catalyzing nitrate reduction (NADH:NR, FH_2:NR, and MV:NR activities) were approximately 70% inhibited at these chloride concentrations. Concentrations of nitrate and artificial substrates (except for MV, which is obtained as the hydrochloride and has an apparent K_m that is too low to measure spectrophotometrically) that were not saturating (Kay & Barber, 1986) were used to avoid obscuring possible competitive inhibition. The mode of chloride inhibition of NADH:NR activity, at saturating NADH concentrations, appeared to be mixed competitive–noncompetitive. Lineweaver–Burk plots (not shown) in the presence of 0–160 mM KCl were linear and intersected in the second quadrant. Secondary plots (Figure 5, upper pannel) indicated a K_I for chloride of 39 mM. Significantly weaker binding to the enzyme–nitrate complex (K_I = 296 mM) was suggested by the ordinate intercept of the secondary plot of $1/V_{max}$ (Figure 5, upper panel).

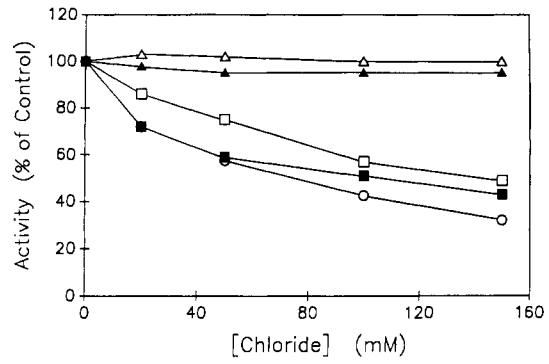


FIGURE 4: Inhibition of nitrate reductase enzymatic activities by chloride. Initial rates of partial enzymatic activities were assayed as described under Materials and Methods in 224 mM MOPS and 0.1 mM EDTA (pH 8.0, μ = 0.2 M) mixed with proportions of 0.2 M KCl and 0.1 mM EDTA to yield the chloride concentrations indicated. (■) NADH:NR, 50 μM NADH, 50 μM NO_3^-; (▲) NADH:FR, 50 μM NADH, 100 μM $K_3Fe(CN)_6$; (▲) NADH:CR, 50 μM NADH, 15 μM cytochrome *c*; (□) FH_2:NR, 100 μM FMN, 50 μM NO_3^-; (○) MV:NR, 75 μM $MV^{+\bullet}$, 50 μM NO_3^-.

Since chloride inhibition of assimilatory NR had not previously been reported, we determined whether the chloride form of the enzyme was fully inactive, or was still capable of catalysis, though at a reduced rate (i.e., hyperbolic or partial mixed-type inhibition). The scheme for hyperbolic inhibition is similar to Scheme I (below) for phosphate activation except that the factor "A" would represent chloride, and turnover of the E·A·S complex (bk_{cat}) is modified by a factor b such that $0 < b < 1$. The appropriate linear graph for partial inhibition is shown in the lower panel of Figure 5 (Segal, 1975). For a partial inhibitor, the intercepts of the two lines on the 1/[chloride] axis should be identical and to the left of the y axis.

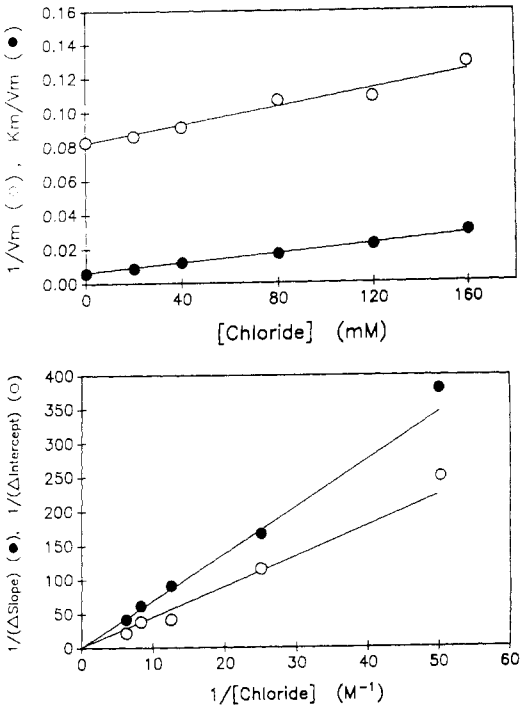


FIGURE 5: Secondary plots of the inhibition of NADH:NR activity by chloride. The effect of chloride on initial rates of NADH:NR activity at pH 8.0 and μ = 0.2 M. Assay media were prepared as described in Figure 4: (○) no chloride; (●) 20 mM chloride; (△) 40 mM chloride; (▲) 80 mM chloride; (□) 120 mM chloride; (■) 160 mM chloride. (Upper panel) Secondary replots of the slope and intercepts appropriate for simple linear mixed competitive–noncompetitive inhibition. (Lower panel) Secondary replot appropriate for partial or hyperbolic inhibition by chloride.

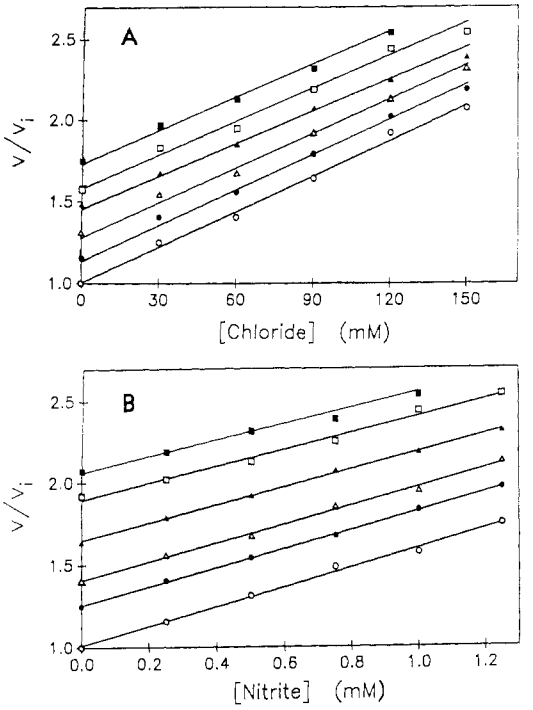


FIGURE 6: Yonetani–Theorell plot of inhibition of NADH:NR activity by combinations of chloride and nitrite. Initial rates of NADH:NR activity were measured in MOPS assay medium containing NADH (160 μM), nitrate (200 μM), and chloride and nitrite as indicated. The total ionic strength was maintained at 0.2 M as described in Figure 4. The basis for the plot is described in the text. (A) (○) no nitrite; (●) 0.25 mM, (△) 0.5 mM, (▲) 0.75 mM, (□) 1 mM, and (■) 1.25 mM nitrite. (B) (○) No chloride; (●) 30 mM, (△) 60 mM, (▲) 90 mM, (□) 120 mM, and (■) 150 mM chloride.

The value of the intercept is then $-y/xK_I$ (Segal, 1975). When the chloride inhibition data were replotted in this form, the lines intersected at the origin, which indicated that $y = 0$ and therefore the chloride form of NR was catalytically inactive (Figure 5, lower panel). Chloride (100 mM) increased the apparent K_I for competitive inhibition by nitrite from 0.36 mM to 3.5 mM (data not shown). Clearly, the presence of chloride altered the inhibition by nitrite, but it was not clear whether chloride binding and nitrite binding were mutually exclusive or if both inhibitors could bind simultaneously. Yonetani–Theorell plots were used to distinguish these possibilities. The Yonetani–Theorell plot for inhibition by mixtures of nitrite and chloride is shown in Figure 6. Mutual exclusivity of chloride and nitrite binding would be indicated by parallel plots when either chloride (Figure 6A) or nitrite (Figure 6B) was the varied inhibitor. Within the limits of accuracy of the experiments, the plots appeared parallel, suggesting that inhibition by chloride and nitrite was essentially mutually exclusive. Had both inhibitors bound to NR simultaneously, the plots would not have been parallel: instead, the gradients of the lines would have increased as the concentration of the second inhibitor was raised. The basis of the plot is that mixtures of inhibitors would, if mutually exclusive, inhibit to an extent determined by the sum of their specific concentrations ($[I]/K_I$), resulting in parallel plots. Simultaneous binding of both inhibitors would lead to less than additive inhibition, and therefore nonparallel plots, due to formation of the doubly inhibited complex.

Other halides were also found to inhibit NADH:NR activity. Fluoride, bromide, and iodide each inhibited by mixed competitive–noncompetitive mechanisms, although the inhibition constants varied widely. The inhibition constants are collected in Table II. The K_I values, representing binding of

Table II: Comparison of Inhibition of NADH:NR Activity by Halides[a]

halide	K_I(E) (mM)	K_I(ES) (mM)	ionic radius (pm)	Pauling electronegativity	$\Delta\Delta G_b$ (kJ mol^{-1})
fluoride	52	580	136	4.0	5.97
chloride	39	296	181	3.2	5.03
bromide	133	425	195	3.0	2.88
iodide	190	266	216	2.7	0.83

[a] The inhibition constants were derived from Lineweaver–Burk plots carried out in the presence of 0–180 mM halide. K_I(E) and K_I(ES) represent respectively the binding constants for halide to NR saturated with NADH and for NR saturated with both NADH and nitrate. The change in Gibbs binding energy for nitrate upon halide binding was calculated as described in the text and is given by $\Delta\Delta G_b$. Values for ionic radius and Pauling electronegativity were taken from Atkins (1978).

halide to free enzyme, for fluoride and chloride were similar. Bromide binding was more than 4-fold weaker, and iodide binding was 5-fold weaker than the binding of chloride. Strength of binding did not correlate well with the ionic radii of the halides (Table II), suggesting that steric hindrance may not be the determining factor in weakening the binding of the larger halides. All the halides appeared from the kinetic analysis to be bound more weakly to the NR–nitrate complex than to free NR (Table II). There was no clear trend in the strength of binding of the different halides to the NR–nitrate complex, again suggesting size was not the determining factor. However, comparison of the strength of binding to free and NR–nitrate complex for each halide revealed that the value of b (the ratio of K_m values in the presence and absence of saturating chloride concentrations) correlated with the electronegativity of the halide ion (Table II), indicating that increasing electronegativity correlated with a shift from nearly

noncompetitive (I^-) to nearly competitive (F^-) inhibition. From the ratio b, which is the ratio of halide binding constants to E (K_E) and ES (K_{ES}), values for the change in halide binding energy caused by nitrate binding may be calculated. From the relations:

$$\Delta G_E = -RT \ln K_E$$
$$\Delta G_{ES} = -RT \ln K_{ES}$$

where ΔG_E and ΔG_{ES} are the free energies of halide binding to free NR and NR–nitrate complex, respectively, then

$$\Delta G_E - \Delta G_{ES} = RT \ln (K_{ES}/K_E)$$

These values are shown in Table II and suggest that halide electronegativity determined the degree of interaction between nitrate and halide binding.

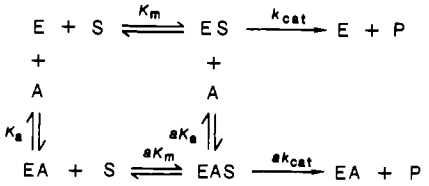
Phosphate Activation. Activation of NADH:NR activity by inorganic phosphate has been previously reported (Nicholas & Scawin, 1956; Howard & Solomonson, 1981) and the site of action localized to the nitrate-reducing partial enzyme activities (Kay & Barber, 1986). Phosphate is not absolutely required for activity and can therefore be considered to be a nonessential activator.

In addition to the specific stimulatory effect of phosphate, nitrate-reducing enzyme activities and the rate of oxidation of the heme prosthetic group are markedly enhanced at high ionic strength by a mechanism that is insensitive to the particular ionic species used (Kay & Barber, 1986). To avoid confusing specific phosphate stimulation with nonspecific ionic strength effects, all kinetic experiments were performed at constant and optimal (μ = 0.2 M) ionic strength as described under Materials and Methods. Since chloride inhibits NR activity, assay conditions were selected that minimized potential Cl^- contamination.

At saturating concentrations of NADH (160 μM), pH 8 and an ionic strength of 0.2 M, increasing concentrations of phosphate increased NR activity, resulting in parallel Lineweaver–Burk plots (not shown). Activity was increased by phosphate at all nitrate and phosphate concentrations examined. Phosphate both increased V_{max} [from 9.3 to 13.0 μmol of NADH min^{-1} (nmol of heme)$^{-1}$] and increased K_m (from 80 to 112 μM) but did not alter the ratio K_m/V_{max} since the slopes of the plots were constant. Thus, the observed stimulation of NADH:NR activity correlated with parallel increases in V_{max} and K_m such that the ratio of K_m to V_{max} remained constant. The same behavior was observed when arsenate replaced phosphate (not shown).

Previous work has shown that *Chlorella* NR follows a random Bi-Bi mechanism in which substrates and products rapidly equilibrate with their binding sites (Howard & Solomonson, 1981). As discussed below, this mechanism permitted the kinetics of the nitrate-reducing active site to be treated like those of a single substrate Michaelis–Menten enzyme, provided the second substrate (NADH) was present at saturating concentrations. Saturation was achieved by addition of 160 μM NADH since the K_m for NADH has been reported to be 4 μM (Howard & Solomonson, 1981). Previous measurements of the degree of heme reduction during catalysis and of partial enzymatic activities have suggested that the slow step of catalysis is associated with nitrate reduction rather than NADH oxidation (Kay & Barber, 1986). Consequently, at saturating concentrations of NADH, a simple kinetic scheme for nonessential activation by phosphate and arsenate may be suggested (Segal, 1975) (Scheme I) in which E, S, ES, A, and P represent, respectively, reduced NR, nitrate, reduced NR–nitrate complex, phosphate or arsenate, and products. The factor a ($a > 1$) represents the extent to which K_m and k_{cat} are altered by activator binding. This scheme would increase both V_{max} and K_m, and yet leave K_m/V_{max} unaltered. Modification of this model, so that activation occurred by a mechanism in which A bound only to ES to enhance k_{cat} (analogous to uncompetitive inhibition, which also generates parallel reciprocal plots), was unable to generate parallel reciprocal plots for nonessential activation, since K_m and V_{max} were necessarily changed by different factors. A possible mechanism to explain how NADH:NR activity may be increased at constant K_m/k_{cat} is proposed below. The concentration dependence of phosphate stimulation of $V_{max,app}$ is shown in Figure 7A. The data points were fitted to the Michaelis form of the rate equation corresponding to Scheme I, derived by using the rapid equilibrium assumption (Segal, 1975), which is given by eq 2:

Scheme I



(1)

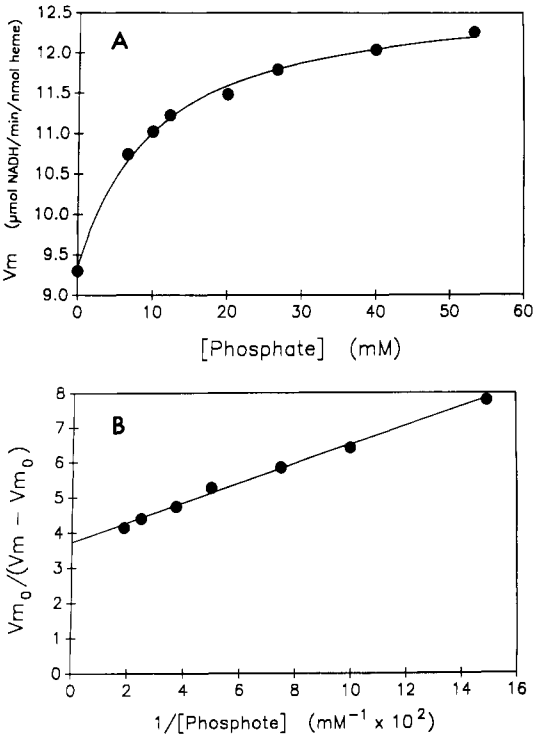


FIGURE 7: Concentration dependence of phosphate stimulation and linearized plot of nonessential activation. Apparent V_{max} values were derived from double-reciprocal plots measured at μ = 0.2 M and pH 8.0 as described in Figure 1. (A) Concentration dependence of V_{max} stimulation by phosphate. (B) Linearized plot of phosphate stimulation of V_{max} plotted according to eq 3 as given under Results.

$$v = \frac{V_{max,app}[S]}{K_{m,app} + [S]} \quad (2)$$

where

$$V_{max,app} = \frac{V_{max}a(K_a + [A])}{aK_a + [A]}$$

and

$$K_{m,app} = \frac{K_m a(K_a + [A])}{aK_a + [A]}$$

Equation 2 was rearranged to a linear form expressing the increase in $V_{max,app}$ caused by phosphate:

$$\frac{V_{max}}{V_{max,app} - V_{max}} = \frac{1}{[A]}\frac{aK_a}{a-1} + \frac{1}{a-1} \quad (3)$$

Values of a (1.40) and K_a (7.4 mM) were derived from the slope and intercept of experimental $V_{max,app}$ data plotted according to eq 3 (Figure 7B). Because of the rapid equilibrium nature of the mechanism of *Chlorella* NR, the observed K_m represents the equilibrium binding constant for nitrate. The change in K_m for nitrate upon saturation with phosphate from 80 to 112 μM (aK_m) corresponded to a change in the Gibbs binding energy for nitrate of 0.83 kJ mol^{-1}.

Nitrite inhibits NADH:NR activity of *Chlorella* NR competitively with respect to nitrate (Howard & Solomonson, 1981). Since phosphate and arsenate appeared to stimulate NADH:NR activity by a mechanism involving the weakening of nitrate binding to NR, it was of interest to examine whether nitrite binding was also weakened by the presence of phosphate. Inhibition by nitrite was competitive with respect to nitrate both in the presence and in the absence of phosphate. The apparent K_m's for nitrate in the presence and absence of phosphate were measured from double-reciprocal plots in the presence of increasing concentrations of nitrite. The plot for a competitive inhibitor corresponds to

$$K_{m,app} = (K_m/K_I)[NO_2^-] + K_m$$

The K_I for nitrite, determined from the x-axis intercept in the absence of phosphate (0.35 mM), was not increased in the presence of phosphate (0.33 mM, data not shown).

The possibility of direct ligation of phosphate to the Mo center was examined by using EPR spectroscopy. However, substitution of phosphate buffer for MOPS buffer did not significantly change the proportions of Signals A and B, neither did Mo(V) spectra obtained in the presence of [^{17}O]phosphate show evidence of ^{17}O superhyperfine interaction. In addition, room temperature EPR potentiometric titration of the Mo-pterin prosthetic group performed in MOPS and phosphate buffers of equivalent ionic strength yielded identical potentials, within experimental error, of +5 and −26 mV for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) redox couples, respectively.

Discussion

The preceding results demonstrate that the interaction of halides with the Mo-pterin center of NR results in both loss of nitrate-reducing activity and alterations in the line shape of the observed Mo(V) EPR spectrum by increasing the proportion of Mo(V) in the form giving rise to signal A, which exhibits superhyperfine coupling of Mo to a single exchangeable proton. In contrast, no direct EPR evidence was obtained to support the existence of a phosphate–Mo complex for *Chlorella* NR, despite the specific activation of nitrate-reducing activities by phosphate.

Very similar EPR parameters have been obtained for the corresponding Mo(V) spectra of spinach NR (Gutteridge et al., 1983). In addition, anions such as nitrate, acetate, phosphate, and fluoride were also found to alter the relative proportions of signals A and B. For the respiratory NR, isolated from *Escherichia coli*, anion binding has been shown to result in additional superhyperfine splittings of the Mo(V) spectrum when F^- was present, indicating direct coordination of the anion to Mo (George et al., 1985).

Specific inhibition of assimilatory NR by halides has not been previously reported. This may be because chloride binds 7-fold more weakly to the NR–nitrate complex than to free enzyme, and so inhibition may be largely overcome by adding sufficient nitrate (Kay & Barber, 1986). Furthermore, in experiments where ionic strength was not adequately controlled, stimulation by increasing ionic strength may have partially offset chloride inhibition. One significant conclusion derived from this work is that the halide complex of NR is catalytically inert. The equilibria, deduced from initial rate kinetics, describing phosphate stimulation and halide inhibition were similar except for the ability of the ternary enzyme–nitrate–anion complex to catalyze nitrate reduction: the phosphate complex was active whereas the halide complex was inactive. All the anions examined weakened nitrate binding but differed in their effect on the binding of the product inhibitor nitrite: halides prevented nitrite binding while phosphate had no effect. This was concluded from Yonetani–Theorell plots, applied to competition between a competitive and a mixed-type inhibitor. Application of the rapid equilibrium assumption to the case where nitrite binding and chloride binding were mutually exclusive gave the equations for the Yonetani–Theorell plots (Figure 6):

$$v/v_i = [Cl^-]\frac{aK_m + [S]}{aK_c(K_m + [S])} + 1 + \frac{[I]K_m}{K_I(K_m + [S])}$$

$$v/v_i = [I]\frac{K_m}{K_I(K_m + [S])} + 1 + [Cl^-]\frac{aK_m + [S]}{aK_c(K_m + [S])}$$

where K_c is the constant for binding Cl^- to the free enzyme (E). In this case, the gradients of the Yonetani–Theorell plots were independent of inhibitor concentrations and so were parallel. Had nitrite and chloride binding occurred simultaneously (with formation of an E·Cl^-·I doubly inhibited complex), then the plot would not have been parallel since the gradient terms would then have been dependent on inhibitor concentrations. In this case, the plots would have intersected to the left of the vertical axis.

The relationship between halide binding and nitrate binding was assessed by comparing the binding constants for nitrate binding to those for the free enzyme and enzyme–halide complex, determined from inhibition studies. The extent to which halide weakened nitrate binding ($\Delta\Delta G_b$, Table II) did not correlate with the size of the halide ion, eliminating size as the determining factor. Instead, the more electronegative the halide, the greater the weakening of nitrate binding observed. These observations are consistent with the usual order of halide–metal ligand binding energies, $F^- > Cl^- > Br^- > I^-$ (Atkins, 1978), and give rise directly to the change from near-competitive (I^-) to near-noncompetitive (F^-) patterns of inhibition that were observed (Table II).

The steady-state kinetic data suggest that nitrate and chloride may bind simultaneously to the Mo-pterin center, while nitrate and nitrite binding and nitrite and chloride binding appear mutually exclusive. Nitrate has been proposed to interact with Mo through two mutually perpendicular coordination sites during catalysis, while nitrite ligation and halide ligation are necessarily monodentate (Cramer et al., 1984). Competitive inhibition by nitrite implied that occupation of the nitrite binding site prevented nitrate binding (Howard & Solomonson, 1981). It is therefore difficult to explain how chloride and nitrate binding could occur simultaneously, while nitrite and chloride binding were exclusive, since occupation of the nitrite binding site by chloride may have been expected to prevent nitrate binding, resulting in competitive inhibition by chloride. Possibly chloride binding to the enzyme–nitrite complex is even weaker than chloride binding to the enzyme–nitrate complex (K_I = 296 mM). If this were correct, then the difference between parallel and nonparallel Yonetani–Theorell plots would become very slight

and difficult to detect. The present data do not therefore fully exclude the possibility of very weak binding of chloride to the enzyme–nitrite complex. EPR analysis did not distinguish these possibilities since both chloride and nitrite promoted formation of the Mo(V) EPR signal A.

There have been several reports of specific stimulation of NADH:NR activity by phosphate (Nicholas & Scawin, 1956; Howard & Solomonson, 1981; Oji et al., 1987, 1988), including a suggestion that stimulation of NR activity by phosphate could represent a possible in vivo regulatory mechanism (Nicholas & Scawin, 1956). In addition to stimulation by phosphate, NADH:NR activities of assimilatory NR from *Chlorella* (Kay & Barber, 1986), spinach (Notton et al., 1988), *Candida nitratophila* (Notton et al., 1987), and barley (Oji et al., 1988) have been shown to be very sensitive to the ionic strength of the assay medium. Thus, failure to maintain constant ionic conditions has probably invalidated the conclusions of several previous studies of phosphate stimulation. In particular, the question of the mechanism of phosphate stimulation and whether K_m, V_{max}, or both are changed by phosphate could not be clearly resolved because of varying ionic strength and/or the presence of halides. The finding that halides inhibited *Chlorella* NR indicated the need to avoid halides while maintaining constant ionic strength. This requirement had been previously unrecognized, with the result that potentially inhibitory concentrations of chloride have often been present in assay media.

The kinetic mechanism of *Chlorella* NR has been reported as rapid equilibrium random Bi-Bi from initial rate studies including product inhibition by nitrite and NAD^+ and dead-end inhibition by adenosine-5′-diphosphoribose (Howard & Solomonson, 1981). Nitrate and NADH may bind in either order, but products are apparently not released until both substrates have bound (Howard & Solomonson, 1981). The binding is rapid equilibrium, with substrates and products equilibrating with their binding sites rapidly compared to the rate of catalysis, so that K_m values are identical with binding constants (K_s). One consequence of the random mechanism is that, at the saturating NADH concentrations used in the present study, the kinetics of inhibitors and activators may be treated as for a single-substrate enzyme, considerably simplifying analysis (Segal, 1975; Cleland, 1973). This would not apply had the mechanism proved to be steady state or sequential. However, the constants measured in the presence of saturating NADH concentrations will represent binding to the NADH-ligated NR and not free NR. In the case of NR from *Chlorella*, NADH binding and nitrate binding do not appear to influence each other, since the binding constants for each substrate are independent of the order of binding and Hill plots indicated no sign of cooperativity (Howard & Solomonson, 1981).

Previous measurements of partial enzymatic activities of *Chlorella* NR and the degree of reduction of the heme during catalysis have provided evidence that the slow step of catalysis is associated with nitrate reduction, rather than with NADH oxidation (Kay & Barber, 1986). Phosphate was previously shown to stimulate only nitrate-reducing enzymatic activities (NADH:NR, FH_2:NR, and MV:NR), and stimulation appeared to be distinct from the effect of ionic strength, since stimulation was strongly dependent on the nature of the anion and high ionic strength alone failed to stimulate MV:NR activity (Kay & Barber, 1986).

At constant ionic strength, in the absence of halides, and at saturating concentrations of NADH, stimulation of NADH:NR activity by phosphate was found to be characterized by parallel increases in V_{max} and K_m such that the ratio V_{max}/K_m remained constant. The simplest model to account for such nonessential activation is shown in Scheme I. The constancy of the ratio V_{max}/K_m may imply that phosphate acts upon NR so as to weaken nitrate binding and that the weakening of nitrate binding is sufficient to explain the stimulation of activity. The principle that weak substrate binding is desirable to increase enzyme activity is well established (Ferscht, 1974). The catalytic advantage of increasing K_m at constant K_m/V_{max} is to increase the proportion of enzyme present in the free form, and thereby increase the rate of catalysis, since the Michaelis–Menten equation may be recast as (Ferscht, 1984)

$$v = [E][S]V_{max}/K_m$$

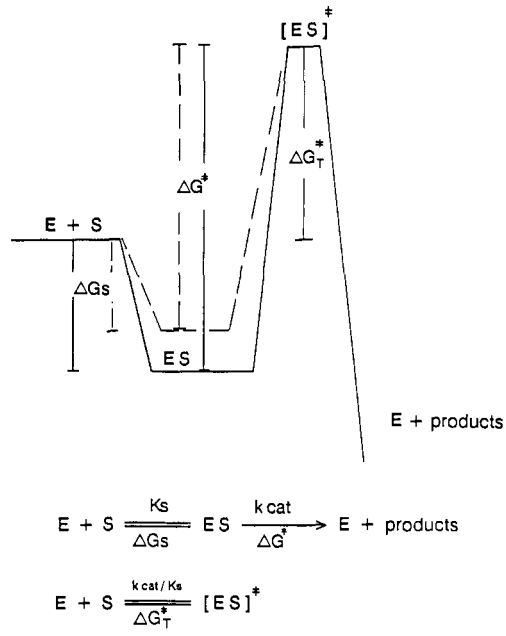


FIGURE 8: Weakening of nitrate binding by phosphate explains stimulation of NR activity. Proposed Gibbs energy profile for the reaction of reduced NR (E) with nitrate (S) where $S > K_m$. (—) No phosphate; (---) +phosphate. The Gibbs energy terms (ΔGs, ΔG^*, G_T^*) may be identified with K_m, k_{cat}, and k_{cat}/K_m as shown below.

In the present study, free enzyme corresponds to NADH-saturated NR. The free energy profile for a Michaelis–Menten reaction when $[S] > K_m$ is depicted in Figure 8. From simple transition-state theory, k_{cat} is determined by the Gibbs energy required to move from the ES complex to the transition state ES^+. Likewise, the K_m is related to the change in Gibbs energy on forming the ES complex (ΔG_B) from free enzyme (E) and nitrate (S). The ratio of k_{cat}/K_m is found from the change in Gibbs energy for the process $E + S \rightarrow ES^*$, reflecting the fact that this ratio represents the second-order rate constant for reaction of free enzyme with substrate. The magnitude of this energy barrier appeared to be unchanged by the presence of phosphate, while k_{cat} and K_m were individually increased by the same factor. This may occur if the binding of NO_3^- is weakened by phosphate by an amount ΔG_{P_i}, as shown by the dashed line in Figure 8, but the stability of the transition state is unaltered. It can be shown that K_m and k_{cat} would be altered in the presence of phosphate or arsenate such that

$$k_{cat(+P_i)} = k_{cat(-P_i)} \exp(\Delta G_{P_i}/RT)$$

$$K_{m(+P_i)} = K_{m(-P_i)} \exp(\Delta G_{P_i}/RT)$$

and so the ratio k_{cat}/K_m would remain constant. The change in nitrate binding energy necessary to increase V_{max} by the

observed factor of 1.4 is relatively small by this mechanism and was caused by a change of only 0.83 kJ mol^{-1} in the affinity of nitrate under these conditions. Clearly, when nonspecific stimulation of NR activity by ionic strength is controlled for, the 40% increase in NR activity observed at saturating phosphate concentrations is unlikely to be large enough to be important physiologically.

The mechanism of activation by phosphate reported here differs from that reported by Oji et al. (1987) for activation of barley NR. These authors also reported increases in K_m-(NO_3^-) and V_{max} in the presence of phosphate, but at low nitrate concentrations, phosphate was inhibitory. The results of the present study demonstrated that phosphate activated *Chlorella* NR at all nitrate concentrations examined. This difference may have been due to the presence of 40 mM chloride in the assay media used for barley NR and to the fact that additional inert salt was not added to the control assays to compensate for changes in the ionic strength upon addition of 25 mM phosphate. Further experiments should establish whether halides also inhibit barley NR and whether a similar mechanism for phosphate activation operates.

No evidence was obtained from EPR to indicate direct ligation of phosphate to Mo in *Chlorella* NR, a result in direct contrast to the results of phosphate binding studies of sulfite oxidase in which direct ligation of phosphate to the Mo–pterin center was directly established by EPR studies (Gutteridge et al., 1980). For NR, no direct ligation was established since (a) Mo(V) samples generated either by potentiometry or by quick-freezing during turnover were identical in the presence and absence of phosphate, (b) ^{17}O-substituted phosphate did not produce detectable splitting or broadening of the Mo(V) spectrum which might have been expected if ^{17}O ligated to Mo, (c) phosphate failed to perturb either the Mo(VI)/Mo(V) or the Mo(V)/Mo(IV) midpoint potentials determined by room temperature EPR potentiometric titration. These three experiments examined different aspects of complex formation. Failure to perturb the Mo(V) line shape by addition of phosphate suggests that if a phosphate complex were formed, then the g values, proton superhyperfine coupling constants, and equilibrium between form A and form B are essentially unchanged by complex formation. The absence of detectable superhyperfine interaction between ^{17}O and Mo(V) might only indicate weak superhyperfine interaction in the complex. The failure of phosphate to perturb the midpoint potentials of the Mo center is not dependent on the structural details of the Mo–phosphate complex but would be predicted from coupling of the redox and phosphate binding equilibria. The only conditions expected not to perturb the midpoint potentials would be either that phosphate binds with equal affinity to Mo(VI), Mo(V), and Mo(IV) or that phosphate does not bind directly to Mo.

While definitive experimental evidence for direct ligation of the Mo–pterin center of NR by phosphate is lacking, it is possible that the observed weakening of nitrate binding by phosphate could arise without direct ligation of phosphate to Mo.

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