Electron Paramagnetic Resonance Studies on the Molybdenum Center of Assimilatory NADH:Nitrate Reductase from *Chlorella vulgaris*

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Larry P. Solomonson†, Michael J. Barber, and William D. Howard4

From the Department of Biochemistry, University of South Florida, College of Medicine, Tampa, Florida 33612

Jean L. Johnson and K. V. Rajagopalan

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The assimilatory nitrate reductase from *Chlorella* contains flavin, heme, and molybdenum as prosthetic groups. The molybdenum in assimilatory nitrate reductase is associated with a pterin moiety (molybdopterin) as evidenced by the ability of the enzyme to donate active molybdenum cofactor to the *Neurospora* nitrate reductase mutant nit-1 and by the oxidative conversion of the pterin to two well characterized fluorescent derivatives. The properties of the molybdenum center have been examined by EPR spectroscopy. A molybdenum V signal, absent in the resting enzyme, is elicited upon reduction with NADH and abolished upon reoxidation with nitrate. Reaction of the reduced enzyme with cyanide also abolishes the molybdenum V signal. The line shape and g values of the signal show pH dependence analogous to those observed previously with hepatic sulfite oxidase. The $g_s$ for molybdenum V at pH 7.0 was 1.977 and at pH 9.0, 1.961. The signal observed at pH 7.0 exhibits interaction with a single exchangeable proton. Potentiometric titration of the molybdenum center at pH 7.0 indicates that the oxidation-reduction potentials of the molybdenum VI/V and molybdenum IV/V couples are -34 and -54 mV, respectively. These potentials are significantly different from the potentials of the molybdenum center of respiratory-type nitrate reductase and in fact quite closely resemble those of hepatic sulfite oxidase. The oxidized enzyme exhibits the EPR signal of a low spin ferric heme which is abolished upon reduction with NADH.

Assimilatory NADH:nitrate reductase (EC 1.6.5.1) from the eucaryotic unicellular green alga, *Chlorella vulgaris*, catalyzes the 2-electron reduction of nitrate to nitrite, an important reaction in the anabolic nitrogen cycle. The purified enzyme is a homotetramer of molecular weight, 360,000 (1). Each subunit contains tightly bound FAD, heme, and molybdenum in the ratio 1:1:1 (1–3).

The molybdoenzymes sulfite oxidase and xanthine dehydrogenase have been shown to possess an unusual sulfur-containing pterin (molybdopterin) which binds the molybdenum in a complex termed the molybdenum cofactor. Molybdopterin in these enzymes has been characterized by its oxidative conversion to two alternative fluorescent derivatives, Form A and Form B (4). The molybdenum cofactor can be released from these enzymes by a variety of treatments and used to reconstitute nitrate reductase activity in extracts of the *Neurospora crassa* mutant nit-1 (5). In studies documented below, we have shown that the *Chlorella* nitrate reductase also contains molybdenum cofactor which can be donated to the apoprotein present in *Neurospora* nit-1. In addition, the presence of the chemical entity molybdopterin in the *Chlorella* enzyme has been demonstrated by oxidative conversion to Forms A and B.

In the group of enzymes comprising molybdenum hydroxylases and sulfite oxidase it has been established that the oxidative hydroxylation of the substrates occurs at the molybdenum center (6). In contrast, the molybdenum center of nitrate reductase is the terminal component of the electron transport chain in the enzyme and carries out a reductive dehydroxylation reaction (6). It was of interest, therefore, to compare the properties of the molybdenum center of nitrate reductase with those of the molybdenum centers of the other enzymes. Direct visualization of the reduced molybdenum center by EPR has proven most useful in this regard as evidenced by the data presented below.

Molybdoenzymes are known to be sensitive to inhibition by cyanide. Two mechanisms of inactivation by this agent have been distinguished. The oxidized forms of molybdenum hydroxylases (xanthine oxidase and dehydrogenase and aldehyde oxidase) are irreversibly inhibited by cyanide via a mechanism involving extraction of a terminal sulfur ligand (7, 8) of the molybdenum and release as thiocyanate (9). Molybdenum enzymes which do not possess the terminal sulfur ligand, which include sulfite oxidase and nitrate reductase, are resistant to cyanide inactivation in their oxidized states. In contrast, the reduced forms of all of the molybdoenzymes are known to be inhibited by cyanide in a manner which is reversed by suitable oxidants (10–13). The assimilatory nitrate reductase of *Chlorella* is especially sensitive to this type of cyanide inhibition, and this has been proposed to be the basis of an *in vivo* mode of regulation of nitrate assimilation (14, 15). The heme center of nitrate reductase has been shown to be unaffected by cyanide, and the site of inhibition has been localized at the molybdenum center (10, 11). The activating effect of oxidants on cyanide-inactivated nitrate reductase has led to the suggestion (16) that the molybdenum center of the reduced cyanide-treated enzyme becomes trapped in an over-reduced state, perhaps in a val-
ence below that normally attained during catalysis. Examination of the reduced molybdenum center in nitrate reductase by EPR has allowed evaluation of the properties of the center with regard to other molybdoenzymes of the two subclasses (with and without terminal sulfur ligands). In addition, the effect of cyanide on the reduced center has been examined, and results consistent with over-reduction of the molybdenum center are presented.

MATERIALS AND METHODS

RESULTS

Molybdenum Cofactor Analyses—A sample of Chlorella nitrate reductase was assayed for molybdenum cofactor activity and compared to a sample of chicken liver sulfite oxidase at an equivalent concentration. Sulfite oxidase donates cofactor to the Neurospora nit-1 extract with close to 100% efficiency. The efficiency of the sample of Chlorella nitrate reductase by atomic absorption demonstrated the presence of molybdenum V. Addition of Fe(CN)$_6$-$0.8$ molybdenum/heme.

Further purification and are seen to be identical for the two proteins (6). The fluorescence spectra of the Form A and Form B derivatives of molybdopterin released from nitrate reductase and sulfite oxidase are shown in Fig. 1. The spectra of Form A were obtained using the boiled enzyme samples without further purification and are seen to be identical for the two molybdoenzymes. The spectra of Form B when assessed directly were found to be slightly perturbed by the presence of FAD released from nitrate reductase. Chromatography on QAE (quaternary aminoethyl)-Sephadex resolved Form B signals attributable to molybdenum V. Addition of Fe(CN)$_6$-$0.8$ molybdenum/heme.

Reduction of the native enzyme with NADH also resulted in the appearance of an overlapping isotropic signal centered at g = 2.004 with a line width of 15 G. The addition of cyanide had no effect on this signal at either pH 7.0 or 9.0 under conditions which abolished the molybdenum V signal. This g = 2.004 transition can be ascribed to the formation of flavin semiquinone. This is consistent with the lack of effect of cyanide on the flavin-associated reactions of nitrate reductase (1,10) and with results obtained with other molybdoflavoproteins (6).

Oxidation-Reduction Potentials of the Molybdenum Center—The behavior of the low pH molybdenum V EPR signal of native nitrate reductase during potentiometric titrations in the presence of dye mediators is shown in Fig. 7. As the applied potential was decreased, the signal appeared with a maximum integrated intensity of approximately 0.38 electron/Mo at around $-50$ mV at pH 7.0. Poising samples of enzyme at more negative potentials resulted in the disappearance of the paramagnetic species. The line shape of the signal was unchanged throughout the course of the titration. Midpoint potentials determined from the experimental data points according to the reduction, scheme, Mo(VI) $\xrightarrow{E_1}$ Mo(V) $\xrightarrow{E_2}$ Mo(IV) were calculated to be $-34$ mV for the molybdenum VI/V and $-54$ mV for the molybdenum V/IV couples.

EPR Spectra of Cytochrome $b_{557}$—The heme component of Chlorella nitrate reductase was visualized by EPR spectroscopy at 15 K. The oxidized enzyme exhibited features at 2200 and 2960 G, g = 2.07 and 2.25, respectively, attributable to a low spin ferric heme (Fig. 8). Brief reduction with NADH led to partial abolition of this signal and appearance of the molybdenum V resonance. Further incubation with NADH resulted in abolition of both signals.
The preceding results represent the first extensive investigation of the spectroscopic properties of the molybdenum center in an assimilatory nitrate reductase. The enzyme from *C. vulgaris* is an excellent subject for such studies for several reasons. The enzyme is obtained as a homogeneous highly active preparation with very nearly stoichiometric complement of cofactors (3). The flavin component is tightly bound, and exogenous FAD is not needed for maximal activity. And, in contrast to preparations of assimilatory nitrate reductase from many other sources, the *Chlorella* enzyme can be purified with polypeptide chains apparently intact.

Previous EPR studies of nitrate reductase have been restricted to the dissimilatory enzyme from *Escherichia coli* (22-24) or related organisms (25). A brief report on the EPR properties of the assimilatory nitrate reductase from *N. crassa* has appeared (26), but the limited amount of sample precluded detailed spectral analyses. It is interesting to note that many of the features of the molybdenum center of *Chlorella* nitrate reductase are more closely related to those of sulfite oxidase than to those of *E. coli* nitrate reductase although some parallels can also be drawn between the assimilatory and dissimilatory nitrate reductases. The molybdenum V EPR signal of the *Chlorella* enzyme exhibits low pH and high pH forms analogous to those reported for sulfite oxidase (18, 27) and *E. coli* nitrate reductase (23) although the pH of the ionizable species responsible for the transition appears to be somewhat basic than the case of *Chlorella* nitrate reductase. Increasing the pH to 9.0 results in complete conversion of the sulfite oxidase molybdenum signal to the high pH form, whereas for *Chlorella* nitrate reductase, a significant portion of the low pH species is still evident at pH 9.6. The pK for interconversion in *E. coli* nitrate reductase is reported to be 8.26 (23).

The *g* values for both low and high pH signals from the *Chlorella* enzyme are lower than those of sulfite oxidase or the dissimilatory nitrate reductase. For comparison, the *g* values of the low pH species in *Chlorella* nitrate reductase, *E. coli* nitrate reductase (23), and sulfite oxidase are 1.977, 1.985, and 1.981, respectively. The corresponding values for the high pH species are 1.961, 1.976, and 1.968. The effects of phosphate and D,O on the molybdenum center of *Chlorella* nitrate reductase are similar to those observed in both sulfite oxidase (18, 27) and *E. coli* nitrate reductase (23).

The differing saturation behavior of the low and high pH molybdenum signals in *Chlorella* nitrate reductase is rather curious. The same phenomenon, i.e., an increased resistance to saturation of the alkaline pH species has been observed with sulfite oxidase, but the difference between low and high pH forms is not nearly so great (data not presented). The saturation behavior of the two species in *E. coli* nitrate reductase was not reported.

Addition of cyanide to reduced *Chlorella* nitrate reductase led to abolition of the molybdenum V EPR signal, both in the low pH and high pH forms. Loss of the molybdenum V signal is in accord with the hypothesis that cyanide promotes reduction of the center as discussed earlier. In addition, this information supports the assignment of the high pH resonance to molybdenum V since cyanide is known to interact at the molybdenum center of this enzyme (10, 11).

The *Chlorella* nitrate reductase differs from both the assimilatory enzyme from *Neurospora* and the dissimilatory enzyme from *E. coli* in that it exhibits no molybdenum V signals in the "resting" state. In addition, we found no indication of any EPR signals ascribable to nonfunctional enzyme species as reported for the *E. coli* enzyme (23).

As shown in Table II, redox potentials of the various molybdoenzymes span a range of approximately 700 mV, with the dissimilatory nitrate reductase as the most positive and the formate dehydrogenase from * Methanothermus fervidum* as the most negative. Based on these oxidation-reduction potentials, the molybdoenzymes examined to date can be divided into two subgroups, the low potential enzymes xanthine oxidase, xanthine dehydrogenase, aldehyde oxidase, and formate dehydrogenase and the high potential group which includes sulfite oxidase and the assimilatory and dissimilatory nitrate reductases. It appears likely that this differentiation derives from the nature of the reaction catalyzed by each enzyme. All of the low potential enzymes catalyze hydroxylation at a carbon atom, whether it be in an aldehyde or in a heterocyclic compound, whereas the high potential enzymes catalyze oxidation or reduction of noncarbon substances. This suggests that the terminal sulfur is an essential requirement for the hydroxylation at carbon centers, possibly through the formation of a thioester-type bond. Although evidence for a terminal sulfur in formate dehydrogenase has not been obtained as yet, the presence of such a moiety in that enzyme would be predicted.

Within the subgroup classified as high potential molybdoenzymes, there exists still a range of actual redox values from near zero up to about 200 mV. It is interesting to note that the potentials of the molybdenum center in *Chlorella* nitrate reductase much more closely resemble those of hepatic sulfite oxidase than those of the dissimilatory nitrate reductase. The higher potential of the *E. coli* nitrate reductase is probably dictated by the fact that the enzyme is the terminal component of a multicomponent electron transport chain and thus has to function as an efficient electron sink.

The heme in *Chlorella* nitrate reductase exhibits optical (3) and EPR spectra which are very similar to those of sulfite oxidase. Moreover, both enzymes function via electron transfer between molybdenum and heme albeit in opposite directions. When sufficient quantities of the *Chlorella* enzyme are available it will be interesting to measure the potential of the heme to ascertain whether the analogies between sulfite oxidase and *Chlorella* nitrate reductase can be extended even further.

**Acknowledgment**—We thank Dr. Robert V. Hageman for assay of molybdenum cofactor activity with the *Neurospora nit-1* mutant.

**REFERENCES**


SUPPLEMENTAL MATERIAL TO:
ELECTRON PARAMAGNETIC RESONANCE STUDIES ON THE MOLYBDENUM CENTER OF ASSIMILATORY NADH: NITRATE REDUCTASE

Table I

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Note: Additional details provided in the original text regarding the experimental methods and results.
**EPR Studies of the Molybdenum Center of Nitrate Reductase**

**Fig. 1.** Fluorescence spectra of Form A (Upper) and Form B (Lower) derivatives of molybdenum-free Chlorella nitrate reductase (---) and chicken liver sulfite reductase (-----). Samples were adjusted to pH 11.6 with NaOH.

**Fig. 2.** EPR spectra of the oxidized and reduced forms of Chlorella nitrate reductase in 0.01 M Tris-HCl, pH 7.0 at 173 K. A) No additives. B) After addition of 1 mM NOC. C) After addition of 100 mM NOC to the reduced sample. EPR spectrometer settings were as follows: microwave frequency, 9.11 GHz; power, 20 milliwatts; modulation, 5 gauss; time constant, 0.5 s. The g-values indicated were calculated to be: 1, 2.004; 2, 1.997; 3, 1.991; 4, 1.985; 5, 1.979; 6, 1.972.

**Fig. 3.** (Left) Effect of pH and additives on the molybdenum EPR signal of nitrate reductase. Conditions were the same as in Fig. 2. A) No additives. B) After addition of 1 mM NOC. C) After addition of 1 mM NOC. D) 0.05 M Tris-HCl, pH 7.0. The g-values indicated were calculated to be: 1, 2.004; 2, 1.997; 3, 1.991; 4, 1.985; 5, 1.979; 6, 1.972.

**Fig. 4.** (Right) Effect of 0.05 M Tris-HCl on the molybdenum EPR signal of nitrate reductase. Conditions were the same as in Fig. 2. A) No additives. B) After addition of 1 mM NOC. C) After addition of 1 mM NOC. D) 0.05 M Tris-HCl, pH 7.0. The g-values indicated were calculated to be: 1, 2.004; 2, 1.997; 3, 1.991; 4, 1.985.

**Fig. 5.** Effect of pH on the low pH form and the high pH form of the molybdenum EPR signal of nitrate reductase. A) 0 pH Tris-HCl, pH 7.0. B) 10 mM NOC. C) Same as B then plus 0.05 M TrisHCl, pH 7.0. D) Same as C then plus 0.05 M NOC.

**Fig. 6.** Effect of substrate on the low pH form and the high pH form of the molybdenum EPR signal of nitrate reductase. A) 0 pH Tris-HCl, pH 7.0. B) 10 mM NOC. C) Same as B then plus 0.05 M Tris-HCl, pH 7.0. D) Same as C then plus 0.05 M NOC.

**Fig. 7.** Potentiometric titration of the molybdenum center of nitrate reductase. "Electrons/HEME" indicates electrons observed at molybdenum per enzyme monomer.
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