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Oxidation-reduction midpoint potentials of the molybdenum center in spinach NADH:nitrate reductase

Michael J. Barber, Brian A. Notton* and Larry P. Solomonson

Department of Biochemistry, University of South Florida, College of Medicine, Tampa, FL 33612, USA and **University of Bristol, Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, England*

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Oxidation-reduction midpoint potentials for the molybdenum center in assimilatory NADH:nitrate reductase isolated from spinach (*Spinacia oleracea*) have been determined at pH 7.0 in the presence of dye mediators using EPR spectroscopy to monitor formation of Mo(V). Values for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples were determined to be −8 and −42 mV, respectively.

Nitrate reductase; Molybdenum; Redox potential; (*Spinacia oleracea*)

1. INTRODUCTION

Spinach NADH : nitrate reductase, a homodimer of 100 kDa subunits, each of which contains an FAD, cytochrome *b*-557 and molybdenum prosthetic groups, catalyzes the rate-limiting step in nitrogen assimilation, the reduction of nitrate to nitrite [1].

EPR studies of the Mo center in both spinach [2] and *Chlorella* [3] nitrate reductase have indicated that the Mo centers in these enzymes are very similar. Each yields a limited number of spectroscopically distinct Mo(V) species, the precise lineshapes of which are dependent upon pH and the presence of anions such as chloride and phosphate. Redox potentials for the molybdenum and heme center have been determined for the *Chlorella* enzyme [3,4], however, only values for the heme center have been reported for the spinach enzyme [5]. While the *b*-type cytochrome in *Chlorella* nitrate reductase exhibits a potential of −168 mV, the equivalent center in the spinach enzyme exhibits a potential of −60 mV. We have determined the redox potentials for the molybdenum center in spinach nitrate reductase to aid comparative studies with the enzyme from *Chlorella* and to determine whether the positive shift in the heme potential, with respect to the *Chlorella* enzyme, is reflected in a similar shift in the potentials of the molybdenum center; it is not.

Correspondence address: M.J. Barber, Department of Biochemistry, University of South Florida, College of Medicine, Tampa, FL 33612, USA

Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; $MV^{\cdot+}$, methyl viologen radical cation

2. MATERIALS AND METHODS

Nitrate reductase was purified from fresh spinach (*Spinacia oleracea*) as described by Fido and Notton [6] and exhibited an A_{280}/A_{412} ratio of better than 2.5. Enzyme activity was monitored using the reduction of nitrate by NADH at 340 nm. Enzyme subunit concentrations were estimated from the absorbance at 412 nm using $E_{mM} = 127$ [6].

Potentiometric titrations were performed as described by Barber and Salerno [7] at 25°C in 50 mM Mops buffer, containing 10 μM FAD and 0.1 mM EDTA, pH 7.0. Enzyme (final concentration 2.2 μM heme) in the presence of the following dye mediators (15 μM each): 2,6-dichloropheno-

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lindophenol (E_0' = 217 mV), 1,2-naphthoquinone (E_0' = 135 mV), phenazine methosulfate (E_0' = 80 mV), 1,4-naphthoquinone (E_0' = 60 mV), methylene blue (E_0' = 10 mV), pyocyanine (E_0' = −60 mV), indigodisulfonate (E_0' = −125 mV), 2-hydroxy-1,4-naphthoquinone (E_0' = −137 mV), anthraquinone-2,7-disulfonate (E_0' = −182 mV) and phenosafranine (E_0' = −225 mV), was reduced using $MV^{\cdot+}$ (20 mM) and reoxidized using $Fe_3(CN)_6^{3-}$ (100 mM). Enzyme was allowed to equilibrate for 10 min at each potential and a sample withdrawn anaerobically, transferred to an Ar-flushed EPR tube and frozen in liquid N_2. Oxidation-reduction midpoint potentials, expressed relative to the standard hydrogen electrode, were obtained by fitting the experimental titration data with theoretical Nernst curves for the intermediate species formed in two consecutive n = 1 reduction processes [8] using a weighted least-squares procedure. This procedure emphasized fitting the theoretical curve to the maximum integrated signal intensity rather than the overall width of the titration curve. Similar procedures have been used to fit titration curves for the molybdenum center of xanthine oxidase which does not exhibit ideal behavior [9].

EPR spectra were obtained using a Varian E109 Century Series spectrometer 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 0.25 mT and an incident microwave power of 5 mW. Double integrations were performed as described by Barber and Siegel [10] using CuEDTA as standard.

3. RESULTS AND DISCUSSION

At high potential, greater than 150 mV, spinach nitrate reductase showed the absence of any Mo(V) EPR signal. As the potential was decreased, an Mo(V) paramagnetic species appeared, increased in intensity, reaching a maximum amplitude at approx. −25 mV and then decreased in intensity as the potential was further decreased. The lineshape of the Mo(V) EPR signal remained unchanged throughout the course of the titration and is shown in fig.1. The spectrum exhibited near axial symmetry and was similar to that previously obtained for spinach nitrate reductase using NADH as the

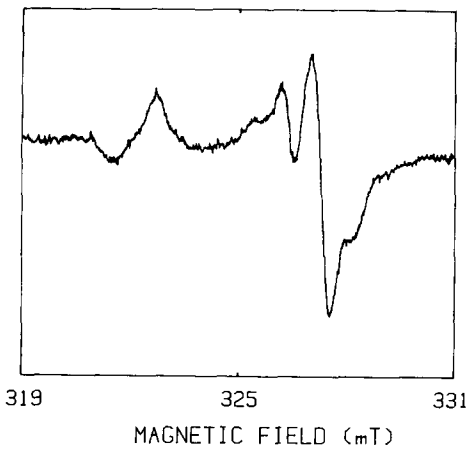

Fig.1. EPR spectrum of the Mo(V) center obtained during potentiometric titration. Nitrate reductase (2.2 μM heme) in 50 mM Mops buffer, containing 10 μM FAD and 0.1 mM EDTA, pH 7.0, was poised at −35 mV using $MV^{\cdot+}$ in the presence of dye mediators and the EPR spectrum recorded as described in section 2. The field scale corresponds to a microwave frequency of 8.986 GHz.

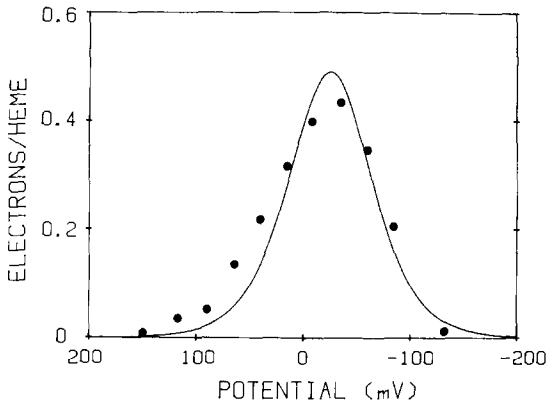

Fig.2. Behavior of the molybdenum center during potentiometric titration of nitrate reductase. Potentiometric titrations were performed and EPR spectra recorded as described in section 2 and Mo(V) integrated signal intensities plotted as a function of the applied potential. The line through the data points corresponds to two consecutive n = 1 reduction processes with midpoint potentials of −8 and −42 mV, respectively.

reductant [2]. In these studies, this Mo(V) signal was identified as being composed of two similar signals, referred to as 'A' and 'B' respectively, with the former species favored by Cl^- binding to the molybdenum.

The variation in the integrated intensity of the Mo(V) EPR signal with applied potential is shown in fig.2, with maximum conversion to the quinquivalent form accounting for 43% of the total heme concentration. The titration was fully reversible with similar behavior observed when titrating the reduced enzyme in the oxidative direction. Behavior of this type is typical of a reduction process involving two, consecutive, one-electron redox steps corresponding to the reactions:

$$\text{Mo(VI)} \overset{E_1}{\rightleftharpoons} \text{Mo(V)} \overset{E_2}{\rightleftharpoons} \text{Mo(IV)}$$

where E_1 and E_2 represent the midpoint potentials for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples, respectively. Values obtained for these couples at pH 7 are given in table 1. Identical behavior was observed for all of the Mo(V) spectral features suggesting that while the different forms of molybdenum may be spectroscopically distinct, Cl^- binding has very little influence on the molybdenum redox potentials.

Oxidation-reduction midpoint potentials for the molybdenum center in nitrate reductase have previously been determined for the assimilatory enzyme from *Chlorella vulgaris* and the dissimilatory enzyme from *E. coli* [11] and are listed in table 1. The potentials for the molybdenum center in spinach nitrate reductase are very similar to the corresponding values for the *Chlorella* enzyme and substantially lower than those for the dissimilatory enzyme. Combining these potentiometric results with previous EPR studies would suggest that the molybdenum centers in spinach and *Chlorella* nitrate reductase are very similar and extends previously noted differences in the molybdenum centers of assimilatory and dissimilatory forms of the enzyme [12].

Table 1

Oxidation-reduction midpoint potentials for the molybdenum center in nitrate reductase (NR)

Species	Buffer	pH	Potential (mV)	
			Mo(VI)/ Mo(V)	Mo(V)/ Mo(IV)
Assimilatory NR, spinach	Mops	7.0	−8	−42
Assimilatory NR, *C. vulgaris*	Mops	7.0	−34	−54
Dissimilatory NR, *E. coli*	Mops	7.1	180	220

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