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ELECTRON PARAMAGNETIC RESONANCE STUDIES OF ^{95}Mo-ENRICHED NADH DEHYDROGENASE ISOLATED FROM IRON-DEFICIENT *AZOTOBACTER VINELANDII*

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SUMMARY

1. An asymmetric EPR signal at $g = 1.95$ is observed under reducing conditions in whole cells and fractions of *Azotobacter vinelandii* grown on an iron-deficient medium. This signal is absent under similar conditions when *A. vinelandii* is grown on a normal amount of iron.

2. Growth of *A. vinelandii* on a low-iron medium using ^{95}Mo in an EPR nuclear isotopic substitution study established that the $g = 1.95$ EPR signal is definitely due to monomeric Mo(V).

3. This Mo(V) signal appears on reduction with NADH, NADPH or acetaldehyde and is associated with an apparently modified NADH dehydrogenase rather than a component of the nitrogenase complex.

4. Based on observed g-values and hyperfine splitting constants from this and related studies, it appears that at the active site of NADH dehydrogenase, Mo(V) could be coordinated with cysteine.

INTRODUCTION

A study of the EPR signals observed under reducing conditions in whole cells** and derived fractions from *A. vinelandii* grown on a low-iron medium revealed an unusual asymmetric EPR signal at $g = 1.95$. *A. vinelandii* is a strict aerobe with a respiratory system quite similar to the mammalian counterpart in terms of substrate turnover rates and composition of respiratory components[1]. Using the criteria of the appearance of the $g = 1.95$ signal by NADH reduction and the direct correlation with NADH–menadione reductase activity, a highly active preparation of NADH dehydrogenase was obtained containing approx. 2 atoms each of molybdenum, iron

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** Under low-iron growth conditions and reducing conditions the $g = 1.94$ EPR signals attributable to iron–sulfur proteins I and II (*cf.* ref. 3) are completely absent in whole cells and successive fractions. On the other hand, whole cells and derived fractions under normal-iron growth showed only the $g = 1.94$ signal and the complete absence of the $g = 1.95$ signal under reducing conditions.

and labile sulfide per mole FMN. The preparation exhibited a single, broadened peak in the analytical ultracentrifuge and a distinct green tinge. The catalytic-center activity* of 16 500 with NADH as substrate and menadione as electron acceptor was in the range of similar NADH preparations (*cf.* ref. 2). Unlike mammalian NADH dehydrogenases this enzyme also catalyzed the oxidation of aldehydes (251 moles acetaldehyde oxidized per mole FMN per min at 25°) and on reduction with specific substrates such as NADH, NADPH or acetaldehyde revealed the same EPR signal at $g = 1.95$ as observed in whole cells. In contrast NADH dehydrogenase isolated by the same procedure from whole cells obtained on normal-iron growth showed a different absorption spectrum with the typical brown color of iron sulfur flavoproteins, a low specific activity with aldehydes and absence of the $g = 1.95$ signal on substrate reduction. Isolation and properties of the NADH dehydrogenase preparations obtained on low-iron and high-iron growth conditions will be presented elsewhere[4].

BRAY AND MERIWETHER[5] studied the electronic environment of molybdenum in xanthine oxidase by the use of EPR isotope substitution techniques using ^{95}Mo as the nuclear isotope. On the basis of their studies with xanthine oxidase as well as complementary model Mo(V) thiol complexes[6] they suggested that sulphur, possibly derived from cysteine, was a ligand of Mo(V) in xanthine oxidase. Recently HUANG AND HAIGHT[7] reported an EPR study of monomeric Mo(V)-cysteine complexes using natural and ^{95}Mo-enriched systems. They confirmed the EPR spectrum of the Mo(V)-cysteine complex first reported by MARTIN AND SPENCE[8] and supported both the BRAY and SPENCE groups in the possibility that cysteine was a ligand for Mo(V) at the active site of molybdenum-containing enzymes.

The EPR signal at $g = 1.95$ could be attributed to paramagnetic Mo(V) because of the measured g-values, relative temperature independence of signal shape and ease of power saturation at relatively low incident microwave powers. The signal strongly resembled the Mo(V) signal of xanthine oxidase[9] and aldehyde oxidase[10]. The unusual properties of the two different types of NADH preparations and the dramatic change in EPR signals observed in whole cells and derived fractions on low-iron and high-iron growth conditions indicated a fundamental change in the redox-active components of the bacterium. Since this difference was primarily reflected in the $g = 1.95$ signal, it was deemed essential to firmly establish the identity of this signal.

This paper describes the experimental results obtained in such a study. Growth of *A. vinelandii* on a low-iron medium in the presence of added ^{95}Mo, isolation of the NADH dehydrogenase at a suitable state of purity, comparison of its reduced EPR signal with a similar fraction grown on low-iron and natural molybdenum should result in a definite intensification of the expected hyperfine structure in the ^{95}Mo EPR sample. Analysis of the hyperfine structure of the ^{95}Mo-enriched NADH dehydrogenase firmly establishes that the EPR signal at $g = 1.95$ is due to Mo(V). A preliminary report on this study has been previously presented[11].

MATERIALS AND METHOD

A. vinelandii (Strain OP, kindly provided by Dr. P. W. Wilson) was grown on

* Moles substrate oxidized per mole FMN per min at 25°.

a high aeration rate in a 10-l glass container using glass-distilled water, a modified medium as previously described[3] but at 3% of the normal iron requirement. 1 mg ^{95}Mo (96.8% enriched), obtained from Oak Ridge National Laboratory, was added as sodium molybdate per l of medium. Radioactive ^{99}Mo (New England Nuclear) was added in trace amounts to the stock ^{95}Mo solution prior to addition to the medium to monitor uptake of molybdenum by whole cells and to determine the relative distribution of molybdenum in the fractionation steps. Whole cells (40 mg protein per ml in 0.025 M potassium phosphate buffer (pH 7.6)) were subjected twice to French pressure cell extrusion and centrifuged for 30 min at 10 000 × *g*. The supernatant was subjected to heat treatment for 10 min at 55° under H_2 and centrifuged for 1 h at 40 000 × *g*. A $(NH_4)_2SO_4$ fractionation was made and the precipitate (20–48% saturation) taken up in a minimum volume of the same buffer, stored under H_2 at 0° and centrifuged for 1 h at 40 000 × *g* before use.

NADH dehydrogenase was purified to the $(NH_4)_2SO_4$ step because unlike the most purified preparations which are highly unstable, limited in amount but show the same $g = 1.95$ signal, the $(NH_4)_2SO_4$ fraction is relatively stable in both the reduced EPR signal and enzymatic activity for at least 48 h under H_2 at 0°. Further the EPR absorption at $g = 1.95$ is quite intense and no interfering EPR signals are observed in either the oxidized or reduced states. This fraction obtained on low-iron or low-iron and added ^{95}Mo growth conditions catalyzes the oxidation of 9–11 μmoles NADH per min per mg protein with menadione as acceptor at 25° using the same assay conditions described by HATEFI AND STEMPEL[2].

EPR spectroscopy was carried out in frequency-matched quartz tubes with a Varian V-4052A X-band spectrometer equipped with a 100-kcycles-field modulation unit, "Fieldial" and an automatic low-temperature accessory. Frequencies were measured with a Hewlett-Packard frequency meter and magnetic field intensities with a Ventron Model G-502 NMR precision gaussmeter previously calibrated with a frequency counter.

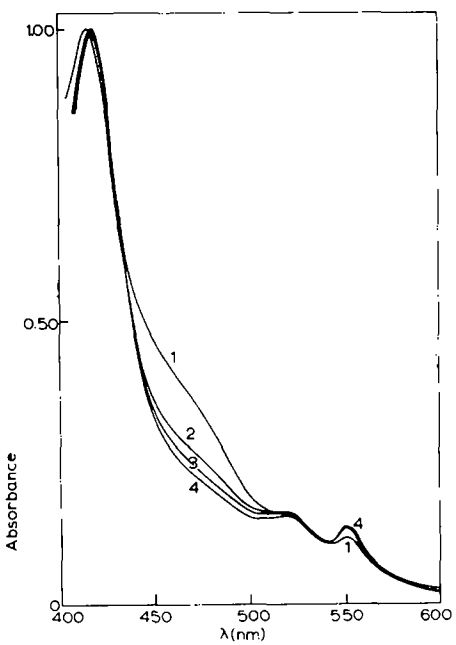
RESULTS AND DISCUSSION

Radioactive measurements indicate that 64% of added ^{99}Mo (and correspondingly ^{95}Mo) was present in the washed whole cells. 90% of the radioactive molybdenum taken up by the whole cells was released in the cell-free extract. The $(NH_4)_2SO_4$ fraction used in the EPR studies contained 19% of the radioactive molybdenum present in the cell-free extract. Part of this molybdenum and the major remaining fraction of radioactive molybdenum is presumably associated with the molybdo iron-protein of the nitrogenase complex. This latter protein was isolated from *A. vinelandii* by BULEN AND LECOMTE[12] and recently crystallyzed by BURNS *et al.*[13] while MORTENSON *et al.*[14] were the first to isolate the molybdo-iron protein from *Clostridium pasteurianum*. The reported EPR spectra[13] of the crystalline protein in both the oxidized and reduced states are quite dissimilar to the EPR spectra observed in preparations of NADH dehydrogenase.

Fig. 1 shows the effect under He of excess NADH, as a function of time, on the absorption spectrum of the same 20–48% $(NH_4)_2SO_4$ fraction used in the EPR studies. Similar absorbance changes are observed on anaerobic addition of NADPH. A pronounced decline in absorbance in the 400–500-nm region is noted indicating

reduction of enzyme chromophores. Cytochromes c_4 and c_5, the major colored contaminants in this fraction, are reduced by NADH dehydrogenase.

The upper trace of Fig. 2 represents the first derivative EPR absorption of the 20–48% $(NH_4)_2SO_4$ fraction after aerobic reduction with excess NADH*. No EPR



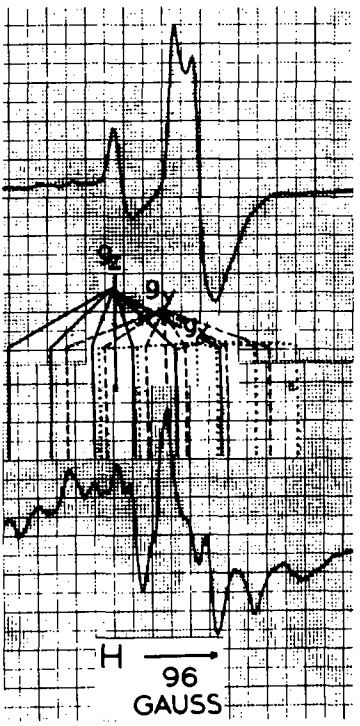


Fig. 1. Effect of NADH on the absorption spectrum of partially purified NADH dehydrogenase obtained from iron-deficient *A. vinelandii*. NADH (final concentration, 0.3 mM) was added under He to an $(NH_4)_2SO_4$ fraction (20–48%) containing 8.4 mg protein per ml. Spectra were recorded on a Cary Model 14 recording spectrophotometer in a 1-cm Thunberg tube (Hellma), total volume 0.8 ml. Spectra 1–4 were recorded at 1, 7, 9.5, and 15 min, respectively.

Fig. 2. Effect of NADH on the EPR spectra of natural molybdenum and ^{95}Mo-enriched samples of partially purified NADH dehydrogenase preparations. The upper trace represents the first derivative EPR absorption of a 20–48% $(NH_4)_2SO_4$ fraction, containing 27 mg protein in 0.3 ml, obtained from cells grown on naturally occurring molybdenum, and reduced aerobically for 3 min at 25° at a final concentration of 2 mM NADH. The lower trace is obtained with a similar fraction (18 mg protein in 0.3 ml) under identical reducing conditions derived from cells grown on ^{95}Mo. EPR conditions: temperature, —176°; modulation amplitude, 4.8 gauss; frequency, 9.302 GHz; incident microwave power, 9 mW. Above 9-mW microwave power, saturation of the molybdenum signal has been observed.

absorption is observed prior to addition of substrate. Peaks are observed at $g = 2.052$, $g = 2.019$, $g = 1.969$, $g = 1.995$ and $g = 1.945$. The peak-to-peak width of the main signal is 43 gauss. The small signal at $g = 2.052$ corresponds to part of the hyperfine structure of Mo(V). Naturally occurring Mo(V) contains 25% of nuclear isotopes ^{95}Mo

* NADH dehydrogenase shows negligible reaction with O_2. EPR measurements are then conveniently carried out in air. The same EPR changes are observed anaerobically.

and ^{97}Mo which have a nuclear spin of 5/2. For a monomeric paramagnetic Mo(V) system 6 hyperfine lines are expected from the relationship $2I + 1$ where I is the nuclear spin. These hyperfine lines are difficult to detect at such a low enrichment and for this reason ^{95}Mo of high enrichment is required for observation of the 6-line hyperfine structure.

The naturally occurring Mo(V) spectrum observed in the upper trace resembles the Mo(V) signals reported for xanthine oxidase[9], xanthine dehydrogenase[15], aldehyde oxidase[10] and the Mo(V)–cysteine complex[8,7]. The g-values and approximate splitting constants are in the same range as those observed for paramagnetic Mo(V) in the described systems. Nevertheless some minor differences in signal shape are detectable indicating slight variation in the symmetry of the Mo(V) complex at the active site of NADH dehydrogenase as compared to the other molybdo–iron-flavoproteins.

TABLE I

EPR CONSTANTS DERIVED FROM ^{95}Mo-ENRICHED COMPLEX METALLO FLAVOPROTEINS AND MODEL SYSTEMS

Substrate	*System*	g_z	g_y	g_x	$g_{av.}$	A_z	A_y	A_x	$Av.$
						(Gauss)			
NADH	NADH dehydrogenase*	2.018	1.977	1.948	1.981	52	47	48	49
Xanthine-γ, δ	Xanthine oxidase, ref. 5	2.025	1.956	1.951	1.977	41	34	37	34
Formaldehyde-α, β		$g_{\parallel}=1.990$		$g_{\perp}=1.971$	1.977	$A_{\parallel}=67$		$A_{\perp}=28$	41
Cysteine	Mo(V), ref. 7	2.029	1.972	1.931	1.977	54	24	34	35

* This study.

The lower trace of Fig. 2 is the EPR absorption of a similar $(NH_4)_2SO_4$ fraction obtained from whole cells of *A. vinelandii* grown on ^{95}Mo of high enrichment. Except for different protein concentrations the EPR spectra were obtained under approximately the same conditions. EPR absorption does not appear unless NADH is added as reductant. There is a distinct intensification of the expected hyperfine structure with a 6-line spectrum observed along each axis in the x, y and z direction of the molybdenum complex. Analysis of the EPR spectrum in terms of g-values and splitting constants* is recorded in Table I. These values are in good agreement with those obtained for xanthine oxidase and the Mo(V)–cysteine complex which have been enriched in ^{95}Mo, suggesting that at the active site of NADH dehydrogenase cysteine could be coordinated with molybdenum.

As mentioned, BRAY AND MERIWETHER[5] first used ^{95}Mo in a study of Mo(V) at the active site of xanthine oxidase. Our study is the first apparent use of ^{95}Mo in a bacterial system and in examining the active site of bound molybdenum in a primary respiratory-chain dehydrogenase. *A. vinelandii* has been previously used for EPR isotopic substitution studies to establish that the $g = 1.94$ EPR signal was due to a reduced iron complex (^{57}Fe, ref. 16) and that a ligand of this iron complex was sulphur (^{33}S, refs. 17, 18).

The results obtained on isotopic substitution with ^{95}Mo establishes that the

* Provisional assignments.

unusual EPR at $g = 1.95$ on low-iron growth conditions is due to Mo(V). In the case then of *A. vinelandii* grown on low-iron a fundamental change in EPR signals, observed under reducing conditions, appears to have occurred since this EPR signal is not present in whole cells or derived fractions when grown on a normal amount of iron. The EPR signal at $g = 1.95$ is readily observable in intact whole cells on low-iron growth and is therefore not due to any degradation artifacts arising from the isolation procedures.

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