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LOW TEMPERATURE MAGNETIC CIRCULAR DICHROISM SPECTROSCOPY AS A PROBE FOR THE OPTICAL TRANSITIONS OF PARAMAGNETIC NICKEL IN HYDROGENASE

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SUMMARY: A partially-purified sample of hydrogenase from Methanobacterium thermoautotrophicum (ΔH strain) has been investigated by optical absorption, magnetic circular dichroism and electron paramagnetic resonance spectroscopy. Variable temperature magnetic circular dichroism studies reveal, for the first time, the optical transitions associated with the Ni(III) center in the oxidized enzyme. Low temperature magnetic circular dichroism spectroscopy provides a new method of assessing both the coordination environment of Ni in hydrogenase and the appropriateness of inorganic model complexes.

Nickel has recently been recognized as an integral component of many bacterial hydrogenases including those from several Methanobacterium and Desulfovibrio species (1). All contain at least one Fe-S cluster in addition to Ni. Since optical transitions localized on Ni are obscured in the UV-visible absorption spectrum by intense S⟶Fe charge transfer transitions from Fe-S centers, information concerning the redox state and coordination of Ni has thusfar been limited to that obtained via EPR[1] and XAS spectroscopy. EPR signals arising from Ni(III) in a tetragonal environment are observed in some form of the oxidized enzymes. EPR studies of Ni(III) oligopeptide complexes suggest that the Ni(III) center of hydrogenases contains one equatorial cysteine sulfur and argue against either axial nitrogen ligands or predominant sulfur coordination (2). In contrast XAS studies have beeen interpreted in terms of 3 or 4 sulfurs in the primary coordination sphere of Ni in the oxidized enzyme (3,4).

1. Abbreviations used are: EPR, electron paramagnetic resonance; XAS, X-ray absorption spectroscopy; MCD, magnetic circular dichroism; CD, circular dichroism.

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The electronic transitions localized on Ni in hydrogenase should provide a sensitive monitor of redox state, coordination number, geometry and ligand type. In this communication we identify the optical transitions associated with the Ni(III) center in the hydrogenase from Methanobacterium thermoautotrophicum (ΔH strain) by using variable temperature MCD spectroscopy.

METHODS:

Enzyme purification: Methanobacterium thermoautotrophicum was grown at 65°C on H_2/CO_2 as previously described (5). All purification procedures were performed aerobically at 4°C using pH 7.6 buffers and hydrogenase activity was measured by the hydrogen evolution assay (6).

Step 1: Crude extract: The crude cell fraction, prepared by lysing the cells, was separated by ammonium sulfate precipitation into an hydrogenase protein fraction and a F_{430} fraction according to Moura et al (7).

Step 2: First DEAE-Biogel column: After dialysis to remove ammonium sulfate, the hydrogenase fraction was applied to a DEAE-Biogel column (6 x 33 cm) and washed with 10 mM Tris-HCl. The enzyme was eluted with a linear gradient (1800 ml of 10 mM Tris and 1800 ml of 250 mM Tris, followed by 1000 ml of 250 mM Tris and 1000 ml of 400 mM Tris) at about 300 mM Tris. The specific activity increased from 20 to 94 μmoles H_2 evolved mg^{-1} min^{-1}.

Step 3: Hydroxylapatite column: Hydrogenase fractions were loaded onto an hydroxylapatite column (4 x 26 cm) and washed with 500 ml of 225 mM Tris. A reverse gradient (800 ml of 225 mM Tris and 800 ml of 10 mM Tris) was applied followed by washing with 1000 ml of 10 mM potassiuum phosphate. The hydrogenase was eluted by a linear phosphate gradient (1000 ml of 10 mM and 1000 ml of 200 mM) at about 100 mM phosphate and had a specific activity of 127 μmoles H_2 evolved mg^{-1} min^{-1}.

Step 4: Second DEAE-Biogel column: After concentrating and exchanging the phosphate buffer for 10 mM Tris, the sample was applied to a DEAE-Biogel column (4.5 x 24 cm) and washed with 500 ml of 10 mM Tris. The enzyme was eluted with a linear gradient (1000 ml of 10 mM Tris and 1000 ml of 250 mM Tris) and the fractions with highest specific activity were combined and concentrated. Specific activity 214 μmoles H_2 evolved mg^{-1} min^{-1}.

Step 5: Sephacryl S-200 column: The sample was absorbed onto a Sephacryl S-200 column (6 x 120 cm) and eluted with 50 mM Tris at a rate of 20 ml/hr. The sample separated into two brown bands and most of the hydrogenase activity was found in the second band. Specific acitivity 417 μmoles H_2 evolved mg^{-1} min^{-1}.

Step 6: Third DEAE-Biogel column: The sample was diluted five times with 5 mM Tris, absorbed onto a DEAE-Biogel column (3 x 20 cm) and washed with 500 ml of 10 mM Tris. The enzyme was eluted with a linear gradient (1000 ml of 10 mM Tris and 1000 ml of 180 mM Tris, followed by 500 ml of 150 mM Tris and 500 ml of 300 mM Tris, followed by 500 ml of 270 mM Tris and 500 ml of 430 mM Tris) at about 320 mM Tris. The enzyme had a specific activity of 712 μmoles H_2 evolved mg^{-1} min^{-1} and an absorption ratio, A_{400}/A_{280} = 0.28. Gel electrophoresis gave two major bands and a few minor bands indicating that the sample was not completely free of contaminating proteins.

Spectroscopic measurements: MCD spectra were recorded using an Oxford Instruments SM3, split-coil, superconducting magnet mated to a Jasco J500C spectropolarimeter. Spectra were recorded digitally using an OKI IF800, Model 30, microcomputer interfaced via a Jasco IF500 interface. Sample temperatures were measured using calibrated carbon-glass resistors (Lake Shore Cryogenics) placed both directly above and below the sample and controlled by a Rh/Fe resistor and heater connected to an Oxford Instruments DTC2 temperature controller. Temperatures below 4.22K were obtained by pumping on a bath of

liquid helium using a two-stage rotary pump connected to a manustat to achieve constant reduced pressure. Magnetic field calibration was carried out with a transverse Hall probe (Lake Shore Cryogenics). MCD spectra are corrected for natural CD and expressed as ΔA, $= A_L - A_R$. Magnetic field, pathlength and Ni(III) concentration, as determined by EPR quantitation, are quoted in the figure legend. All hydrogenase samples used in this work contained 50% v/v ethylene glycol to enable optical quality glasses to be formed on freezing. Absorption spectra were recorded in 1mm quartz cuvettes using a Cary 219 UV-visible spectrometer. EPR spectra were recorded on a Varian E-line, X-band spectrometer interfaced to an Apple IIc microcomputer and fitted with an Air-Products Helitrans low-temperature cryostat. Spectra were quantified at 70K and 1mW microwave power using 1mM CuEDTA as standard.

RESULTS AND DISCUSSION:

In agreement with previous studies, as isolated Methanobacterium thermoautotrophicum hydrogenase exhibits a rhombic EPR signal (g = 2.300, 2.230, 2.014), see Fig. 1 (8). The signal is observable up to 120K and undergoes power saturation above 2mW microwave power at 13K. Quantitation at 70K against a CuEDTA standard gave a spin concentration of $120 \pm 10\ \mu M$. No other EPR signals were observed in the temperature range 5-120K. Analogous signals have been observed in the majority of Ni-containing hydrogenases investigated to date (1), and they are attributed to Ni based on hyperfine splittings observed for samples enriched with ^{61}Ni. Since these signals are

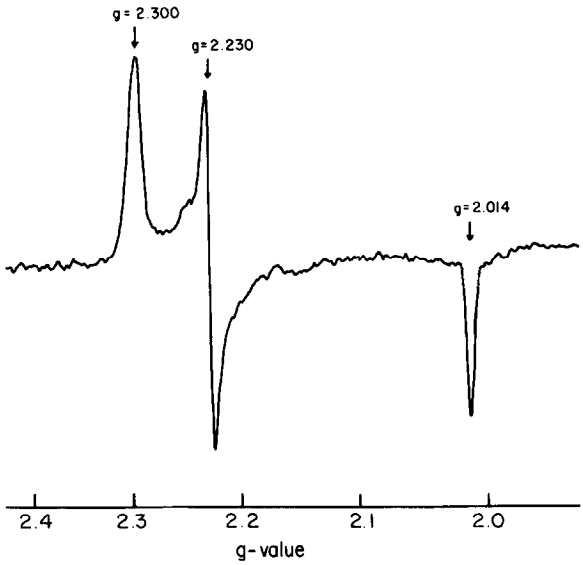

FIG. 1. EPR Spectrum of Partially-Purified Hydrogenase from Methanobacterium thermoautotrophicum. Conditions: temperature, 70 K; microwave power, 1 mW; microwave frequency, 9.018 GHz; modulation amplitude, 0.63 mT. Sample in .15 M Tris/HCl pH 7.5 buffer with 50% v/v ethylene glycol.

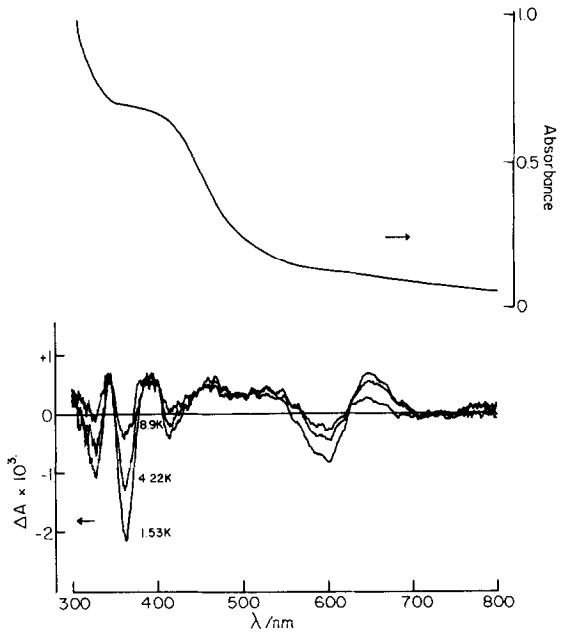

FIG. 2. Room Temperature Absorption amd Low Temperature MCD Spectra of Methanobacterium thermoautotrophicum Hydrogenase. Upper panel: room temperature absorption, 1 mm pathlength. Lower panel: MCD spectra at 1.53, 4.22, and 8.9 K; magnetic field, 4.5 T; pathlength, 1.67 mm; Ni(III) concentration, 120 ± 10 μM (from EPR spin quantitation). Sample as in Fig. 1.

lost on reduction, they are considered to originate from Ni(III) rather than Ni(I). The observed g-value anisotropy ($g_{\perp} > g_{\parallel}$) is consistent with tetragonal as opposed to square planar geometry for a low spin (S=1/2) Ni(III) center (9).

Room temperature absorption and low temperature MCD spectra of as isolated Methanobacterium thermoautotrophicum hydrogenase, in the range 300-800nm, are shown in Fig. 2. The broad, featureless absorption spectrum with a shoulder at approximately 400nm is typical of that exhibited by Fe-S proteins containing [4Fe-4S] or [3Fe-xS] centers. Optical transitions from the Ni are obscured by the broad envelope of intense S⟶Fe charge transfer bands. The low temperature MCD spectra show temperature-dependent transitions in the regions 300-460nm and 530-670nm which must originate from a paramagnetic chromophore. The form of the spectrum is quite distinct from that of any known paramagnetic Fe-S center and no such center was apparent in the EPR data. Since Ni is the only known paramagnetic chromophore in this form of the enzyme, the observed transitions are assigned to the Ni(III) center. This is

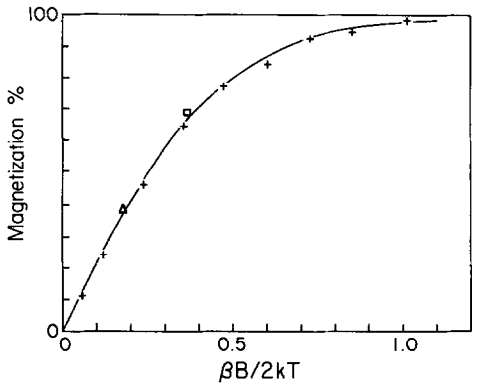

FIG. 3. MCD Magnetization Plot for Methanobacterium thermoautotrophicum Hydrogenase. Wavelength, 650-600nm peak-to-trough; (+) 1.53K, 0 to 4.5 T; (□) 4.22 K, 4.5 T; (△) 8.9 K, 4.5 T. Solid line represents theoretical magnetization curve based on the EPR determined g-values for the Ni(III) center: $g_{\perp}$ = 2.27, $g_{||}$ = 2.01; polarization ratio (11), $m_z/m_+ = -1$.

confirmed by MCD magnetization plots constructed at 650, 600, and 363nm. The data were similar at all three wavelengths and a typical plot is shown in Fig. 3. The experimental points are in good agreement with theoretical data formulated as described in Ref. 10, using the EPR-determined g-values for the Ni(III) center. The theoretical data does not depend critically on the polarization of the transition for an S=1/2 ground state with small g-value anisotropy (11). The magnetization data indicate that the observed EPR spectrum and the optical transitions originate from the same S=1/2, ground state.

Low temperature MCD spectroscopy clearly provides an effective method of selectively identifying electronic transitions associated with the Ni(III) center in Methanobacterium thermoautotrophicum hydrogenase. Furthermore, recent studies in this laboratory indicate that an analogous Ni(III) MCD spectrum is observed in as isolated Desulfovibrio gigas hydrogenase, however, in this case it is superimposed on the MCD signal from a paramagnetic, oxidized [3Fe-xS] center[2]. In light of the optical spectra reported for Ni(III) complexes (12) and the recent XAS results (3,4), we tentatively assign transitions in the regions 530-670nm and 300-460nm to d-d and S⟶Ni charge

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transfer bands respectively. More detailed assignments and diagnostic use of the MCD spectra to determine the Ni coordination environment, must await MCD studies of structurally well characterized Ni(III) complexes. At present, low temperature MCD provides a valuable spectroscopic criteria for assessing the appropriateness of inorganic analog complexes.

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