A Spectroelectrochemical Study of Factor F_{430} Nickel(II/I) from Methanogenic Bacteria in Aqueous Solution

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Abstract: The nickel(II) ion in factor F_{430} pentacarboxylic acid, both in the native form and in the 12,13-di-epimer, in aqueous solution can be reversibily reduced in a one-electron step to the monovalent state by the action of titanium(III) citrate. The $3d^9$ Ni(I) is quantitatively detected by EPR spectroscopy as a tetragonally distorted octahedral system with the hole in the $d(x^2 - y^2)$ orbital resulting in an $S = {}^1/_2$ spin with $g_\parallel = 2.244$, $g_\perp = 2.061$, ($g_\parallel = 2.238$, $g_\perp = 2.057$ for the di-epimer), subject to isotropic superhyperfine interaction from the four in-plane nitrogen ligands with $A = 1.0$ mT. The UV–visible spectrum of the reduced native F_{430} has peaks (extinction coefficients in mM^{-1} cm^{-1}) at 268 (24.0), 378 (34.7), and 715 nm (2.3). In the reduced di-epimer these values are 265 (23.7), 376 (27.6), and 710 nm (1.6). Complete reduction is achieved only at alkaline pH; the process has an apparent pK of 8.9 (native) or 9.4 (di-epimer). The Ni(II)/Ni(I) couple in F_{430} has a reduction potential $E_{m,10.4} = -0.65$ (native) or -0.62 V (di-epimer). Three-pulse stimulated ESEEM spectroscopy on the reduced complexes reveals a single, near isotropic proton resonance at ≈13 MHz. In ^2H_2O solution this line is replaced by a quadrupole-split deuterium resonance around ≈2 MHz, indicating that the Ni(I) in both native and 12,13-di-epimeric F_{430} is weakly coordinated by water. The absence of a strong axial bond with water would leave the ability of the $d(z^2)$ pair for nucleophilic attack on substrate carbon largely intact.

Introduction

Factor F_{430}[2] is a hydrocorphinoid nickel(II) complex[3-5] involved in the last step of methanogenesis as a cofactor of methyl coenzyme M reductase, the enzyme that catalyzes the reductive cleavage of methyl coenzyme M to coenzyme M and methane.[6] Several lines of evidence suggest that the monovalent state of nickel is relevant for catalytic action. Cyclic voltammetry of a derivative of coenzyme F_{430} (the pentamethyl ester, F_{430}M) in tetrahydrofuran or dimethylformamide showed a single, reversible one-electron wave at -1.32 V *vs* the ferrocene/ferrocenium couple.[7] Reduction of the nickel in F_{430}M in tetrahydrofuran by sodium amalgam has been characterized by UV–visible and EPR spectroscopy.[7] Remarkably, a rather different EPR spectrum was reported for F_{430} in aqueous solution, reduced by dithionite.[8] EPR signals from cell suspensions of *Methanobacterium thermoautotrophicum* strain Marburg indicate that the nickel in factor F_{430} is redox-active under physiological conditions and that the reduced state can occur in two forms.[9] Neither of the EPR signals from these two forms compared with the signal reported[8] for isolated aqueous Ni(I)–F_{430} reduced by dithionite. However, one of the signals was similar to that of Ni(I)–F_{430}M in THF (*cf.* ref 7). On the basis of EPR studies on whole cells, a reaction mechanism has been proposed in which the methyl coenzyme M reductase can be active only if the nickel is present in the Ni(I) form and, therefore, needs a reductive activation if the nickel is present as Ni(II).[9] Reductive activation was shown to be a prerequisite for methyl coenzyme M activity in crude cell extracts[10] and with purified components of the methyl coenzyme M reductase system.[11] The physiological reducing system could be replaced by Ti(III) citrate as artificial electron donor.[11-13]

In the present study we give a quantitative EPR analysis of the reduced factor F_{430} pentacarboxylic acid and of its 12,13-di-epimer, two forms isolated from the cell extracts.[14-16] We compare our results with signals of the pentamethyl ester F_{430}M, of whole cells of *M. thermoautotrophicum*, and of purified methyl coenzyme M reductase. Furthermore, we have determined the reduction potential by EPR spectroelectrochemical titrations in alkaline aqueous solution. We have also characterized the reduced F_{430} with ESEEM and UV–visible spectroscopy.

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(2) Abbreviations used: F_{430}, nickel tetrapyrrole pentacarboxylic acid cofactor F_{430}; F_{430}M, nickel tetrapyrrole pentamethyl ester cofactor F_{430}; methyl coenzyme M, 2-(methylthio)ethanesulfonic acid; HSHTP, *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine; Mops, 3-[*N*-morpholino]propanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane; Ches, 2-[*N*-cyclohexylamino]-ethanesulfonic acid; Caps, 3-[cyclohexylamino]-1-propanesulfonic acid; MCR, methyl coenzyme M reductase; ESEEM, electron spin echo envelope modulation; MCD, magnetic circular dichroism.

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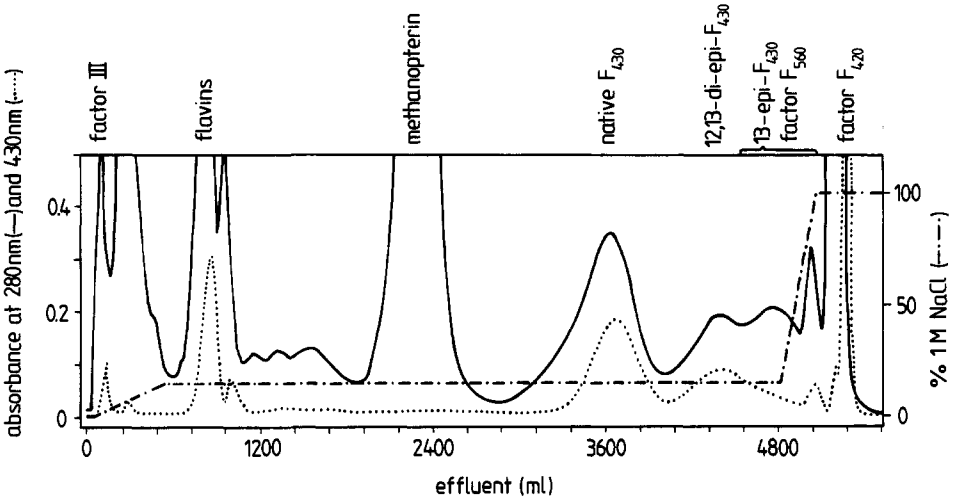

Figure 1. Elution of cofactors from the QAE-Sephadex A-25 column.

Materials and Methods

Cells of *Methanobacterium thermoautotrophicum*, strain ΔH, were generously provided by Dr. J. T. Keltjens, Department of Microbiology, University of Nijmegen. Frozen cells (275 g wet weight) were resuspended in 300 mL of demineralized water, and the cell suspension was cooled to 0 °C. The pH of the suspension was adjusted to 2 by slow addition of 0.5 M $HClO_4$ under continuous stirring at 0 °C. The suspension was stirred for another 45 min. Precipitates were removed by centrifugation for 30 min at 27 000*g* at 4 °C. The pellet obtained was washed with 200 mL of 15 mM $HClO_4$, and the suspension was again centrifuged. This step was repeated four times. The supernatants were combined and adjusted to pH 7 with 1 M $KHCO_3$. Precipitated $KClO_4$ was removed by centrifugation. The supernatant was adjusted to pH 4.5 with 5 M HCl. Cofactors were concentrated using Sep-Pak C-18 cartridges (Millipore Corp., Milford, MA). Cofactors were eluted from the cartridges with 50% methanol. The methanol fractions were combined and flash evaporated to dryness. The dry material was resuspended in 30 mL of 50 mM potassium phosphate, pH 6. About 8 mL of this fraction was applied (4 mL/min) to a QAE-Sephadex A-25 column (1.6 × 100 cm) equilibrated with 50 mM potassium phosphate pH 6 at 4 °C. Effluent was monitored at 280 and 436 nm. Cofactors were eluted with the following NaCl gradient: 0 M (40 mL), 0–0.15 M (480 mL), 0.15 M (4320 mL), 0.15–1 M (240 mL), and 1 M (480 mL). Factor III (corrinoid) eluted first, followed by flavins, methanopterins, native F_{430}, 12,13-di-epi F_{430}, 13-epi F_{430}, F_{560}, and F_{420} (see Figure 1). Native F_{430} eluted between 3000 and 3850 mL, 12,13-di-epi F_{430} between 3900 and 4800 mL. Fractions containing native F_{430} or 12,13-di-epi F_{430} were combined, NaCl was added up to about 1 M, and the pH was adjusted to 4.5. Cofactors were concentrated with Sep-Pak C-18 cartridges. Purity was checked with UV–visible spectroscopy. Factor F_{430} was stored under N_2 at −20 °C. Due to severe shrinking of the QAE-Sephadex A-25 column support by the NaCl gradient, the column had to be repacked between individual preparative runs.

The concentration of Ni(II)–F_{430} was based on a determination of the nickel content using a Perkin-Elmer ICP 5500 atomic absorption spectrometer equipped with an HG-400A graphite furnace atomizer.

Titanium(III) citrate was prepared from $TiCl_3$ (ampule) and sodium citrate as previously described.[17] Reduction of factor F_{430} in different buffers was done by filling an argon-purged 250-μL syringe first with 175 μL of buffer, followed by 25 μL of ≈5 mM factor F_{430} and 50 μL of 100 mM Ti(III) citrate pH 9. The solution was then transferred to an anaerobic EPR tube where mixing occurred. The samples were kept at room temperature for 15 min before being frozen in liquid nitrogen. All buffers contained 0.1 mM methyl and benzyl viologen because reduction of factor F_{430} by Ti(III) citrate was much faster in the presence of these two dyes. The 15-min incubation time was chosen after establishment of the time course of the reaction: reduction of factor F_{430} was essentially completed after 2–3 min and remained so for at least 20 min; incubation for longer times resulted in a slow diminishing of the EPR signal (see below) of reduced factor F_{430}. Redox potentiometry according to the method of Dutton[18] used 0.1 mM end concentration of methyl and benzyl viologen as redox mediators. Ti(III) citrate was used as titrant.

Deuterium oxide, 99.8 atom % ^2H, and sodium deuterioxide 30 wt % solution in ^2H_2O, 99+ atom % ^2H, both from Janssen Chimica (Beerse, Belgium), were used to prepare deuterated Caps buffer p^{2}H 10.4. Native and 12,13-di-epimer F_{430} in H_2O were freeze-dried and anaerobically redissolved in deuterated Caps buffer plus viologen mediators. Ti(III) citrate in water was freeze-dried and anaerobically redissolved in deuterated Caps buffer and was then mixed with the F_{430}, kept at ambient temperature for 15 min, and frozen in liquid nitrogen. UV–visible and EPR spectroscopy was used on samples redissolved in nondeuterated buffer to ensure that the freeze-drying and redissolution did not cause any structural change (*e.g.*, di-epimerization) in the samples.

Continuous-wave EPR spectra were obtained with a Bruker ER-200 D spectrometer with field modulation at a frequency of 100 kHz and with periferal instrumentation and data acquisition as described before.[19] $S = {}^1/_2$ EPR spectra subject to inhomogeneous broadening in field space were simulated with a program written in FORTRAN with options for automated minimization, filtering in Fourier space, minimization on second derivatives (W. R. Hagen, unpublished results).

Electron Spin Echo Envelope Modulation (ESEEM) experiments were performed at X-band on a home-built machine[20] operating in the three-pulse stimulated echo mode: p90-τ-p90-t-p90-echo. The pulse duration of a 90° pulse (p90) was 30 ns. Waiting time τ was fixed at 200 ns, while t was stepped from 420 to 5420 ns in 10-ns intervals. The electron spin echo envelopes were high-pass filtered, apodized, and transformed to the Fourier amplitude mode. Data acquisition and processing was performed on a PC-AT computer with a program written in TURBO-C (courtesy J. J. Shane, unpublished results).

UV–visible spectra were obtained with an SLM Aminco DW-2000 spectrophotometer. Suba-seal capped quartz cuvettes of 1-mm path length were made anaerobic and filled with solutions prepared in a syringe as described for the reduction in EPR tubes. Data were corrected by subtraction of the spectrum of a solution without F_{430}.

Results

EPR Spectroscopy of Aqueous Ni(I)–F_{430}. In the isolated cofactor F_{430} in aqueous solution as well as in the purified enzyme, the nickel is Ni(II);[21,22] however, the catalytically active form of the cofactor has been proposed to carry Ni(I).[23] We have now found that the nickel in F_{430} in alkaline aqueous solution can be virtually quantitatively reduced by the action of Ti(III) citrate, resulting in an EPR spectrum very similar to one of the signals

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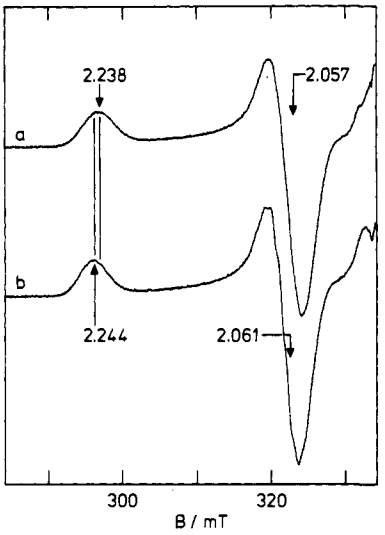

Figure 2. EPR spectrum of ≈0.5 mM 12,13-di-epi F_{430} (trace a) and native F_{430} (trace b) in which the nickel was quantitatively reduced to Ni(I) with 10 mM Ti(III) citrate plus 0.1 mM each benzyl viologen and methyl viologen in aqueous solution buffered with 100 mM Caps, pH 10.4. The sample was frozen in liquid N_2 after 15 min of incubation at ambient temperature. The high-field ramp is the onset of the Ti(III) signal with all g values <2. EPR conditions: microwave frequency, 9.30 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; microwave power, 0.8 mW; temperature, 66 K. The spectra are averages of nine traces.

observed in whole cells.[9] The Ni(I) spectra are presented in Figure 2 for the native F_{430} and di-epi F_{430}. As Ti(III) is $3d^1$ and Ni(I) is $3d^9$, the sign of the spin–orbit coupling for the two paramagnets is opposite, and therefore their g values are on opposite sides of the free-electron value. This ensures that EPR spectral interference from excess Ti(III) citrate is insignificant. Double integration of the Ni(I) signal versus an external cupric standard gives 1.0 $S = {}^1/_2$ spins per F_{430} as determined by nickel atomic absorption. The Ni(I) signal is readily observable at least over a temperature range from 17 to 110 K, with microwave power saturation setting in at approximately 0.08, 1.3, and 5 mW for T = 17, 66, 110 K, respectively.

Contrary to the spectra from sodium amalgam-reduced F_{430}M in tetrahydrofuran[7] and, especially, to a comparable signal from whole cells,[9] superhyperfine splittings from the in-plane nitrogens are only just barely detectable in Figure 2, particularly for the di-epimerized F_{430}, as a number of slight inflections in the perpendicular feature. This is not surprising as both the replacing of water by organic solvent and, especially, the magnetic diluting by "placing" a cofactor in a diamagnetic protein matrix generally leads to some decrease in the inhomogeneously broadened EPR line width as a result of g-strain attenuation.[24]

In view of this low resolution we have analyzed the spectra by computer simulation on the basis of a restricted parameter set, namely, we have assumed an axially-symmetric g-tensor, a single isotropic line width, and a single isotropic value for all the superhyperine splittings from the corphinoid nitrogens (*i.e.*, a total of four adjustable parameters). The result of minimized simulation for native F_{430} is given in Figure 3, trace b. The simulation parameters are $g_\perp = 2.061$, $g_\parallel = 2.224$, $W_{iso} = 0.92$ mT (W is one standard deviation of a Gaussian line shape), and $A_{iso}(^{14}N) = 1.0$ mT. The residuals between traces a and b are of the order of the experimental noise. In other words, the simulation is optimal, and no further information can be extracted on, *e.g.*, a possible rhombicity of the g-tensor or on any anisotropy in the line width or in the ligand hyperfine splittings. For the 12,13-di-epi F_{430} the parameters are $g_\perp = 2.238$, $g_\parallel = 2.057$, $W_{iso} = 1.04$ mT, and $A_{iso}(^{14}N) = 1.05$.

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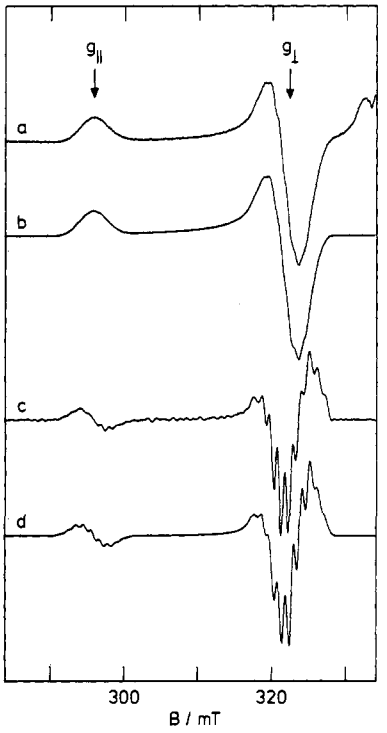

Figure 3. (a) EPR spectrum of native F_{430} Ni(I) from Figure 2. (b) Computer simulated spectrum (see text for parameters). (c) Fourier-filtered second-derivative spectrum. (d) Simulation (see text for explanation).

In order to check whether the assumption of detectability of superhyperfine splittings was a realistic one, the second-derivative EPR spectrum was calculated from the experimental spectrum as a method to emphasize poorly resolved structure. Differentiation brings along a considerable increase in noise of higher frequencies. Therefore, the second-derivative spectrum was subjected to discrete Fourier transformation. Subsequently, all high-frequency components were eliminated with a cutoff filter just above the typical frequency components for nitrogen hyperfine, and the spectrum was back-transformed to magnetic field space.[25] Note that the original data were first edited to eliminate the onset of the Ti(III) signal in order to prevent the creation of wraparound errors by the Fourier transformation. The result of this procedure is presented in Figure 3, trace *c*. On top of the residual noise, a ligand hyperfine pattern is clearly visible, especially in the perpendicular feature.[26] Finally, trace d is obtained by subjecting the simulation in trace b to the same differentiation/filtering procedure. The second derivatives of the filtered data and the simulation are very similar, indicating that our simple model of four identical isotropic nitrogen hyperfine interactions is a reasonable one. Contrary to what Jaun and Pfaltz reported for F_{430}M Ni(I) in THF, here no improved fit is obtained by allowing for a finite rhombicity in the g-tensor.

ESEEM Spectroscopy of Aqueous Ni(I)–F_{430}. A considerable amount of spectroscopic data[16,21,22,27–29] indicates that the Ni(II) in native F_{430} in frozen solution at cryogenic temperatures is S

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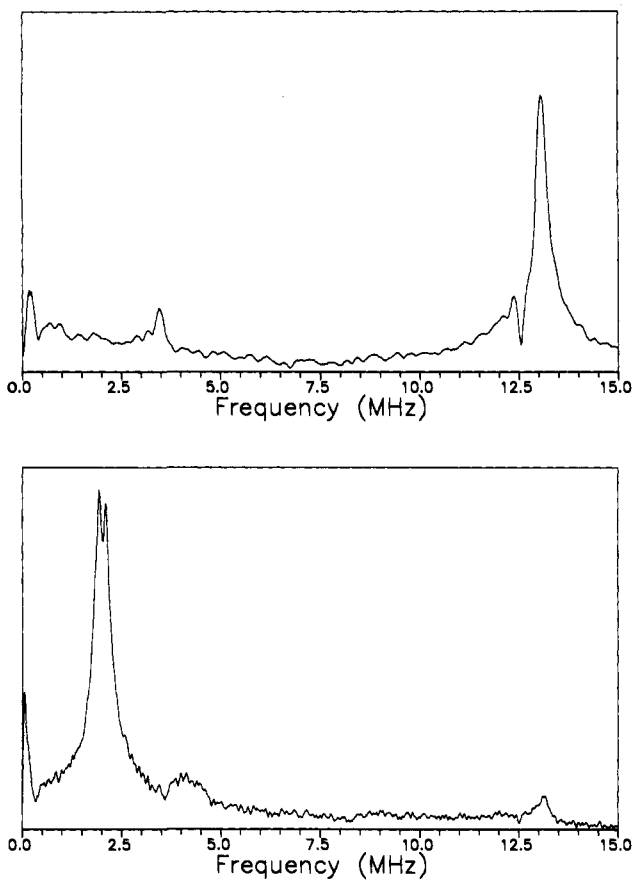

Figure 4. ^1H (upper trace) and ^2H (lower trace) FT-ESEEM spectra of native F_{430} measured at the approximate $g_\perp$ position of the EPR spectrum (*cf.* Figure 3a). Conditions: magnetic field, 309 mT; microwave frequency, 9.03 GHz; τ = 200 ns.

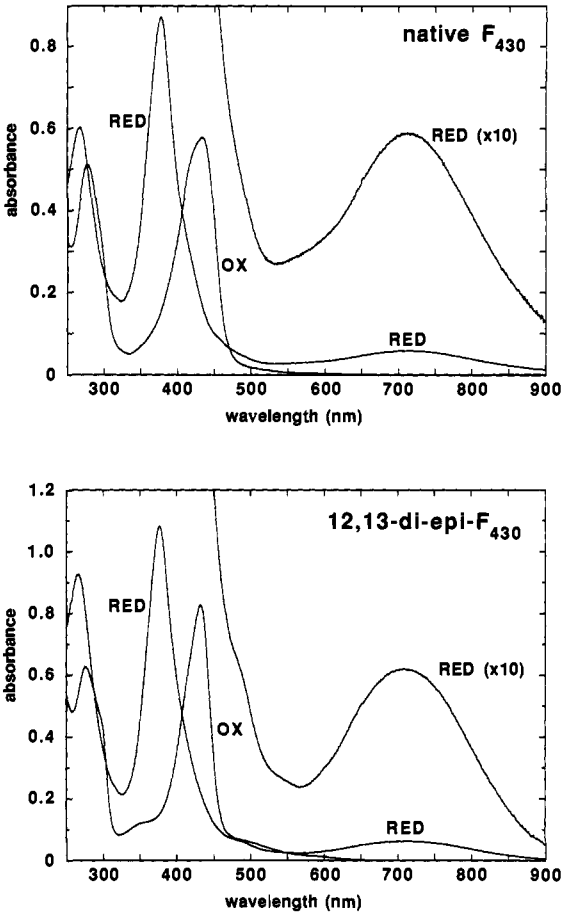

Figure 5. UV–visible spectra of 0.25 mM native (upper panel) and 0.41 mM 12,13-di-epimeric (lower panel) F_{430} pentacarboxylic acid, in 100 mM Caps, pH 10.4, in the presence of 0.1 mM methyl viologen and 0.1 mM benzyl viologen. The optical path was 0.10 cm. The reduction was by Ti(III) citrate (final concentration 4 mM) at ambient temperature (20 min). The spectra are corrected for contributions from blank solutions, i.e., without F_{430} but with mediators and reductant.

= 1, tetragonally coordinated with two water molecules as the axial ligands. Since the catalytically active form of F_{430}, either free or enzyme-bound, is presumed to have the nickel as Ni(I), it is of importance to see whether the description of the coordination of the nickel in the oxidized, "resting" F_{430} can be carried over to the activated, reduced form. Thus far Ni(I)–F_{430} has been studied only in F_{430} derivatives dissolved in nonaqueous solution.[7,30-32]

When factor F_{430} Ni(I) is prepared in deuterated buffer, the resulting EPR spectra are indistinguishable from those of Figure 2, except for a slightly increased resolution of the nitrogen superhyperfine pattern for the di-epimer (not shown). This hints toward a weak electron–nuclear coupling to the protons (deuterons) of coordinating water molecules. However, CW-EPR is less suited to study this effect in view of the inhomogeneous line width and also because of the predominantly in-plane σ-character of the unpaired electron in the $d(x^2 - y^2)$ orbital. ESEEM spectroscopy is particularly suited for the detection of these weak couplings.[33]

We have measured the three-pulse stimulated echo at the two turning points in the EPR spectrum (corresponding to the principal values of the axial g-tensor) for native and di-epimeric F_{430} Ni(I) in H_2O and ^2H_2O. The example of native F_{430} in water detected at $g_\perp$ is given in Figure 4. For both native and 12,13-di-epimeric F_{430} a single modulation signal is observed at the free proton nuclear Larmor frequency corresponding to the magnetic field of the measurement (12.3 MHz for $g_\parallel$ and 13.1 MHz for $g_\perp$). When the experiment is repeated in deuterated water the proton signal is replaced with a single quadrupole doublet centered at the deuterium Larmor frequency (1.78 MHz at $g_\parallel$ and 2.02 MHz at $g_\perp$). The quadrupole splitting of 0.16 MHz corresponds exactly to three times the quadrupole parameter, $K = e^2qQ/4h$, of ^2H in ^2H_2O. The observed proton and deuterium modulation signals are typical for paramagnetic species solvated by many uncorrelated water molecules at relatively large distances (4–8 Å). For these systems the ^2H ESEEM feature is dominated by a first-order quadrupole splitting given by the perpendicular component of the (axial) quadrupole tensor, which amounts to 3K.[34] For a particular g-principal direction and a particular hydrogen isotope, the ESE modulation depth is similar for native *vs* di-epimer F_{430}. It appears that at cryogenic temperatures the two forms of the cofactor have similar water solvation to the Ni(I).

UV–Visible Spectroscopy of Ni(II)/Ni(I) F_{430}. A facile reduction of the F_{430} chromophore was observed optically (Figure 5) under the same reaction conditions as employed for EPR spectroscopy. Comparison of the kinetics of the reduction by parallel experiments with UV–visible and EPR spectroscopy showed a good correspondence between the appearance of the Ni(I) $S = {}^1/_2$ EPR signal and the bleaching of absorbance at ≈433 nm. Similar observations were made by Jaun and Pfaltz[7] for sodium amalgam reduction of F_{430}M in THF solution.

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Table I. Comparison of UV–Visible Spectral Data from the Ni(II) and Ni(I) Forms of Aqueous F_{430} Pentacarboxylic Acid (pH 10.4) and of Pentamethyl F_{430} in Tetrahydrofuran

	native F_{430}		di-epi F_{430}		$F_{430}M$ in THF[a]	
	λ (nm)	ε (mM^{-1} cm^{-1})	λ (nm)	ε (mM^{-1} cm^{-1})	λ (nm)	ε (mM^{-1} cm^{-1})
Ni(II)	275	15.4	278	20.4	275	16.9
	432	20.3	434	23.0	440	19.9
Ni(I)	265	23.7	268	24.0	278	20.4
	376	27.6	378	34.7	382	29.6
	710	1.6	715	2.3	754	2.5

[a] Data taken from ref 7.

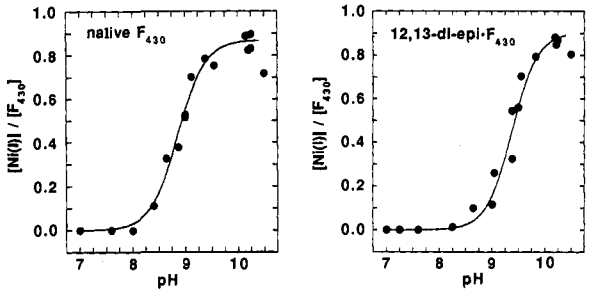

Figure 6. Reduction of the nickel(II) in native factor F_{430} (left-hand panel) and the 12,13-di-epimer (right-hand panel) by Ti(III) citrate plus viologens at different pH values. The reduction was carried out as described in the Materials and Methods section. The following buffers were applied: 100 mM Mops, pH 6.12 and 7.02; 100 mM Tris, pH 8.04 and 8.91; 50 mM Tris/50 mM glycine pH 9.80; 50 mM Ches/50 mM Caps, pH 9.57, 10.27, and 10.97. The pH values in the figure are values of solutions of 100 mM Ti(III) citrate pH 9 and buffer in a ratio of 1:4 measured in an anaerobic glovebox. Nickel(I) concentration was measured with EPR spectroscopy: microwave frequency, 9.30 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 2 mW; temperature, 110 K.

Although an accurate determination of isobestic points and extinction coefficients was not feasible due to substantial optical contributions of mediators and reductant, the overall UV–visible characteristics of reduced aqueous F_{430} (Figure 5 and Table I) were similar to those of reduced $F_{430}M$ in THF. The main difference is the wavelength shift of the 754-nm absorbance to 715 nm in aqueous solution with a further shift to 710 nm for the di-epimer.

Oxidation of the Ni(I) species by short exposure to air (not shown) resulted in >95% restoration of the ≈433-nm peak. Thus in our hands a full and essentially reversible reduction of Ni(II) to Ni(I) in F_{430} is possible under protic conditions, in contrast to previous observations.[7] To our knowledge this represents the first demonstration of a reversible Ni(II)/Ni(I) conversion in an aqueous nickel complex with quantitative detection of the Ni(I) species by EPR spectroscopy.

pH Dependence of Aqueous F_{430} Reduction. The pH dependence of the reduction of Ni(II) in F_{430} was studied by carrying out the EPR experiment of Figure 2 with a range of buffers in the pH range 7–11. Thus F_{430} was incubated with excess Ti(III) citrate for 15 min, and the extent of nickel reduction was determined from the Ni(I) EPR amplitude. The results are presented in Figure 6. There is no reduction at low pH values. The native F_{430} bulk is half reduced at pH 8.86; for the 12,13-di-epi-F_{430} the corresponding value is pH 9.38. These findings could be accounted for by any combination of the following effects: (1) a pH dependence of the reduction potential of the Ti(IV)/Ti(III) citrate couple; (2) a pH dependence of the reduction potential of the Ni(II)/Ni(I) F_{430} couple; and (3) a difference in the Ni(II)/Ni(I) reduction potentials of native and di-epimeric F_{430}.

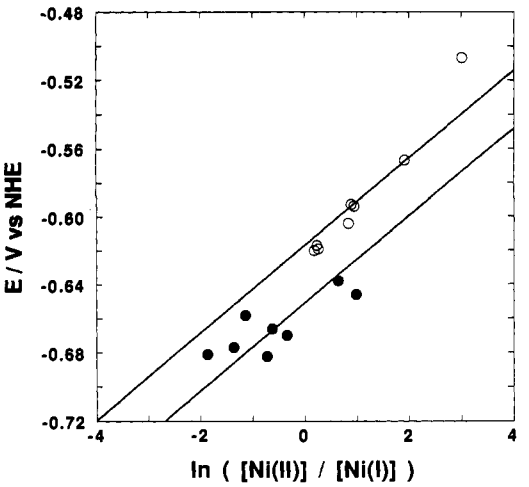

Figure 7. Mediated redox titration at 25 °C of aqueous F_{430} in 100 mM Caps, pH 10.4, with Ti(III) citrate as the reductant in the presence of 0.1 mM each of benzyl viologen and methyl viologen. The concentration of reduced F_{430} was determined as [Ni(I)] by quantitative EPR spectroscopy (conditions identical to those for Figure 6). The solid symbols are for 83 μM native F_{430}; the open symbols are for 262 μM 12,13-di-epi F_{430}. The solid lines are Nernst $n = 1$ curves.

In the pH range used, the Ti citrate takes up a single proton upon reduction, $E_{m,7.0} \approx -0.50$ V and $\Delta E/\Delta pH = -0.06$ V at 25 °C.[35]

The Ni(II) in F_{430} could only be reduced quantitatively by Ti(III) citrate in basic aqueous solution. Therefore, redox titrations were carried out at pH 10.4. In the titration cell a stable potential lower than –0.68 V *vs* NHE was never reached, even after addition of Ti(III) citrate in excess. At this potential only some 80% reduction of the nickel was achieved. The amount of Ni(I) was calculated by measuring the intensity of the spectral features at $g = 2.06$ and 2.24 and correlating those with intensities of a sample where Ni(I) was quantified by double integration of the spectrum. A reduction potential of –0.65 V *vs* NHE was calculated from a Nernst plot (Figure 7). For the di-epimerized F_{430}, the midpoint potential was –0.62 V.

The difference in $E_{m,10.4}$ between native and di-epimer F_{430} is in the "wrong" direction to explain the pH shift for half reduction as observed in Figure 6. This would seem to indicate that the reduction potential of the di-epi F_{430} is less—if at all—pH dependent than the E_m of native factor F_{430}; the latter, in its turn, changes less rapidly with pH than the E_m of the Ti(IV)/(III) couple, *i.e.*, with less than –60 mV per pH unit. It is difficult to imagine any feature of oxidized, native factor F_{430}[5] that could ionize with a pK ≈ 9–10 except for water ligands axially coordinating to the Ni(II). These ligands would then have to be absent in di-epimer F_{430}; this is consistent with previous spectroscopic studies (see below). We have been unable to verify this conclusion with redox titrations done at pH values significantly less than 10.4. Namely, under these conditions the final extent and the reproducibility of mediated F_{430} reduction proved to be insufficient.

Discussion

The nickel is Ni(II) in purified *M. thermoautotrophicum* methyl coenzyme M reductase as well as in the isolated cofactor F_{430} in aqueous solution.[21,22] It has, however, been proposed that the nickel changes oxidation state during or preceeding catalysis and, specifically, that the active species for the reductive cleavage of carbon–sulfur bonds involves monovalent nickel.[7,9,23] Jaun and Pfaltz have shown that the nickel in a derivative of F_{430} (the pentamethyl ester $F_{430}M$) can be essentially quantitatively reduced

(35) Zehnder, A. J. B. Ph.D. Thesis, Federal Institute of Technology (ETH), Zürich, Switzerland, 1976.

to Ni(I) by sodium amalgam, with no apparent reduction in the corphinoid ring system, when the cofactor is dissolved in certain organic solvents.[7] The reduced $F_{430}M$ exhibits d^9, $S = {}^1/_2$ EPR of close to axial symmetry ($g = 2.065, 2.074, 2.250$), with isotropic superhyperfine splitting from four equivalent nitrogen ligands.

It has not been clear until now whether the specific electronic structure of the Ni(I), as observed in $F_{430}M$ in organic solvent, is of biological relevance. Albracht *et al.* have reported an EPR signal for isolated F_{430} plus dithionite in aqueous solution which differs significantly from the signal just described, namely with the following properties:[8] $g \approx 2.03(5), 2.21$, no resolved nitrogen splittings, and an integrated intensity corresponding to only 1% of the F_{430} concentration. The matter is further complicated by a subsequent report from Albracht *et al.* of an EPR signal (labeled "MCR-red1" in ref 9) observed in whole cells, and recently in partially purified enzyme,[36] which is more similar (though not identical) to the signal from $F_{430}M$ in organic solvent, namely: $g \approx 2.052, 2.24$, with resolved splittings apparently from a number of nitrogen ligands.

The signals that we have detected and analyzed here are rather different in their EPR characteristics from the one previously reported for aqueous F_{430} reduced with dithionite.[8] This, combined with the fact that we recover 100% of the F_{430} as EPR detectable Ni(I) where the dithionite reduction experiment yielded only 1%, leads us to conclude that the dithionite-induced signal does probably not reflect intact F_{430} cofactor. On the other hand, the signals reported on here are similar to the MCR-red1 signal found in whole cells of *M. thermoautotrophicum*,[9] the only significant difference within experimental error being the different line width. The MCR-red1 signal has recently also been detected in partially purified, highly active enzyme; however, a low signal-to-noise ratio required a high modulation amplitude, and therefore, nitrogen splittings were not observed.[36] All these signals have slightly, but significantly, lower g values than those reported for the $F_{430}M$ in THF.[7] These observations indicate that either the MCR-red1 signal in whole cells is from free F_{430} or the monovalent nickel in F_{430}, as it is observed in the MCR-red1 signal, is coordinated in a manner essentially identical to that in the free F_{430} in water.

From previous spectroscopic studies (EXAFS,[16,27,28] UV–visible,[27] resonance Raman,[27–29] MCD[21,22]) it has been concluded that the Ni(II) in native F_{430} in frozen solution at cryogenic temperatures is $S = 1$, tetragonally coordinated with two water molecules as the axial ligands. At ambient temperature this structure appears to be in equilibrium with a ruffled-planar four-coordinated one with diamagnetic Ni(II).[22,28,29] These studies also indicate that in di-epimeric F_{430} the Ni(II) is predominantly four-coordinated and diamagnetic,[16,27,28] although in a 50% glycerol glass at low temperature a significant amount may be six-coordinated, high-spin Ni(II).[22] By MCD spectroscopic comparison it has been inferred that the Ni(II) in methyl coenzyme M reductase is six-coordinated, $S = 1$, with two oxygens in axial position.[21,22]

The bearings of these studies from a mechanistic point of view are less obvious as they have been obtained with oxidized, "resting" cofactor. The catalytically competent form of the cofactor is presumably that in which the nickel is Ni(I). The picture that emerges from our present EPR plus ESEEM spectroscopic studies on the reduced cofactor is different from the picture just outlined for the oxidized cofactor. The two forms of the reduced F_{430}, native and 12,13-di-epimer are very similar with respect to their coordination of the Ni(I). Both forms *lack* strongly bound axial ligands; they do, however, through their Ni(I) both weakly sense several protons (or deuterons) of water at a distance of 4–8 Å, *i.e.*, they appear to weakly (and probably fluxionally) coordinate a number of water molecules.

This coordination model concurs with the results of recent activity studies; the isolated factor F_{430} is a catalyst in reactions of potential technological relevance, *e.g.*, reductive dehalogenation.[17] The dechlorination of 1,2-dichloroethane is catalyzed both by the cobalt-containing cofactor cobalamin and by factor F_{430}. The Co cofactor is somewhat more active than the Ni cofactor, and the products of reduction by both cofactors are ethene and chloroethane. The product ratio, R = [chloroethane]/[ethene], is over 1 order of magnitude greater for F_{430} compared to cobalamin.[17] The production of chloroethane requires entry and activation of a water molecule; it also requires a nucleophilic attack on 1,2-dichloroethane carbon. The weakly Ni(I)-coordinated water that we have found in ESEEM spectroscopy would appear to be an appropriate candidate as the weakness of its binding leaves the potentiality of the electron pair in the $d(z^2)$ orbital as a nucleophile largely intact.

The redox properties of factor F_{430} in the active methyl coenzyme M reductase have not been established yet. On the basis of the data presented above, it is expected that the holoenzyme will have a potential similar to the $E_m = -0.62$ to -0.65 V of the free factor in aqueous solution. However, the enzyme may exhibit considerable ability to modulate its E_m via changes in the axial coordination of the nickel, *e.g.*, through conformational changes of the protein. The biological relevance of such an option may be understood as follows. In *in vitro* systems of methanogenic bacteria, an ATP-dependent reductive preactivation of the methyl coenzyme M reductase is required for activity.[9,10] The enzyme system responsible for the reduction of inactive methyl coenzyme M reductase has been resolved in three protein fractions: components A2, A3a, and A3b.[37] Component A3a, a protein with a molecular mass of 500 kDa, is probably involved in electron transfer from component A3b, which has methyl viologen linked hydrogenase activity, to methyl coenzyme M reductase. It has been hypothesized[37] that the effect of MgATP on component A3a is analogous to the effect of MgATP on the Fe-protein of the nitrogenase complex (*cf.* ref 38): the binding of ATP is a prerequisite for electron transfer between the two components (either between A3a and methyl coenzyme M reductase or between the nitrogenase Fe-protein and MoFe-protein). The binding and the hydrolysis of ATP in both cases is envisioned to be related to a protein conformational change and a consequential change in redox potential. The exact order of events is not established in either of the two systems.

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