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Direct Determination of the Number of Electrons Needed To Reduce Coenzyme F430 Pentamethyl Ester to the Ni(I) Species Exhibiting the Electron Paramagnetic Resonance and Ultraviolet–Visible Spectra Characteristic for the MCR State of Methyl-coenzyme M Reductase

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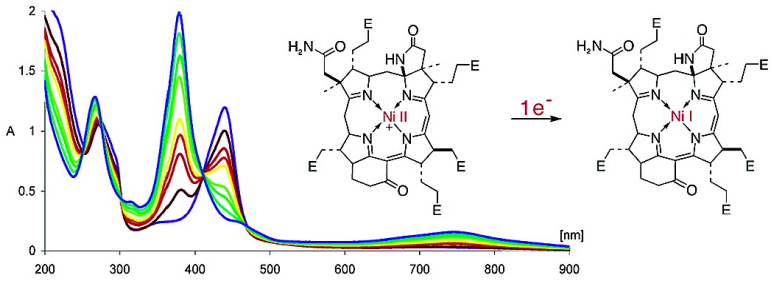

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Direct Determination of the Number of Electrons Needed To Reduce Coenzyme F430 Pentamethyl Ester to the Ni(I) Species Exhibiting the Electron Paramagnetic Resonance and Ultraviolet−Visible Spectra Characteristic for the MCR_{red1} State of Methyl-coenzyme M Reductase

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Abstract: The UV−visible and electron paramagnetic resonance (EPR) spectra of MCR_{red1}, the catalytically active state of methyl-coenzyme M reductase, are almost identical to those observed when free coenzyme F430 or its pentamethyl ester (F430M) are reduced to the Ni(I) valence state. Investigations and proposals concerning the catalytic mechanism of MCR were therefore based on MCR_{red1} containing Ni^{I}F430 until, in a recent report, Tang et al. (*J. Am. Chem. Soc.* **2002**, *124*, 13242) interpreted their resonance Raman data and titration experiments as indicating that, in MCR_{red1}, coenzyme F430 is not only reduced at the nickel center but at one of the C=N double bonds of the hydrocorphinoid macrocycle as well. To resolve this contradiction, we have investigated the stoichiometry of the reduction of coenzyme F430 pentamethyl ester (F430M) by three independent methods. Spectroelectrochemistry showed clean reduction to a single product that exhibits the UV−vis spectrum typical for MCR_{red1}. In three bulk electrolysis experiments, 0.96 ± 0.1 F/mol was required to generate the reduced species. Reduction with decamethylcobaltocene in tetrahydrofuran (THF) consumed 1 mol of $(Cp^*)_2Co$/mol of F430M, and the stoichiometry of the reoxidation of the reduced form with the two-electron oxidant methylene blue was 0.46 ± 0.05 mol of methylene blue/mol of reduced F430M. These experiments demonstrate that the reduction of coenzyme F430M to the species having almost identical UV−vis and EPR spectra as MCR_{red1} is a one-electron process and therefore inconsistent with a reduction of the macrocycle chromophore.

Coenzyme F430, the hydrocorphinoid nickel complex **1**,[1−4] is the prosthetic group of methyl-coenzyme M reductase (MCR),[5] which catalyzes the transformation of methyl-coenzyme M (**3**) and coenzyme B (**4**) into methane and the mixed disulfide (**5**) of coenzyme B and coenzyme M in methanogenic archaea.[6−8] Although high-resolution X-ray structures of several inactive Ni(II) forms of the enzyme have been determined,[9−11] the catalytic mechanism for this unusual enzymatic reaction remains largely unknown.[10,12−15]

The enzyme can be isolated in two different electron paramagnetic resonance- (EPR-) active states designated as MCR_{ox1} and MCR_{red1} by changing the H_2/CO_2 gas mixture under which the cells are grown to either N_2/CO_2[16,17] or H_2,[16,18] respectively, before harvesting. In frozen solution, both forms exhibit nearly axial EPR spectra with $g_{||} > g_{\perp}$, indicating a ground state with a predominant $d_{x^2-y^2}$ configuration, but the *g*-anisotropy of the MCR_{red1} signal is much larger than that of MCR_{ox1}.[19] Because the EPR and UV−vis spectra of MCR_{red1} are nearly identical to those observed when either coenzyme F430 pentamethyl ester (**2**) is reduced with NaHg/tetrahydrofuran (THF)[20] or coenzyme

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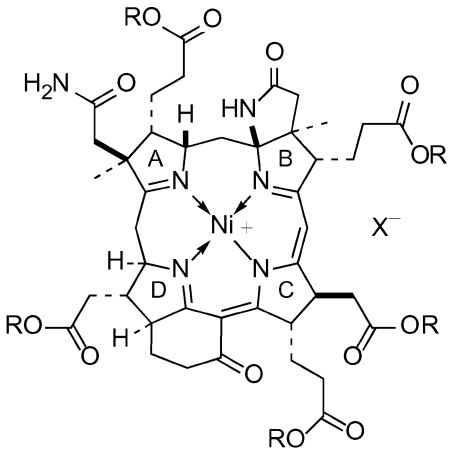
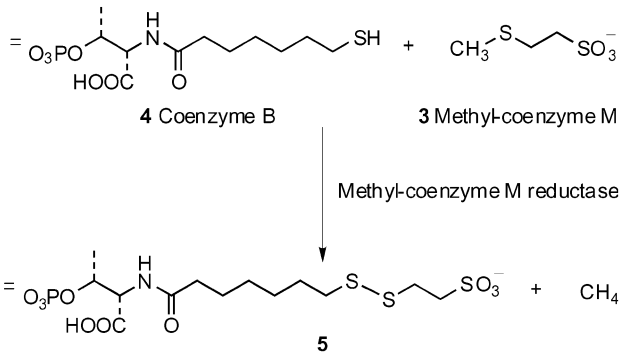


1 Coenzyme F430 (R=H)
2 Ni^{II}F430M (R=Me, X=ClO_4)



4 Coenzyme B **3** Methyl-coenzyme M **5**

F430 (**1**) is reduced by Ti^{III}citrate in H_2O at pH 10,[21] MCR_{red1} was assigned to an enzyme form containing coenzyme F430 in the Ni(I) valence state.[22] In the absence of similar reference spectra from solution studies of isolated coenzyme F430 derivatives and because both Ni(I) and Ni(III) complexes with $d_{x^2-y^2}$ configurations are known, the assignment of the valence state of nickel for MCR_{ox1} is not so obvious. The generation of MCR_{ox1} from $MCR_{ox1-silent}$ [an EPR-inactive Ni(II) state] upon γ-irradiation at low temperature (cryoreduction) and EPR/electron−nuclear double resonance (ENDOR) data have been interpreted in terms of a nickel +1 valence state for MCR_{ox1}.[23,24] On the other hand, the UV−vis spectrum, X-ray absorption data,[25] and theoretical calculations[26] were interpreted as being consistent with a formal Ni(III) valence state with a $d_{x^2-y^2}$ configuration for MCR_{ox1}.

In the presence of the two substrates, MCR_{red1} is highly active and produces methane, whereas MCR_{ox1} is completely inactive. However, MCR_{ox1} can be converted into active MCR_{red1} with Ti^{III}citrate at pH 9 and 60 °C,[22] and in the presence of coenzyme M and coenzyme B, MCR_{red1} can be converted to MCR_{ox1} by oxidation with polysulfide.[27] Since no other redox-active transition metals were found in MCR by X-ray diffraction, assigning a Ni(I) valence state to MCR_{ox1} leads to the apparent paradox that an inactive Ni(I) form (MCR_{ox1}) is activated by the strong reductant Ti(III) to give an active Ni(I) form (MCR_{red1}) without change in the valence state of the nickel of coenzyme F430.

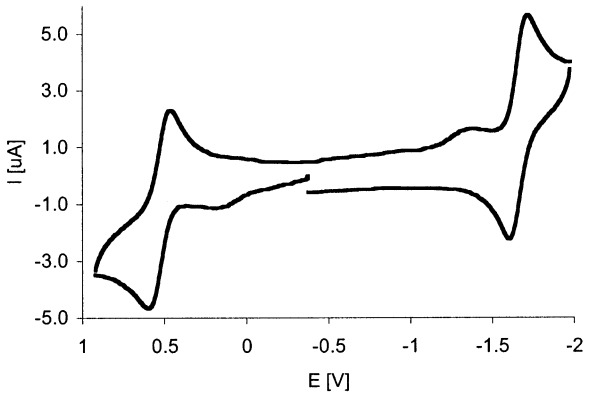


Figure 1. Cyclic voltammogram of Ni^{II}F430M (c = 1.0 mM) in dry propionitrile/0.1 M $TBAPF_6$ under nitrogen. Conditions: Pt electrode, sweep rate 0.1 V/s, potential scale vs Fc^+/Fc.

On the basis of resonance Raman studies of MCR_{ox1} and MCR_{red1} as well as titrations of the enzyme and free coenzyme F430 with Ti^{III}citrate, Ragsdale and Bocian and co-workers[28] have recently proposed that MCR_{red1} contains the cofactor in a form in which not only the nickel is reduced to Ni(I) but one of the C=N double bonds in rings B or D is hydrogenated as well. According to this proposal, MCR_{ox1} would correspond to the Ni(I) species that—compared to the silent form—is reduced at the metal only and its activation with Ti(III) would correspond to a reduction of the macrocycle. The authors argue that although in our original work with coenzyme F430 pentamethyl ester[20] the cyclic voltammogram showed a single one-electron reduction wave, the EPR and UV−vis spectra were obtained on samples that had been reduced chemically with NaHg in THF and that it could therefore not be excluded that an additional reduction on the macrocycle had occurred.

This prompted us to determine, by three independent experiments, the number of electrons needed to reduce **2** to the Ni(I) species exhibiting the UV−vis and EPR spectra seen for MCR_{red1}. We chose to use the pentamethyl ester (Ni^{II}F430M, **2**) rather than the free coenzyme (Ni^{II}F430, **1**) because, in our hands, the Ni(I) form of coenzyme F430 is not stable at neutral pH in the protic solvents needed to dissolve **1**.

Results and Discussion

1. Cyclic Voltammetry, Spectroelectrochemistry, and Bulk Electrolysis of 2. The cyclic voltammogram of Ni^{II}F430M (**2**) in propionitrile/0.1 M $TBAPF_6$ at a platinum electrode is shown in Figure 1. Starting at −0.4 V and sweeping first to +0.9 V, then to −2.0 V, and back to −0.4 V gave reversible one-electron waves for the Ni(II)/Ni(I) and Ni(III)/Ni(II) couples. Within experimental error, both waves show the same peak currents.

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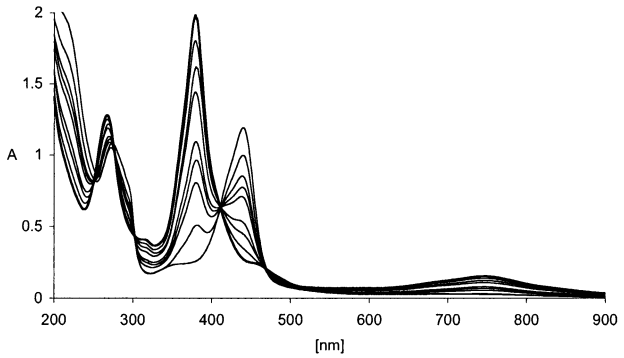

Figure 2. Changes in the UV−vis spectrum of Ni^{II}F430M in dry acetonitrile/0.1 M $TBAPF_6$ during reduction to Ni^{I}F430M (bold line) in a thin-layer spectroelectrochemical cell (l = 0.03 cm).

Table 1. Bulk Electrolysis of Ni^{II}F430M

F430M (μmol)	solvent/electrolyte	E^a (V)	Q_{total} (C)	$Q_{electrolyte}$ (C)	$Q_{F430M}{}^b$ (C)	z (F/mol)
1.6	acetonitrile/0.1 M $TBAPF_6$	stepwise: −1.476, −1.526, −1.576	0.143	0.010	0.133	0.86
2.6	acetonitrile/0.1 M $TBAPF_6$	−1.526	0.254	0.015	0.239	0.95
2.7	propionitrile/0.1 M $TBAPF_6$	stepwise: −1.576, −1.726	0.300	0.015	0.285	1.09

[a] Versus Fc^+/Fc. [b] $Q_{F430M} = Q_{total} - Q_{electrolyte}$.

Provided that the diffusion coefficients are similar for all three redox forms involved, this signifies that within the time scale of cyclic voltammetry, oxidation and reduction of Ni^{II}F430M involve the same number of electrons.

Figure 2 shows the UV−vis spectra recorded while stepping the potential through the reduction wave in a thin-layer spectroelectrochemical cell with a platinum grid electrode. The isosbestic points at 301, 412, and 467 nm demonstrate a clean transformation of Ni^{II}F430M into a single reduced species that exhibits a UV−vis spectrum indistinguishable from that observed after chemical reduction with NaHg in THF[20] or ZnHg in dimethylformamide (DMF). This spectrum is also observed for active methyl-coenzyme M reductase in the MCR_{red1} form and, although with a slight shift of the band at long wavelength from 750 to 710 nm, for coenzyme F430 (**1**) after reduction by Ti^{III}citrate in aqueous solution.[21] Since this band is most likely due to a charge-transfer transition, some variation of its position with solvent is to be expected. The position of this band in MCR_{red1}[29] is actually closer to that of reduced **2** in acetonitrile or THF than to that of reduced **1** in water,[21] in keeping with the highly hydrophobic environment of F430 in the enzyme as revealed by the X-ray structures.[9]

The results of bulk electrolysis experiments in acetonitrile or propionitrile with 0.1 M $TBAPF_6$ are summarized in Table 1. In each of the three experiments, the net charge transferred to Ni^{II}F430M was within 10% of 1 F/mol, which proves that, under these conditions, the reduction is a one-electron process. The UV−vis and EPR spectra of the reduced species are shown in Figures 3 and 4, respectively. The UV−vis spectrum of the electrochemically reduced form in propionitrile or acetonitrile with 0.1 M $TBAPF_6$ shows the same strong bands at 269 and 380 nm and the characteristic weak band at 750 nm as the spectrum reported earlier for the product of chemical reduction

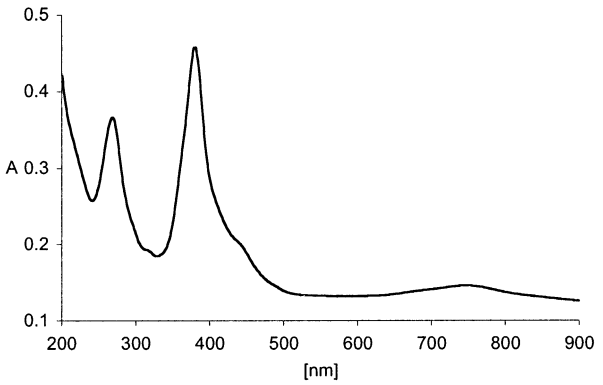

Figure 3. UV−vis spectrum of Ni^{I}F430M generated by bulk electrolysis in dry acetonitrile/0.1 M $TBAPF_6$ (l = 0.03 cm).

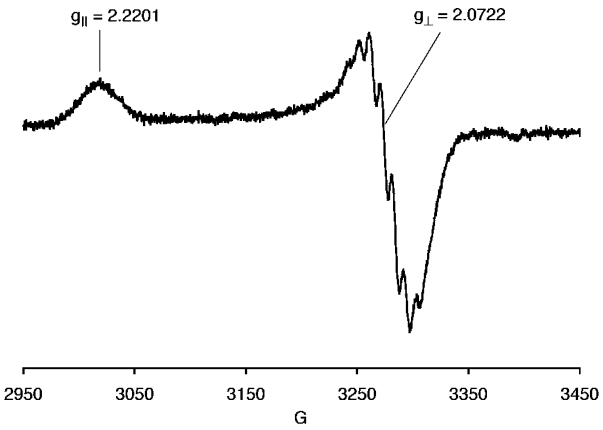

Figure 4. X-band EPR spectrum of Ni^{I}F430M generated by electrolysis in acetonitrile/0.1 M $TBAPF_6$ (frozen solution, T = 130 K; microwave frequency, 9.512 GHz; microwave power, 2.0 mW; field modulation amplitude, 5.00 G; modulation frequency, 100.00 kHz; 10 accumulations).

of Ni^{II}F430M by NaHg in THF. The $g_{||}$ and $g_{\perp}$ values in the EPR spectrum (Figure 4) are within experimental error the same as for the NaHg/THF reduced sample reported earlier, reduced **1** in water and MCR_{red1}. The superhyperfine splitting of the $g_{\perp}$ line due to the four ^{14}N nuclei of the macrocycle is much better resolved in the spectrum obtained from the electrolysis experiment than in any other spectrum of a free Ni^{I}F430 derivative reported so far. Interestingly, a similarly well-resolved EPR spectrum was recently observed for the MCR_{red1m} form of the enzyme (MCR_{red1} in the presence of methyl-coenzyme M but in the absence of coenzyme B).[29]

2. Stoichiometric Titration of Ni^{II}F430M with Decamethylcobaltocene. In view of the highly negative potential of the Ni(II)/Ni(I) couple of coenzyme F430 and because, at sufficiently negative potentials, the macrocyclic π-system must eventually be reduced as well, the choice of suitable chemical reducing agents to cleanly generate the Ni(I) form is limited. By cyclic voltammetry in acetonitrile, we sometimes observed a second, irreversible reduction wave just before the onset of electrolyte reduction (E_{pc} ca. −2.4 V), which we tentatively assign to reduction of the macrocycle chromophore of the Ni(I) form. In our hands, NaHg in solvents such as THF and acetonitrile gives clean reduction to Ni(I) if the contact time is carefully controlled. Prolonged contact, however, leads to over-reduction under attack of the ligand chromophore. Liquid zinc amalgam (1.5% w/w), on the other hand, does reduce

(29) Mahlert, F.; Grabarse, W.; Kahnt, J.; Thauer, R. K.; Duin, E. C. *J. Biol. Inorg. Chem.* **2002**, *7*, 101−112.

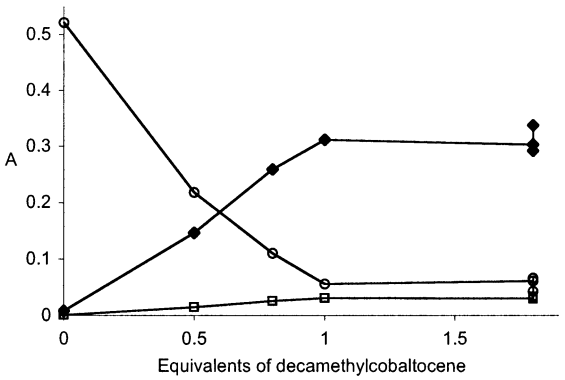

Figure 5. UV−vis absorbance of $Ni^{II}F430M$ at 440 nm (○), and of $Ni^{I}F430M$ at 755 nm (□, ◆ × 10) as a function of added equivalents of decamethylcobaltocene, in THF (l = 0.1 cm). The data points at 1.8 equiv were obtained in three parallel experiments.

$Ni^{II}F430M$ in DMF cleanly to the Ni(I) form without overreduction, even after several days of contact at room temperature. However, we have not been able to use ZnHg in any nonhydroxylic solvent other than DMF because, with all other solvents tested, liquid 1.5% ZnHg separated into Zn powder and Hg. Reduction with an excess of cobaltocene in THF afforded only ca. 2% reduction because its potential is slightly less negative than that of the Ni(II)/Ni(I) couple of F430M as determined by cyclic voltammetry under identical conditions.

Decamethylcobaltocene [$(Cp^*)_2Co$] has a potential that is 0.56 V more negative than that of cobaltocene[30] and proved to be a reductant ideally suited for reduction of $Ni^{II}F430M$ without overreduction. The results of a titration of $Ni^{II}F430M$ in THF with increasing aliquots of a decamethylcobaltocene solution [generated from a standard solution of $(Cp^*)_2CoPF_6$ in THF by reduction with 0.87% NaHg] are displayed in Figure 5. Whereas the bands of $(Cp^*)_2Co$, $(Cp^*)_2Co^+$, and $Ni^{I}F430M$ overlap in the region below 400 nm, the bands of $Ni^{II}F430M$ at 440 nm and $Ni^{I}F430M$ at 750 nm are isolated and were used to determine the stoichiometry of the reaction. The observed absorbance changes were proportional to the added mole equivalents of $(Cp^*)_2Co$ up to 1 mol equiv. Between 1.0 and 1.8 mol equiv of $(Cp^*)_2Co$, however, the absorbance at 440 and 750 nm did not change within experimental error. Since decamethylcobaltocene is a one-electron reductant, this shows that only one electron is taken up by $Ni^{II}F430M$ upon generation of the reduced species exhibiting the spectrum shown in Figure 3. Acidic aqueous workup in the air and characterization of the reoxidized material by UV−vis and TLC proved that it was identical with the $Ni^{II}F430M$ used as starting material, allowing us to exclude any nonreversible change in the structure of the macrocycle upon reduction.

3. Stoichiometry of the Reoxidation of $Ni^{I}F430M$ with Methylene Blue. When $Ni^{II}F430M$ in DMF was completely reduced over ZnHg, separated from the amalgam, and oxidized back with an equimolar aliquot of standard solution of the two-electron oxidant[31] methylene blue in DMF, the remaining absorbance of the band of methylene blue at 665 nm corresponded to a consumption of 0.46 ± 0.05 mol equiv of methylene blue/mol of $Ni^{I}F430M$ (see Figure 6).

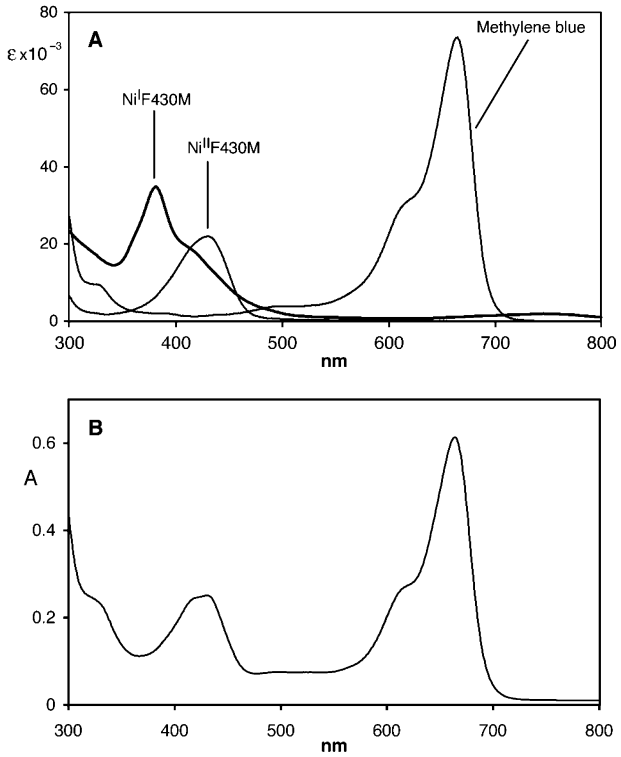

Figure 6. UV−vis spectra in DMF (l = 0.1 cm) of (A) methylene blue (oxidized from), $Ni^{I}F430M$ (bold line), and $Ni^{II}F430M$; (B) result of equimolar reaction between $Ni^{I}F430M$ and methylene blue.

Conclusion

The three experiments described above consistently and independently prove that *a one-electron reduction* of the corresponding Ni(II) form of the coenzyme (such as present in the "silent" forms of the enzyme) generates the species exhibiting the UV−vis and EPR spectra characteristic for the active MCR_{red1} state of methyl-coenzyme M reductase. This observation is inconsistent with a concomitant reduction of the macrocycle by two additional electrons as reported by Tang et al..[28]

The fact that these experiments were done with the pentamethyl ester **2** rather than with the native pentacarboxylate **1** has no bearing on this conclusion because the original assignment of MCR_{red1} to an enzyme form containing coenzyme F430 with nickel in the +1 valence state without changes in the macrocycle constitution rests on the close similarity of the UV−vis and EPR spectra of MCR_{red1} to those of both $Ni^{I}F430M$ in organic solvents and $Ni^{I}F430$ in water.

Since the proposed additional reduction of the hydrocorphinoid chromophore, which would have elegantly explained why reduction by Ti(III) is needed to apparently generate one Ni(I) form (MCR_{red1}) from another one (MCR_{ox1}), must now be ruled out, the assignment of the Ni(I) valence state to MCR_{ox1} may have to be reconsidered. Whereas the EPR and ENDOR data of MCR_{ox1} clearly show that it is a $S = {}^1/_2$ species with a predominant $d_{x^2-y^2}$ configuration,[23] its UV−vis and X-ray absorption (XAS) spectra are more similar to those of the "silent" Ni(II) forms than to those of MCR_{red1}.[25] Considering the fact that the known Ni(III) form of F430M in the weakly coordinating solvent acetonitrile[32] has a d_{z^2} configuration, the EPR spectrum of MCR_{ox1} would only be compatible with a

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(31) Zutic, V.; Svetlicic, V.; Lovric, M.; Ruzic, I. *J. Electroanal. Chem.* **1984**, *177*, 253−268.
(32) Jaun, B. *Helv. Chim. Acta* **1990**, *73*, 2209−2217.

formal Ni(III) valence state if at least one strong donor ligand occupying an axial position would induce a change of the ground-state configuration from d_{z^2} to $d_{x^2-y^2}$. Extended X-ray absorption fine structure (EXAFS) results[25] and the generation of MCR_{ox1} by low temperature γ-irradiation from $MCR_{ox1-silent}$,[24] which has the thiol(ate) sulfur of coenzyme M coordinated in the axial position, indicate that this axial ligand would have to be coordinating to the nickel center via sulfur.

Experimental Section

1. Materials. $Ni^{II}F430M{\cdot}ClO_4$ (**2**) was prepared from coenzyme F430 that had been extracted from cells of *Methanothermobacter marburgensis* as described earlier.[1] Traces of halogenated solvents (which react very rapidly with $Ni^{I}F430M$) and of water were eliminated from F430M samples by 3-fold precipitation from dry THF with dry toluene. The sample was then dried at 10^{-3} mbar overnight. All experiments were done with material from a single batch of $Ni^{II}F430M{\cdot}ClO_4$. The concentrations and mole equivalents for **2** are based on photometry at 430 nm in methanol ($\epsilon = 22\,000$).[1] This molar extinction coefficient was redetermined with the batch used in our experiments by determination of the nickel content with ICP-MS.

Tetrabutylammonium hexafluorophosphate ($TBAPF_6$, Fluka) was triply recrystallized from EtOAc/EtOH (2:1), dried at rt for 3 days at 10^{-3} mbar, and dried again overnight before use. $(Cp^*)_2CoPF_6$ (Aldrich) was used without further purification. Methylene blue (Fluka) was dried at rt and 10^{-3} mbar. Its extinction coefficient in DMF was determined as $\epsilon = 73\,500$ at $\lambda_{max} = 665$ nm. Alumina (alumina N, Super I; ICN Biomedicals) was activated for 24 h at 150 °C and 10^{-3} mbar and stored under a nitrogen atmosphere. THF was triply distilled under nitrogen from potassium and then degassed by three freeze−pump−thaw cycles. DMF was freshly distilled under vacuum at ca. 10 mbar and 40 °C over a fractionating column (110 cm) packed with glass beads, using a reflux ratio of 10:1 and taking the middle 30%, degassed by three freeze−thaw cycles, and stored in a drybox (Vacuum Atmospheres). All other chemicals were obtained from Fluka and were used without further purification.

NaHg Amalgam 0.87% (w/w). Sodium (120 mg) was added to mercury (1 mL, 13.6 g) placed in a 50 mL flask under nitrogen. The piece of sodium was dipped below the mercury surface, whereby the amalgam formed spontaneously.

ZnHg amalgam 15% and 1.5% (w/w). In a two-necked flask a mixture of mercury (ca. 9 g, 0.67 mL, 45 mmol) and zinc powder (1.3 g, 20 mmol) was covered with 5% acetic acid (ca. 5 mL) and heated to the boiling point under nitrogen while the flask was shaken manually until the metal mixture had become homogeneous. After cooling to rt the amalgam solidified. The aqueous phase was removed and the amalgam was rinsed several times with water and then stored under 2% acetic acid. Liquid 1.5% ZnHg was prepared from 15% ZnHg by dilution immediately before use. To 10 g of 15% ZnHg covered with 2% acetic acid mercury (90 g, 6.7 mL) was added and the mixture was treated as described for 15% ZnHg. The liquid ZnHg was washed several times with water, then with methanol, and dried in a stream of nitrogen.

UV−vis spectroscopy was performed on a Perkin-Elmer λ 20 spectrophotometer.

2. Cyclic Voltammetry and Bulk Electrolysis of $Ni^{II}F430M$. All potentials are given vs the ferrocenium/ferrocene (Fc^+/Fc) couple measured in situ together with the analyte.[33]

Apparatus. A BAS 100W (Bioanalytical Systems) electrochemistry system, with cyclic voltammetry (CV) and bulk electrolysis modes, was used. The cell was inside a drybox (O_2 < 4 ppm) with the electrode cables connected via a plug in the wall of the drybox to the external potentiostat. A four-compartment electrolysis cell with two bridging compartments between the working electrode and counterelectrode compartments was used in order to minimize diffusion between the counterelectrode and working electrode (volume of each compartment ca. 2.0−2.5 mL, D5-glass fritted disks as diaphragms). The working electrode for bulk electrolysis and the counterelectrode were semicircular platinum grid electrodes (ca. 8 × 10 mm, 0.5 mm mesh); an additional small Pt wire-tip electrode (3 mm, ø = 0.3 mm) was used for the CV experiments. A silver wire coated with AgCl (anodized in 0.1 M HCl) inside a Luggin capillary served as the quasi reference electrode.

Bulk Electrolysis. $TBAPF_6$ (0.774 g) was dissolved in acetonitrile or propionitrile (20 mL) freshly filtered over activated alumina. The electrolyte solution was degassed by three freeze−pump−thaw cycles and transferred under vacuum into the drybox. Inside the drybox, F430M (2.0 μmol) was dissolved in the electrolyte to give a 1.0 mM solution, the electrolysis cell was filled with electrolyte solution and, in the working electrode compartment, with F430M solution, and the cyclic voltammogram was recorded with the Pt-wire working electrode (conditions for CV as given in the caption of Figure 1). Bulk electrolysis was carried out with vigorous stirring by a magnetic stir bar inside the working electrode compartment. The potentials were set as given in Table 1, and electrolysis was continued until the current had dropped to 1% of its initial value. The net charge transferred was determined by current integration and was corrected by subtraction of the background charge transfer as determined by a second experiment with pure electrolyte solution under otherwise identical conditions (always <10% of the charge transferred to $Ni^{II}F430M$). After electrolysis, aliquots of the solution in the working electrode compartment were transferred by a gastight syringe to a UV−vis cell ($l = 0.03$ cm) and two EPR tubes, which were capped with septum caps and taken out of the drybox where the EPR tubes were immediately frozen in liquid N_2 and the UV−vis and EPR spectra were recorded. X-Band EPR spectra were taken on a Bruker EMX with N_2 flow cryostat set to 130 K. Conditions: see caption to Figure 4. The *g*-values were measured with a NMR gaussmeter and calibrated by co-measurement of solid DPPH ($g = 2.0037$) sealed in a quartz capillary (0.5 mm) that was attached to the outside of the EPR tube.

3. Spectroelectrochemistry of the reduction of $Ni^{II}F430M$ (2) was measured in a home-built two-compartment spectroelectrochemistry cell (Suprasil quartz, $l = 0.03$ cm) with a fine Pt mesh electrode (optical density = ca. 0.11). The reference cell contained pure electrolyte and an identical Pt mesh. The counterelectrode compartment was separated from the working electrode compartment by a D4-fritted glass disk. The counterelectrode was a Pt wire; the quasi-reference electrode was Ag/AgCl (0.1 mm, isolated by a Teflon capillary) reaching to the bottom of the UV−vis cell. The BAS 100W potentiostat was used for stepwise electrolysis. The cell was dried in an oven before use. $TBAPF_6$ (0.387 g) was dried at 10^{-3} mbar overnight, flushed with nitrogen, and dissolved in acetonitrile (10 mL) freshly filtered over activated alumina inside a glovebox. The cell was mounted in the glovebox, closed with septum caps, flushed with argon, filled with electrolyte and F430M (**2**) (ca. 1 μmol) solutions, and degassed with a stream of argon through a fine needle for 10 min. With the cell in the optical path of the spectrophotometer, electrolysis was started at a potential of −1.275 V and then the potential was decreased stepwise to −1.48 V. The reduction was followed by means of UV−vis spectroscopy.

4. Stoichiometry of the Reduction of $Ni^{II}F430M$ (2) by Decamethylcobaltocene in THF. All reactions were carried out in a drybox (O_2 < 8 ppm). Experiment 1: $(Cp^*)_2CoPF_6$ (1.255 mg, 2.645 μmol) was dissolved in dry THF (5.00 mL), NaHg amalgam (0.87%, ca. 0.5 mL) was added and the mixture was stirred for 3 h. $Ni^{II}F430M$ (0.425 mg, 0.386 μmol) was dissolved in dry THF (1.30 mL) and 150 μL (0.44 pmol) of the solution was placed in every one of three UV−vis cells, to which 150 μL of the obtained $(Cp^*)_2Co$ (0.79 pmol, 1.8 equiv) solution was added. Experiment 2: $(Cp^*)_2CoPF_6$ (0.710 mg, 1.50 μmol) was dissolved in dry THF (6.50 mL), NaHg amalgam (0.87%, ca. 0.5

(33) Gritzner, G.; Kûta, J. *Pure Appl. Chem.* **1984**, *56*, 462−466.

mL) was added, and the mixture was stirred for 3 h. Ni^{II}F430M (0.595 mg, 0.54 μmol) was dissolved in dry THF (1.80 mL) and solutions both of the coenzyme and of the obtained $(Cp^*)_2Co$ were mixed in UV−vis cells as follows: (1) 150 μL (0.45 pmol) of Ni^{II}F430M and 100 μL (0.23 pmol, 0.5 equiv) of $(Cp^*)_2Co$; (2) 150 μL (0.45 pmol) of Ni^{II}F430M and 150 μL (0.35 pmol, 0.8 equiv) of $(Cp^*)_2Co$; (3) 120 μL (0.36 pmol) of Ni^{II}F430M and 160 μL (0.37 pmol, 1.0 equiv) of $(Cp^*)_2Co$.

The cells were closed with septum caps and taken out of the drybox, and the UV−vis spectra were measured within 20 min. Spectra of solutions of Ni^{II}F430M, $(Cp^*)_2Co$, and $Cp^*_2Co^+$ [obtained by reoxidation of the $(Cp^*)_2Co$ solution with air] were also recorded.

After the experiment, all Ni^{I}F430M samples were combined, treated with 0.1 M $NaClO_4$/0.01 M $HClO_4$, and extracted with chloroform; the organic layer was evaporated at reduced pressure and the residue was analyzed by UV−vis spectroscopy in methanol and TLC on silica gel 60 with 2-propanol−ammonia (4:1) as a mobile phase to confirm that the recovered material was identical to the starting material Ni^{II}F430M.

5. Stoichiometry of Reoxidation of Ni^{I}F430M by Methylene Blue in DMF. The whole experiment was carried out in a drybox (O_2 < 8 ppm). Ni^{II}F430M (0.660 mg, 0.600 μmol) was dissolved in DMF (2 mL) and vigorously shaken for ca. 15 min with 1.5% ZnHg amalgam (ca. 0.5 mL). Methylene blue (0.432 mg, 1.350 μmol) was dissolved in DMF (5 mL), which had been stirred for 2 h over 1.5% ZnHg in order to destroy any residual electrophiles/oxidants. In each of five UV cells, 130 μL (0.39 pmol) of Ni^{I}F430M solution and 145 μL (0.39 pmol, 2 equiv) of methylene blue were mixed, the cells were closed with septa and taken out of the drybox, and the UV−vis spectra were recorded immediately. UV−vis spectra of the starting Ni^{II}F430M solution, of Ni^{I}F430M after reduction with ZnHg, and of the methylene blue solution were obtained as controls. To one of the five cells containing reoxidized F430M and excess methylene blue, ca. 80 μL of 1.5% ZnHg was injected through the septum cap, and the cell was vigorously shaken. This reduced both the methylene blue in excess and Ni^{II}F430M and quantitatively restored the original spectrum of Ni^{I}F430M.

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Supporting Information Available: Charge vs time curve for electrolysis of Ni^{II}F430M, UV−vis spectra of the titration of Ni^{II}F430M with $(Cp^*)_2Co$, and tables with aliquots of stock solutions used for the reactions of Ni^{I}F430M with methylene blue and Ni^{II}F430M with $(Cp^*)_2Co$. This information is available free of charge via the Internet at http://pubs.acs.org.

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