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Reactivity of [1,4,7,10,13-Pentaazacyclohexadecane-14,16-dionato(2-)]nickel(II) toward Derivatives of Methyl-Coenzyme M

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The reactivity of [1,4,7,10,13-pentaazacyclohexadecane-14,16-dionato(2-)]nickel(II), $Ni^{II}L$, toward $H_3CSCH_2CH_2SO_3^-$, methyl-coenzyme M, possesses striking similarities to the active site chemistry of methyl-coenzyme M reductase, which contains the nickel tetrapyrrole species F_{430}. The initial rate of substrate conversion for a series of substrate analogues of the formula $RX(CH_2)_nY^-$ is reported, where the R and X groups have the greatest influence on reactivity. When $R = CH_3$ and $X = S$, little change in reactivity is observed when $n = 2$, 3, or 6 or when $Y^- = SO_3^-$, CO_2^-, or PO_3^-. These results are consistent with the two-site model for substrate binding at F_{430}, the proposed enzyme active site.

Introduction

Barker first proposed that carbon dioxide metabolism by methanogenic bacteria proceeds through a series of four two-electron reduction steps.[1] The conversion of CO_2 to methane involves several unusual coenzymes and related components[2] including methyl-coenzyme M reductase,[3] which contains the nickel tetrapyrrole containing factor,[4] F_{430}, shown in Figure 1. This enzyme catalyzes the formation of methane from $H_3CSCH_2CH_2SO_3^-$, methyl-coenzyme M, during the final methane evolution step during CO_2 metabolism.[1,2,5] The essential role of F_{430} in methane formation was demonstrated by Ankel-Fuchs and Thauer, who reported the in vitro catalysis of $H_3CSCH_2CH_2SO_3^-$, methyl-coenzyme M, to methane and coenzyme M by purified methyl-coenzyme M reductase under reducing conditions.[6] Since F_{430} exists in both the Ni(I) and Ni(II) state in *Methanobacterium thermoautotrophicum*,[6] the role of the nickel oxidation state in methyl-coenzyme M catalysis is of interest.

In a recent communication we reported that [1,4,7,10,13-pentaazacyclohexadecane-14,16-dionato(2-)]nickel(II),[7] $Ni^{II}L$, shown in Figure 1, converts methyl-coenzyme M to methane and coenzyme M in a stoichiometric process.[8] The addition of an oxidant such as I_2 or NaClO makes the reaction catalytic in nickel ion. Since aqueous solutions of nickel(II) acetate, nickel(II) tetraethylenepentamine, or nickel(II) 1,4,8,11-tetraazacyclotetradecane-5,7-dionate(2-) do not convert methyl-coenzyme M to methane under similar conditions, the reactivity of $Ni^{II}L$ does not typify nickel(II) chemistry. The ligand (L) may activate Ni(II) toward methyl-coenzyme M, since the aqueous solution Ni(II/III) redox couple of this complex is one of the lowest reported values in the literature (0.48 V vs NHE).[7] The identification of nickel complexes capable of generating methane from methyl-coenzyme M has proved difficult, and nickel coordination complexes used as spectroscopic models for the enzyme active site have no demonstrated reactivity toward the natural substrate.[9] Thus, $Ni^{II}L$ is a potential model for the active site chemistry of NiF_{430}. We report a series of experiments that examine the mechanism of substrate cleavage and analyze our results in terms of the proposed mechanism in Figure 2.

Experimental Section

Materials and Methods. All chemicals (Aldrich) and solvents were purified as stated or according to literature procedures. Distilled water was used in all synthesis and catalytic reactions. NMR spectra were collected on a Bruker multinuclear 300 MHz or a Varian AM 360 spectrometer. Vibrational spectra were obtained on an IBM IR-90 FT instrument using either a 9 cm path length cell with KBr windows and a vacuum stopcock or a 17-cm cell fitted with IRTRAN-2 windows and a vacuum stopcock. UV-visible spectra were obtained on an IBM 9400-UV/Vis spectrometer. GC analysis of the reaction gas was performed on a Hewlett-Packard 5750 GC fitted with a glass column ($^1/_8$ in. × 8 ft) packed with Chromosorb 104, a Hastalloy C rotary valve, and a flame ionization detector. Mass spectra of gas samples were obtained either by injecting samples with a gastight syringe into a Hewlett-Packard HP-5988A GC-MS fitted with a Supelco SP-1000 30-m capillary column or by using the service of the MIT Chemistry Spectroscopy Facility where the sample was inserted into the argon inlet port of a FAB mass spectrometer. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratories. All coenzyme M derivatives were made as the ammonium salt unless otherwise indicated, and their purity was assayed by ^1H NMR spectroscopy.[11]

Kinetic Studies. In a typical reaction $Ni^{II}L$ (0.267 mmol) in 20 mL of deoxygenated distilled H_2O was placed into a two-neck round-bottom

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(5) (a) Walsh, C. T.; Orme-Johnson, W. H. *Biochemistry* **1987**, *26*, 4901. (b) Rouviere, P.; Wolfe, R. S. *Novel Biochemistry of Methanogens*, in press. (c) Daniels, L.; Zeikus, J. G. *J. Bacteriol.* **1978**, *136*, 75.
(6) (a) Jaun, B.; Pfaltz, A. *J. Chem. Soc., Chem. Commun.* **1986**, 1327. (b) Albracht, S. P. J.; Ankel-Fuchs, D.; van der Zwaan, J. W.; Fontijn, R. D.; Thauer, R. K. *Biochim. Biophys. Acta* **1986**, *870*, 50. (c) Ankel-Fuchs, D.; Thauer, R. K. *FEBS Lett.* **1986**, 171. The rate in the purified methyl-coenzyme M reductase system is 6×10^{-3} (mol of CH_4) (g of protein)$^{-1}$ h^{-1}. (d) Albracht, S. P. J.; Ankel-Fuchs, D.; Böcher, R.; Ellermann, J.; Moll, J.; van der Zwaan, J. W.; Thauer, R. K. *Biochim. Biophys. Acta* **1988**, *955*, 86. This paper reports several EPR signals attributed to F_{430}. The EPR signals of Ni(I) and Ni(III) are expected to be very similar.
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(9) Juan, B.; Pfaltz, A. *J. Chem. Soc., Chem. Commun.* **1988**, 293. If the reaction rate of the pentamethyl ester of Ni^IF_{430} is similar to that of $Ni^{II}L$, it is possible that insufficient methane would be generated to be detected.
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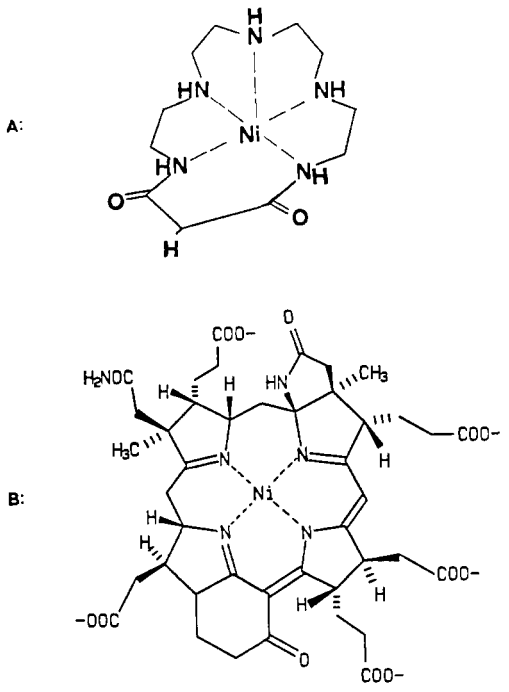

Figure 1. Structure of (A) $Ni^{II}L$ and (B) F_{430}.

$$LNi(II) + CH_3\text{-}S\text{-}CoM \rightleftharpoons LNi(II)S(CH_3)CoM \quad (1)$$

$$H_2O + LNi(II)S(CH_3)CoM \rightarrow LNi(III)\text{-}S\text{-}CoM + CH_4 + OH^- \quad (2)$$

$$LNi(III)\text{-}S\text{-}CoM + OH^- \rightarrow {}^1/_2O_2 + LNi(II) + HS\text{-}CoM \quad (3)$$

$$LNi(II) + HS\text{-}CoM \rightleftharpoons LNi(II)S(H)CoM \quad (4)$$

$${}^1/_4O_2 + HS\text{-}CoM \rightarrow {}^1/_2H_2O + {}^1/_2(CoM\text{-}S\text{-}S\text{-}CoM) \quad (5)$$

$$CH_3\text{-}S\text{-}CoM + {}^1/_2H_2O \rightarrow CH_4 + {}^1/_4O_2 + {}^1/_2(CoM\text{-}S\text{-}S\text{-}CoM) \quad (6)$$

$$HS\text{-}CoM + I_2 \rightarrow 2HI + {}^1/_2(CoM\text{-}S\text{-}S\text{-}CoM) \quad (7)$$

Figure 2. Proposed mechanism for substrate cleavage by $Ni^{II}L$ (Scheme I). HS-CoM is coenzyme M, $HSCH_2CH_2SO_3^-$. The overall charge on the nickel complexes is unknown and has been omitted.

flask equipped with a magnetic stir bar and a rubber septum, and the flask was connected to a gas-uptake manometer. The reaction was initiated upon the addition of methyl coenzyme M (0.800 mmol) to the reaction vessel under a heavy flow of argon. The amount of product formed was calculated from the gas-uptake manometer and compared favorably (±4%) to an analysis of the components present in the reaction mixture. The reaction mixture was freeze-dried and dissolved in D_2O, and its ^1H NMR spectrum was integrated. The methyl and ethylene peaks of the reaction mixture indicate the substrate to product ratio. The gas-phase products were collected in an evacuated 200-mL glass bulb fitted with two airtight valves and analyzed by FT-IR spectroscopy, GC, or GC–MS.

1,4,7,10,13-Pentaazahexadecane-14,16-dione (L) was prepared according to the literature procedure[8] with appropriate modifications[13] in 10% yield. Anal. Calcd for $C_{11}H_{23}N_5O_2{\cdot}H_2O$: C, 47.98; H, 9.14; N, 25.43; O, 17.45. Found: C, 48.02; H, 8.72; N, 24.8; O, 18.46 (by difference). Mp: 173–174 °C.[8] ^1H NMR ($CDCl_3$): δ 7.90 (s, 2 H, amide), 3.30 (m, 4 H, amide ethylene), 3.20 (s, 2 H, malonyl methylene), 2.85 (m, 12 H, ethylene), 2.00 (b s, 3 H, amine). ^1H NMR (D_2O): δ 3.2 (m, 4 H, amide ethylene), 2.6 (m, 4 H, amide ethylene), 2.5 (b s, 8 H, ethylene). The malonyl methylene was found to exchange with the solvent. ^{13}C NMR ($CDCl_3$): δ 169.0 (amide carbonyl), 49.0 (amide ethylene), 48.5 (ethylene), 48.0 (ethylene), 43.0 (ethylene), 38.5 (malonyl methylene).

[1,4,7,10,13-Pentaazacyclohexadecane-14,16-dionato(2−)]nickel(II) ($Ni^{II}L$). $Ni^{II}(OAc)_2{\cdot}H_2O$ (0.406 g, 1.63×10^{-3} mol) was added under a vigorous flow of argon to a stoichiometric amount of 1,4,7,10,13-pentaazacyclohexadecane-14,16-dione (0.420 g, 1.63×10^{-3} mol) dissolved in 30 mL of deoxygenated EtOH and stirred at reflux for 2 h. The resulting green solution was cooled to room temperature and rotoevaporated to a green solid and the solid was dried in vacuo for 12 h. The nickel complex was recrystallized from 10 mL of EtOH by adding 50 mL of dry Et_2O. Pure $Ni^{II}L$ (0.53 g) was filtered out and dried in vacuo. Yield: 93%. Solid $Ni^{II}L$ is hygroscopic, and dilute $Ni^{II}L$ aqueous solutions decompose over a period of days. $Ni^{II}L$ was stored in an inert-atmosphere box or in a desiccator after sealing the vial under argon. Anal. Calcd for $C_{11}H_{21}N_5O_2Ni{\cdot}H_2O$: C, 39.78; H, 6.99; N, 21.09; Ni, 16.82; O, 15.32. Found: C, 40.10; H, 6.90; N, 20.30; Ni, 17.04; O, 15.66 (by difference). FAB mass spectrum [*m/e* (relative intensity ion)]: 332 (6800, M^+), 333 (1800, M + 1). UV–visible spectrum, pH 9.5 borate buffer [nm (ϵ)]: 260, 290 (1664), 340 (120). Solution magnetic moment μ = 2.6 μ_B/Ni by the Evans method.[14]

Coenzyme M Disulfide. An aqueous solution of coenzyme M (0.5 g, 3 mmol) was titrated with I_2-saturated EtOH until a yellow color persisted. The water was stripped and the resulting white solid recrystallized from hot water and acetone. Yield: 95%. ^1H NMR (D_2O): δ 3.13 (m, 4 H), 2.88 (m, 4 H).

Methyl-Coenzyme M.[11] 2-Mercaptoethanesulfonic acid sodium salt (0.50 g, 3 mmol) was dissolved in 125 mL of NH_4OH(aq), and the solution was deoxygenated with argon for 15 min and cooled to 0 °C before methyl iodide (0.566 g, 4 mmol) was syringed into the reaction vessel. The solution was stirred for 12 h in the dark and the solvent stripped by rotoevaporation to yield a white solid. This solid was twice recrystallized from a minimum amount of H_2O upon slow addition of acetone and then dried in vacuo: 90–95% pure. Additional recrystallization resulted in 99+% methyl-coenzyme M at 85% yield. ^1H NMR (D_2O): δ 2.97 (m, 2 H), 2.67 (m, 2 H), 1.95 (s, 3 H). The solid was stored under argon to prevent oxidation of the thioether.

Alkyl-Coenzyme M. Alkyl-coenzyme M derivatives were made according to the procedure for methyl-coenzyme M with the following modifications: allyl-coenzyme M was formed by stoichiometric addition of allyl iodide to coenzyme M. Allylamine was removed by extracting the aqueous solution with $CHCl_3$. The product was 90% pure by ^1H NMR spectroscopy in 85% yield. ^1H NMR (D_2O): δ 5.70 (m, 1 H), 5.05 (d of d, 2 H), 3.12 (d, 2 H), 3.05 (m, 2 H), 2.69 (m, 2 H). ^{13}C NMR (D_2O): δ 154, 138, 71, 53, 44. *n*-Propyl-coenzyme M was obtained 90% pure in 83% yield. ^1H NMR (D_2O): δ 3.05 (m, 2 H), 2.65 (m, 2 H), 2.48 (t, 2 H), 1.5 (m, 2 H), 0.88 (t, 3 H). ^{13}C NMR (D_2O): δ 71, 53, 46, 42, 32. Cyclopropylmethyl-coenzyme M was obtained from cyclopropylmethyl bromide, 92% pure in 82% yield. ^1h NMR (D_2O): δ 3.05 (m, 2 H), 2.83 (m, 2 H), 2.42 (d, 2 H), 0.9 (m, 1 H), 0.45 (m, 2 H), 0.1 (m, 2 H). ^{13}C NMR (D_2O): δ 71, 56.5, 45.5, 30.5, 25, 23. Cyclobutyl-coenzyme M was obtained by adding coenzyme M to bromocyclobutane to produce a yellow solution after several minutes. The reaction was stirred in the dark at room temperature for 24 h, brought to reflux for 30 min, cooled, and rotoevaporated to a crude solid. The solid was purified with 500μ preparative cellulose chromatography plates eluting with 3.5:1:1 methanol/dioxolane/NH_4OH(aq) to yield 0.32 g of pure 2-(cyclobutylthio)ethanesulfonate. ^1H NMR (D_2O): 3.05 (m, 2 H), 2.8 (m, 2 H), 1.85 (m, 1 H), 0.95 (m, 2 H), 0.25 (m, 4 H). ^{13}C NMR (D_2O): 62, 47, 35, 20, 13.

Trifluoromethyl-Coenzyme M.[11] Trifluoromethyl iodide (12 g, 61 mmol) was condensed into a solution of coenzyme M (1.00 g, 6 mmol) in 20 mL of deoxygenated NH_4OH(aq) cooled by a dry ice/CCl_4 bath in a vessel connected to a gas bubbler. The solution was irradiated with UV light (200 W Xe/Hg lamp) for 2 h at −29 °C. The solution was stirred for an additional 2 h and warmed slowly to 0 °C. The entire procedure was repeated with additional F_3CI to increase the yield, and the solvent was stripped by rotoevaporation to produce a white solid. ^1H NMR spectroscopy indicated about 15% trifluoromethyl-coenzyme M was present. The products were separated by preparative silica gel plates eluted with acetone. The top band was removed, extracted, rotoevaporated, and purified by preparative cellulose chromatography plates, eluted with 3.5:1:1 methanol/dioxolane/NH_4OH(aq). The product was dried in vacuo and was 98% pure in 16% yield based on starting coenzyme M. ^1H NMR (D_2O): δ 3.2 (m, 2 H), 3.0 (m, 2 H). ^{13}C NMR (D_2O): 68, 41, 28. ^{19}F NMR (D_2O; shift reported versus 2,2,2-trifluoroethanol): 32 ppm.

2-Difluoromethyl-Coenzyme M.[11] 2-Mercaptoethanesulfonic acid (2.00 g, 0.012 mol) in 5 mL of dioxane was added to 1.8 g of NaOH in 10 mL of H_2O, and the solution was heated to 60 °C. Difluoromethane (Freon 22, Matheson) was passed through the solution for 5 h, the solution rotoevaporated, and the resulting white solid dissolved in 5 mL of H_2O and shaken with an I_2-saturated hexane solution to convert un-

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reacted coenzyme M to the disulfide. The product was isolated from cellulose (0.897 g, 47% yield). ^{1}H NMR (D_2O): δ 7.10 (t, 1 H, J = 56 Hz), 3.35 (m, 2 H), 3.21 (m, 2 H). ^{19}F NMR (D_2O): δ 91.5 (d, J = 56 Hz).

2-(Dimethylsulfonio)ethanesulfonic Acid. 2-Mercaptoethanesulfonic acid sodium salt (0.50 g, 3 mmol) was dissolved in 125 mL of deoxygenated NH_4OH(aq) at 0 °C, and methyl iodide (2.20 g, 15 mmole) was added. The reaction mixture was stirred for 12 h in the dark. A white solid present when the solvent was removed was twice recrystallized from a minimum amount of distilled water upon slow addition of acetone and dried in vacuo. The air-sensitive product was 90–95% pure. ^{1}H NMR (D_2O): δ 3.3 (m, 2 H), 2.8 (m, 2 H), 2.75 (s, 6 H). ^{13}C NMR (D_2O): δ 66, 45, 42.

2-Methoxyethanesulfonic Acid. 2-Hydroxyethanesulfonic acid (3.00 g, 21 mmol) was dissolved in 20 mL of aqueous ammonia, and the solution was purged with argon for 15 min and cooled to 0 °C. Methyl iodide (3.73 g, 26 mmol) was added and the reaction warmed to room temperature while it reacted for 14 h. The solvent was rotoevaporated and the resulting solid dissolved in a minimum amount of water and precipitated by acetone. The 99% pure 2-methoxyethanesulfonic acid was dried in vacuo. Yield: 80% based on starting 2-hydroxyethanesulfonic acid. ^{1}H NMR (D_2O): δ 3.72 (t, 2 H), 3.3 (s, 3 H), 3.1 (t, 2 H). ^{13}C (D_2O): 62, 53, 45.

2-(Methylamino)ethanesulfonate.[15] 2-Bromoethanesulfonate (11.00 g, 52 mmol) was added to 390 mL of deoxygenated 40% (aq) methylamine solution and the mixture stirred for 10 days in the dark under argon. The solvent was rotoevaporated to form a white solid 88% pure. Recrystallization from EtOH increased purity to 95%. Yield: 4.27 g, 53%. ^{1}H NMR (D_2O): 3.3 (m, 2 H), 3.11 (m, 2 H), 2.61 (s, 3 H).

3-(Methylthio)propanesulfonate. 1-Bromo-3-chloropropane (22.00 g, 0.1397 mol) was added to 215 mL of (2:3) ethanol/water containing sodium sulfite (13.00 g, 0.1031 mol), and the mixture was refluxed 1.5 h, cooled, and reduced to 100 mL by rotoevaporation. Thiourea (6.945 g, 91 mmol) was added to the solution, which was refluxed 45 min. The solvent was removed, NH_4OH(aq) added, and the solution slowly heated to reflux for 2 h. *Caution*! At 60 °C a vigorous reaction can occur. The reaction was cooled and the solvent flashed off. The solid was taken up in a minimum amount of water and the mixture column chromatographed (Dowex 50W-X8, Na^+ form). The eluted solution was chromatographed by silica gel TLC developed with acetone. The fractions containing 3-mercaptopropanesulfonate were reduced to a white solid and dried in vacuo. Yield: 12.5 g (52%). ^{1}H NMR (D_2O): 2.95 (m, 2 H), 2.6 (m, 2 H), 1.9 (m, 2 H). ^{13}C (D_2O): 59, 39, 33.

3-Mercaptopropanesulfonate (0.72 g, 4.2 mmol) was added to 15 mL of deoxygenated NH_4OH(aq) at 0 °C followed by the addition of methyl iodide (0.81 g, 5.6 mmol) and the solution stirred in the dark for 14 h at room temperature. The solvent was removed and the resulting white solid was recrystallized from water/acetone. Yield: 0.53 g, 67%. ^{1}H NMR (D_2O): 2.95 (m, 2 H), 2.6 (t, 2 H), 2.05 (s, 3 H), 1.9 (m, 2 H). ^{13}C NMR (D_2O): 59, 58, 39, 33.

6-(Methylthio)hexanesulfonate. 1-Bromo-6-chlorohexane (10.00 g, 0.050 mol) and Na_2SO_3 (4.66 g, 0.037 mol) were refluxed for 2 h in 77 mL of deoxygenated 3:2 H_2O/EtOH. The solution was reduced to 35 mL, thiourea added (2.47 g, 0.033 mol), and reflux continued for 2 h under argon. The solution was rotoevaporated to form a white solid, 50 mL of NH_4OH(aq) was added, the solution was refluxed for 2 h under argon, and the solvent was removed. The solid was purified by column chromatography (Dowex 50W-X8 Na form). Fractions containing the product were collected and dried (7.12 g, yield 72%). The thiol was methylated according to the procedure given for methyl-coenzyme M (4.83 g, 69% yield). ^{1}H NMR (D_2O): δ 2.7, (t, 2 H), 2.45, (t, 2 H), 1.95 (s, 3 H), 1.25–1.65 (m, 8 H).

2-(Methylthio)propionic Acid.[11] 2-Mercaptopropionic acid (3.65 g, 34 mmol) was added to 20 mL of NH_4OH(aq), and the solution was deoxygenated and cooled to 0 °C. Methyl iodide (5.86 g, 2.57 mL, 41 mmol) was added and the reaction allowed to warm to room temperature in the dark for 14 h. The solvent was stripped in a rotoevaporator to yield 2-(methylthio)propionic acid. One recrystallization of the white solid from water and acetone produces 93.5% pure product. ^{1}H NMR (D_2O): δ 2.75 (m, 2 H), 2.5 (m, 2 H), 2.15 (s, 3 H). ^{13}C (D_2O): 187, 46, 40, 26.

2-(Methylthio)ethanephosphonic Acid. 2-Chloroethanephosphonic acid (1.00 g, 6.9×10^{-3} M) was dissolved in 6.5 mL of deoxygenated 95% ethanol. Thiourea (0.53 g, 6.9×10^{-3} M) was added to the solution, which was refluxed for 8 h. The solvent was removed, 3 mL of NH_4OH(aq) was added, and the mixture was refluxed for 4 h. After the NH_4OH was removed the resulting solid was chromatographed (Sephadex SP-C25 column, NH_4^+ form). The fraction containing the desired product was collected, dried, and recrystallized from acetone/water. The thiol was methylated with methyl iodide. Yield: 0.28 g, 42%. ^{1}H NMR (D_2O): δ 3.05 (m, 2 H), 2.81 (m, 2 H), 1.95 (s, 3 H).

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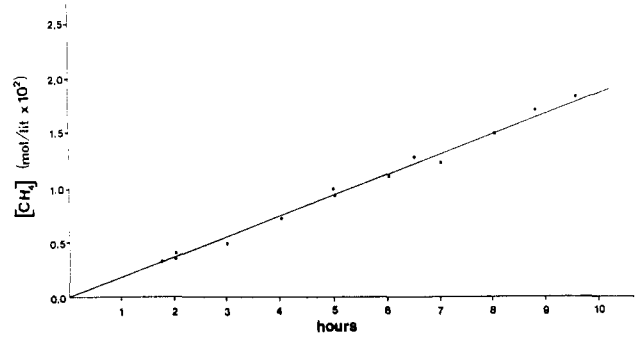

Figure 3. Evolution of CH_4 (mol × 10^2) versus time (h). [$Ni^{II}L$] = 1.36×10^{-2} M and [$H_3CSCH_2CH_2SO_3^-$] = 4.08×10^{-2} M, pH 8.5 at 20 °C.

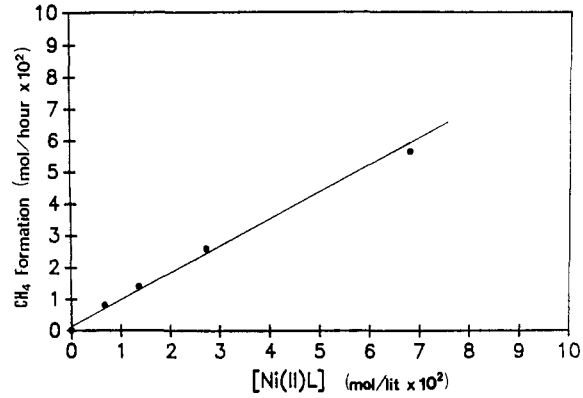

Figure 4. Effect of $Ni^{II}L$ concentration on the initial rate of methyl-coenzyme M cleavage. [$H_3CSCH_2CH_2SO_3^-$] = 1.36×10^{-1} M, pH 8.5 at 20 °C.

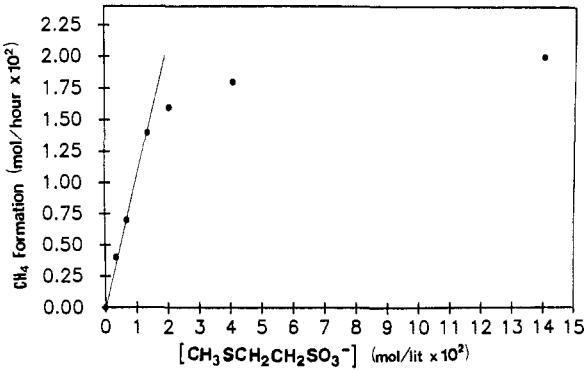

Figure 5. Effect of [$H_3CSCH_2CH_2SO_3^-$] on the initial rate of methane evolution. [Ni(II)L] = 1.36×10^{-2} M, pH 8.5 at 20 °C.

Results

Mechanistic Studies. All kinetic data were collected under conditions where the reaction is stoichiometric. They were gathered in the absence of added oxidant, I_2 or NaClO, and were determined prior to the initial one-quarter of the first equivalent of substrate consumed. All reported rates were determined within the first 10 h on the reaction, and Figure 3 indicates the rate of evolved CH_4 is linear during this period. The kinetic rate law for methyl-coenzyme M cleavage is first-order in $Ni^{II}L$ concentration (Figure 4) and first-order in substrate concentration below a 3:1 substrate to $Ni^{II}L$ ratio. As the ratio of the substrate to $Ni^{II}L$ increases, the rate becomes zero-order in methyl-coenzyme M (Figure 5). The pH of the reaction in freshly distilled water increases from 7.2 to 7.6; however, the initial rate of methane formation by $Ni^{II}L$ is not first-order in proton concentration. The rate at pH 4.00, 5.95, 7.70, and 9.00 is 25, 22, 20, and 15 mmol of CH_4/(mol of $Ni^{II}L$ h), respectively. The conformation of the

Table I. Substrate Analogues of Methyl-Coenzyme M, $CH_3-S-(CH_2)_2-SO_3^-$ versus $R-X-(CH_2)_n-Y^-$

R	X	n	Y	enzyme activity rate × 10^{-3}	$Ni^{II}L$ rate × 10^2
methyl	S	2	SO_3	1.02[a]	1.89[b]
difluoromethyl	S	2	SO_3	1.80	8.89
trifluoromethyl	S	2	SO_3	inhibitor	inhibitor
propyl	S	2	SO_3	no binding	1.60
allyl	S	2	SO_3	0.01[c]	1.51
cyclopropylmethyl	S	2	SO_3	no binding	0.41
cyclobutyl	S	2	SO_3	not determined	no binding
dimethyl	S	2	SO_3	no binding	2.70
methyl	O	2	SO_3	weak inhibitor	no binding
methyl	N	2	SO_3	weak inhibitor	weak inhibitor
methyl	S	3	SO_3	no binding	1.41
methyl	S	6	SO_3	not determined	2.21
methyl	S	2	CO_2	0.12	1.95
methyl	S	2	PO_3	no binding	1.91
	Br	2	SO_3	inhibitor	weak inhibitor
$SCH_2CH_2SO_3^-$	S	2	SO_3	inhibitor	no inhibition

[a] k(cat)/h based on methyl-coenzyme M reductase as 10% of total protein and two active sites per protein. Data taken from ref 11a. [b] Initial rate is in moles of CH_4 per mole of $Ni^{II}L$ per hour. Under conditions where the reaction is first-order in [$Ni^{II}L$] and [methyl-coenzyme M] the rate constant is 0.69 (mol of CH_4) (mol of Ni mol of substrate h)$^{-1}$. All $Ni^{II}L$ rates are ±0.10 × 10^{-2}. [c] Substrate is an inhibitor of methyl-coenzyme M.

nickel complex is pH dependent, and $Ni^{II}L$ demetalates outside of the pH range examined.[7]

The evolution of dioxygen has been detected during $Ni^{II}L$-induced cleavage of methyl-coenzyme M with the O_2 indicator $[Cp_2TiCl]_2ZnCl$ (Cp = cyclopentadienyl) in toluene.[8] This observation was confirmed by running the reaction in 48.7% ^{18}O-enriched H_2O and analyzing the gas evolved by mass spectroscopy. Along with m/z peaks corresponding to methane and argon, peaks were observed at m/z 34 and 36 having an approximate 2:1 intensity ratio (the m/z 32 peak includes background atmospheric O_2). The amount of O_2 gas evolved was quantified by determining the mass balance of the reaction of methyl-coenzyme M and $Ni^{II}L$ in H_2O under 1 atm of argon with no added oxidant. The ^1H NMR spectrum of the reaction mixture indicated 1.10 turnovers of substrate had been converted to coenzyme M disulfide (0.57) and coenzyme M (0.53). The total gas evolved was measured and corresponded to 1.18 turnovers of CH_4, and the excess gas volume observed was assumed to be due to the O_2 evolved.

Methane evolved until $\simeq$1.2 mol of methyl-coenzyme M was consumed per mole of $Ni^{II}L$ and the green solution containing $Ni^{II}L$ and methyl-coenzyme M became brown with a UV-visible spectrum identical with that of $Ni^{II}L$ titrated with coenzyme M.[8] Oxidants such as O_2, I_2-saturated hexane, or 5% NaClO(aq) convert a solution of coenzyme M to the disulfide. When 5% NaClO(aq) or I_2-saturated hexane was added to the reaction mixture, coenzyme M was converted to coenzyme M disulfide and $Ni^{II}L$ was regenerated. $Ni^{II}L$ formation was confirmed by the reappearance of its UV-visible spectrum. Upon the addition of methyl-coenzyme M, the regenerated green solution evolved methane at the same initial rate as that of a fresh solution of $Ni^{II}L$.[8] A reaction titrated with NaClO over a period of 1 week resulted in seven substrate turnovers with a 30% loss in $Ni^{II}L$ activity.[10]

Substrate Analogues. The reactivities of a variety of substrate analogues have been tested with cell-free extracts of methyl-coenzyme M reductase.[11] The nomenclature to describe substrate modifications is illustrated in eq 8. The variation in methyl-

$$CH_3-S-(CH_2)_2-SO_3^- \qquad R-X-(CH_2)_n-Y^- \qquad (8)$$

coenzyme M substrate involves either the alkyl group (R), the heteroatom (X), the methylene chain length (n), or the acid residue (Y^-). The initial reaction rates of the substrates with $Ni^{II}L$ are listed in Table I. No added oxidant is present, and all reactions were measured during the initial one-quarter of a turnover. The ability of inactive substrates to inhibit the reaction of methyl-coenzyme M was examined as well. Changes in the alkyl (R) group and the heteroatom (X) can alter the reactivity of the substrate to a significant degree, while the methylene chain length (n) and the acid residue (Y^-) have very little influence.

The effect of the alkyl (R) group is significant. As the chain length of primary alkyl substituents is increased from R = methyl to allyl, propyl, and cyclopropylmethyl, the rate decreased to a small extent, while alkyl groups such as R = cyclobutyl were unable to bind to $Ni^{II}L$. No shift in the UV-visible spectrum of the reaction mixture containing allyl-coenzyme M was observed. 2-((Trifluoromethyl)thio)ethanesulfonic acid was not a substrate for the nickel complex; however, this substance did inhibit methane formation from methyl-coenzyme M, resulting in a 20% decrease in the initial rate (initial ratio of [$Ni^{II}L$]:[$F_3CSCH_2CH_2SO_3^-$]:[$H_3CSCH_2CH_2SO_3^-$] was 1:2:8). The ^{19}F NMR spectrum of the reaction mixture demonstrated only a single ^{19}F-containing species in solution. The addition of 0.1 M HCl to the nickel complex extracted from the reaction mixture did not result in gas evolution, suggesting no stable organonickel species had formed.

Although methyl-coenzyme reductase cleaves substrate analogues at the R-X bond in every instance, $Ni^{II}L$ does not. FAB mass spectrometry and GC-MS analysis of the gas-phase products from 2-((cyclopropylmethyl)thio)ethanesulfonic acid indicate the presence of cyclopropylmethyl mercaptan and cyclopropylmethane but no butene. When R = HCF_2, the FT-IR and mass spectra of the gas phase identified HCF_2SH as the sole product derived from the substrate. ^{19}F NMR spectra indicated that the only fluorine-containing species remaining in solution was the unreacted substrate.

The reactivity of $Ni^{II}L$ upon heteroatom substitution of the substrate exhibited the same pattern as methyl-coenzyme M reductase. When X = O or N, 2-methoxyethanesulfonic or 2-(methylamino)ethanesulfonic acid, respectively, no methane, methanol, or methylamine was observed. When these substrates were present in 10-fold excess over $Ni^{II}L$ and in 5-fold excess over methyl-coenzyme M, little or no inhibition of the natural substrate was observed. 2-Bromoethanesulfonic acid (RX = Br) did not react with $Ni^{II}L$ and was not an inhibitor of methane formation from methyl-coenzyme M, while $[(CH_3)_2S^+CH_2CH_2SO_3^-]$NaI was the most reactive substrate tested.

Variation of the methylene chain length had a minor effect on the reactivity of the substrate. The initial rate of methyl-coenzyme M (n = 2) was between the rates observed for the n = 3 and 6 analogues. Within experimental error, the nature of the acid residue Y = SO_3^-, CO_2^-, or PO_3^- did not change the rate of methane formation induced by $Ni^{II}L$.

Discussion

Since the nickel tetrapyrrole containing factor, F_{430}, has been proposed as the active site of methyl-coenzyme M reductase, it has been investigated extensively.[1-6,11,17-19] F_{430} extracted at low temperature can be reconstituted with the apoenzyme to yield active methyl-coenzyme M reductase.[6,16] The 10 K Ni EXAFS of the purified enzyme is consistent with the coordination of the four pyrrole nitrogens and the presence of a second-row donor atom in the axial position.[17] Although low-temperature resonance Raman experiments are consistent with a six-coordinate nickel ion, the spectrum of the nickel chromophore in methyl-coenzyme M reductase is dissimilar to known five- or six-coordinate Lewis

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base adducts of F_{430}.[18] Thus, the nickel ion in methyl-coenzyme M reductase is considered to be at least five-coordinate and might weakly coordinate a sixth ligand, which can dissociate prior to binding the thio sulfur atom of methyl-coenzyme M. The assignment of F_{430} as the active site of methyl-coenzyme M reductase is supported by the observation of methyl-coenzyme M cleavage by $Ni^{II}L$ to generate coenzyme M and methane.

Kinetic studies of methyl-coenzyme M reductase exhibit in vitro rates less than 1% of the whole cell rate.[2,11] The loss of activity occurs upon cell breakage and not during protein purification.[19] Since the cause of enzyme inactivation is unknown, caution must be exercised in drawing an analogy between reactivity studies of the whole cell and the cell-free protein extract. Evidence for a methanoreductosome has been presented recently where up to 20 methyl-coenzyme M reductase units form a large hollow sphere.[5,20] The enhanced rate of methane formation in the whole cell might result from elevated local concentrations of substrate present in methanoreductosomes.

Several mechanisms proposed for the active-site chemistry of methyl-coenzyme M reductase invoke the initial reduction of Ni(II).[5,11] The leading literature mechanism for methyl-coenzyme M reductase involves the one-electron reduction of $Ni^{II}F_{430}$ to form $Ni^{I}F_{430}$, which homolytically cleaves methyl-coenzyme M to form methyl-$Ni^{I}F_{430}$. Protonation of the acid-labile organonickel species yields methane and regenerates $Ni^{II}F_{430}$ and is consistent with the hydrolysis of highly air-sensitive alkyl-Ni(I) tetraazamacrocycles.[21] The rationale for this initial reduction step is as follows: (a) desulfurization by Raney nickel is a precedent;[8,22] (b) reductive conditions are thought to be present at the enzyme active site; (c) approximately 20% of the nickel present in whole cell methanogens is detectable by EPR spectroscopy and exhibits a signal similar to that of the pentamethyl ester derivative of $Ni^{I}F_{430}$.[6] The coenzyme M radical that is formed is thought to combine with *N*-(7-mercaptoheptanoyl)-*O*-phosphothreonine, HS-HTP, to form a heteroatom disulfide, which is reduced to regenerate coenzyme M and HS-HTP.[23] However, the pentamethyl ester derivative of $Ni^{I}F_{430}$ in DMF is reported to be incapable of effecting the cleavage of methyl-coenzyme M,[9] although it does exhibit the reactivity expected for Ni(I).[21] Thus, the formation of Ni(I) might not in itself be the sole requirement for nickel ion activation.

Several aspects of the catalytic conversion of methyl-coenzyme M by $Ni^{II}L$[8] are analogous to the enzyme active site chemistry. Methane and coenzyme M are the sole products derived from the substrate, there is no ^2H isotope effect in D_2O, and the thiol product ultimately is converted to a disulfide. However, we believe these observations support a mechanism involving a Ni(II/III) rather than a Ni(I/II) redox couple for the following reasons: (a) Dioxygen formed in the reaction indicates that redox chemistry occurs. A $Ni^{I}L$ species is incompatible with the presence of O_2. (b) The reaction becomes catalytic in nickel ion when oxidizing species such as I_2 or NaClO are in solution. In addition, the initial reaction rate increases when the reaction is observed under 1 atm of O_2 instead of 1 atm of argon.[8] (c) Added reductant, 1 atm of H_2 or $Na_2S_2O_4$, does not increase the initial rate.[8] (d) The magnetic susceptibility of the reaction mixture indicates the predominant oxidation state of the nickel ion is divalent throughout the course of the reaction.[8] (e) The unusual reactivity of $Ni^{II}L$ toward methyl-coenzyme M can be understood, since it has one of the lowest Ni(II/III)L redox couples reported for an azamacrocycle ligand.[7,8]

The amount of O_2 was quantified in order to determine to what extent Scheme I (Figure 2) represents the reaction pathway. The amount of coenzyme M disulfide, the volume of O_2 evolved, and the amount of O_2 dissolved in solution[24] account for 75 ± 5% of the O_2 expected in Scheme I. We believe this number represents a lower limit of the O_2 evolved, since $Ni^{II}L$ is reported to bind O_2 in solution[7] and slowly decomposes in aqueous solution in the presence of O_2.[8] Thus, it is not unreasonable to expect these side reactions to consume most or all of the unaccounted O_2.

The stoichiometric dealkylation of thioethers is known to occur in the presence of d^8 metal ions where the order of reactivity parallels ion lability, $Ni^{2+} > Pd^{2+} > Pt^{2+}$.[25] The proposed mechanism for S-dealkylation by Ni(II) salts involves sulfur coordination to nickel, which makes the weakened carbon–sulfur bond subject to nucleophilic attack. The resulting mercaptan binds to nickel and makes the reaction stoichiometric. However, nucleophilic attack by water on a $Ni^{II}L$–methyl-coenzyme M complex would be expected to form methanol, a product not observed in this reaction. The extent to which the stoichiometric dealkylation of thioethers in DMF by nickel(II) halides can be analogized to the decomposition of methyl-coenzyme M by $Ni^{II}L$ in aqueous solutions may be limited. Scheme I (Figure 2) presents a set of balanced equations consistent with the catalysis of methyl-coenzyme M to form CH_4 and coenzyme M disulfide and dioxygen.[8] The formation of $Ni^{II}L$–coenzyme M, step 4, is thought to prevent more than about one substrate turnover. The role of the oxidant is to convert coenzyme M to the disulfide and regenerate $Ni^{II}L$ by driving the equilibrium in step 4 to the left. An oxidant such as NaClO, which can be maintained in high concentration in the aqueous phase, was found to be more efficacious than an I_2/hexane solution or O_2.

Whether the reactivity of $Ni^{II}L$ with methyl-coenzyme M serves as a direct analogy to F_{430} type chemistry is unknown. We were interested if the reactivities of $Ni^{II}L$ and methyl-coenzyme M reductase toward a series of methyl-coenzyme M derivatives used to evaluate the role of steric and electronic properties at the enzyme active site would be similar. Table I compares the initial reaction rates for various substrates with cell-free extracts of methyl-coenzyme M reductase and $Ni^{II}L$.[11] Although the enzyme is fairly intolerant to increased steric requirements of substrate analogues, it was expected that $Ni^{II}L$ would be more tolerant toward an increase in the steric bulk of the alkyl (R) substituent.

The methyl-coenzyme reductase active site does not accommodate alkyl (R) groups bulkier than allyl. For example, methyl-coenzyme M reacts at a rate approximately 50% faster than the ethyl analogue, while allyl reacts at about 1% of the rate of the natural substrate.[11] Allyl-coenzyme M is a potent inhibitor of the enzyme toward the natural substrate, although the UV–visible spectrum is unchanged, suggesting that an allyl–nickel species is not present. The propyl and isopropyl analogues do not inhibit methane formation from methyl-coenzyme M, indicating that sterically demanding R groups prevent substrate binding at the enzyme active site. The reactivity of whole-cell extracts of methyl-coenzyme M reductase are consistent with observations of whole-cell methanogens.[11]

$Ni^{II}L$ cleaves alkyl-coenzyme M derivatives with R groups as sterically demanding as cyclopropylmethyl. In contrast to the enzyme, $Ni^{II}L$ converts allyl-coenzyme M to propene at an initial rate only 20% slower than that of methyl-coenzyme M. No reaction is observed when R is sulfur-bound by the secondary carbon in cyclobutyl-coenzyme M. Since cyclobutyl-coenzyme M does not inhibit the decomposition of methyl-coenzyme M, it probably is unable to bind to $Ni^{II}L$. Although a sterically demanding sulfonium salt such as $[(CH_3)_2S^+CH_2CH_2SO_3^-Na]^{1-}$

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is not cleaved by the enzyme and inhibits the natural substrate,[11] it was the most active substrate toward $Ni^{II}L$. Evidently, a sufficiently large R group can prevent effective substrate binding to the nickel ion.

The reactivity of cyclopropylmethyl-coenzyme M is of particular interest because it can serve as an internal clock to measure the rate of C–S bond cleavage. The gaseous products contain cyclopropylmethane but no butene. This suggests that either homolytic cleavage of the C–S bond does not occur or this step is fast compared to the half-life of the free radical ($2.7 \times 10^8\ s^{-1}$).[26] However, rearrangement of a cyclopropylcarbinyl is several orders of magnitude slower than the free radical.[27] The observation of a small quantity of cyclopropylmethyl mercaptan gas was unexpected, since the cleavage of an internal C–S methylene bond has no precedence in methyl-coenzyme M reductase chemistry. It is possible the steric interaction of the R group with $Ni^{II}L$ forces the substrate to adopt a conformation that favors the cleavage of either C–S bond.

Trifluoromethyl-coenzyme M cannot be decomposed by either the enzyme or $Ni^{II}L$ although it does inhibit methyl-coenzyme M in both $Ni^{II}L$ and the enzyme. Apparently, $F_3CSCH_2CH_2SO_3^-$ coordinates to the nickel ion but the relatively strong F_3C–S bond is not cleaved. No spectroscopic or chemical evidence exists to support the formation of a F_3C–Ni bond during these reactions. When difluoromethyl-coenzyme M is added to methyl-coenzyme M reductase, difluoromethane is evolved at nearly twice the rate of methane.[11] In contrast, the FT-IR and mass spectra of the gas-phase products indicate $Ni^{II}L$ generates HF_2CSH from $HF_2CSCH_2CH_2SO_3^-$ and the relatively strong HF_2C–S bond evidently can direct cleavage to the internal C–S bond. It is an intriguing possibility that the enzyme active site can promote the selective cleavage of the alkyl group.

The identity of the heteroatom (X) strongly influences the reactivity of the substrate toward $Ni^{II}L$ and the enzyme. In the enzymatic system the rate of methane formation, Te > Se > S, parallels the affinity of the heteroatom toward the metal ion and the decrease in C–X bond strength.[11] 2-Methoxyethanesulfonic acid is not a substrate for $Ni^{II}L$ or methyl-coenzyme M reductase and does not inhibit either catalyst in the presence of methyl-coenzyme M. 2-(Methylamino)ethanesulfonic acid is not a substrate for the enzyme or $Ni^{II}L$ although it weakly inhibits both systems. This indicates the substrate weakly coordinates to the catalysts, but once bound, the C–N bond is not dealkylated. Although 2-bromoethanesulfonic acid is a powerful inhibitor of methyl-coenzyme M reductase, it does not appreciably affect the rate of methyl-coenzyme M decomposition by $Ni^{II}L$.

The enzyme is very sensitive to the length of the methylene chain length. When $n > 3$, the substrate analogue is inactive and fails to inhibit methane evolution from added methyl-coenzyme M. This result raises the possibility that a substrate is self-activated by the adoption of a cyclic conformation at the enzyme active site. For example, a five-membered cyclic structure of $H_3CSCH_2CH_2SO_3^-$ could be result from direct donation of electron density of the sulfonate group into the empty d orbitals of the heteroatom. This could explain the inactivity of substrates containing X = O or NH. Alternatively, a six-membered ring would result from a hydrogen-bonded S–H–OSO_2 species. However, the reactivity of $H_3CS(CH_2)_6SO_3^-$, which is not expected to form the entropically disfavored eight- or nine-membered ring, indicates substrate self-activation does not occur.

Variation of the acid residue (Y^-) influences the reactivity of methyl-coenzyme M reductase. When Y^- is $-CO_2$, the initial rate is only 12% of the natural substrate while the phosphonate derivative is inactive and is not an inhibitor of methyl-coenzyme M.[11] Since 2-bromoethanesulfonic acid is a potent inhibitor of methanogenesis while 2-chloroethanephosphonic acid is not, it is possible that the phosphonate derivative is metabolized or binds to a protein site that prevents it from reacting with methyl-coenzyme M reductase. In contrast, the initial rate of methane evolution by $Ni^{II}L$ where Y = $-SO_3$, $-PO_3$ or $-CO_2$ is within one standard deviation of experimental error. This observation argues against substrate activation by the acid residue. These results are consistent with the two-site binding model proposed for methyl-coenzyme M reductase because the nature of the acid residue is expected to influence the reactivity of the enzyme and not $Ni^{II}L$.

Conclusion

The reactivity of $Ni^{II}L$ toward methyl-coenzyme M analogues supports the assignment of F_{430} as the active site in methyl-coenzyme M reductase. The effect of the heteroatom and the alkyl substituent group in the substrate analogues can be explained by the known reactivity of nickel coordination complexes. Our observations suggest the ligand plays a key role in nickel activation by adjusting the redox couple of the metal ion and argue against substrate self-activation by the formation of a cyclic structure. Cleavage of the methylene–sulfur bond by $Ni^{II}L$ occurs only when the alkyl substituent is sterically demanding or when it possesses a strong external C–S bond. This suggests the conformation of the active site in the enzyme can influence the site of C–S bond cleavage. The reactivity of methyl-coenzyme M analogues is consistent with the two-site binding model proposed for methyl-coenzyme M reductase that involves the coordination of the heteroatom by the nickel ion and the binding of the acid group by a positively charged species such as a protein residue.[11] This second substrate binding site can influence the overall reaction rate by enhancing the formation constant of the nickel–substrate complex. It is noteworthy that according to Scheme I, step 3, the evolution of O_2 by nickel would be suppressed if an electron rather than a hydroxide ion were the reducing equivalent. Although the enzyme and $Ni^{II}L$ chemistry is expected to differ in this respect, caution should be exercised in making a direct analogy between the mechanism of $Ni^{II}L$ and $Ni^{II}F_{430}$. While a Ni(II/III) redox couple appears to be operative in substrate decomposition by $Ni^{II}L$, no direct evidence to support this pathway in the enzymatic reaction is available. Finally, it is possible to envision alternative mechanisms to Scheme I including those which involve Ni(IV). We favor a $Ni^{III}L$ pathway at present only because this species has been generated electrochemically and $Ni^{IV}L$ has not.[7]

The 5 and 7 orders of magnitude slower rates for $Ni^{II}L$ compared to those for enzyme extracts and the whole cell, respectively, can be ascribed to several causes: (a) The nickel redox couple could be optimal in the native system, but this is unlikely for $Ni^{II}L$. (b) Two-site substrate binding in the enzyme shifts the equilibrium in step 1 of Scheme I (Figure 2) far to the right with respect to $Ni^{II}L$. (c) The rate of electron transfer in the enzyme is more rapid than the attack of water on $LNi^{II}S(CH_3)CoM$. (d) Inhibition by coenzyme M in the natural system is far less important than that in $Ni^{II}L$.

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