Substrate Analogues as Mechanistic Probes of Methyl-S-coenzyme M Reductase†

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ABSTRACT: Methyl-S-coenzyme M reductase catalyzes the ultimate methane-yielding reaction in methanogenic bacteria, the reductive cleavage of the terminal carbon–sulfur bond of 2-(methylthio)ethanesulfonic acid. This protein has previously been shown to contain 2 equiv of a tightly bound nickel corphinoid cofactor, denoted cofactor F430, that may play a role in catalysis. Prior to this study, only one substrate analogue, ethyl-S-coenzyme M, had been demonstrated to be processed to a product by anaerobic cell extracts from *Methanobacterium thermoautotrophicum* strain ΔH. In this investigation, we have synthesized three additional substrate analogues that serve as substrates as well as five previously unknown inhibitors. Steady-state kinetic techniques were developed in order to assess relative rates of processing for these substrates and inhibitors by use of anaerobic cell extracts from *M. thermoautotrophicum*. With this assay system, a K_M of 0.1 mM and a k_{cat} of 17 min^{-1} were determined for methyl-S-coenzyme M as substrate. Methyl-seleno-coenzyme M was converted to methane with a k_{cat} threefold higher than that of methyl-S-coenzyme M, but k_{cat}/K_M was unchanged. The carbon–oxygen bond of 2-methoxyethanesulfonic acid was not cleaved to yield methane, but this analogue acted as an inhibitor with a K_I of 8.3 mM. Methyl reductase catalyzed reductive cleavage of difluoromethyl-S-coenzyme M to yield difluoromethane as the sole product, but trifluoromethyl-S-coenzyme M and trifluoromethyl-seleno-coenzyme M were inhibitors and not substrates. Allyl-S-coenzyme M, cyano-S-coenzyme M, and (difluoromethyl sulfoxide)-coenzyme M were shown to be tight binding inhibitors, but no spectroscopically detectable intermediates were observed upon incubation of these analogues with methyl reductase.

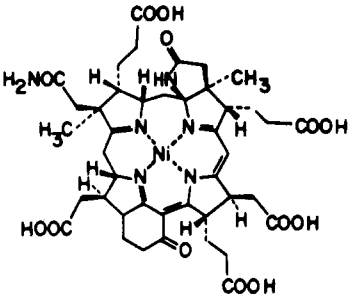
Methyl-S-coenzyme M reductase catalyzes the last two-electron reductive step in the overall eight-electron reduction of carbon dioxide to methane that is carried out by methanogenic bacteria. This complex enzyme system generates methane by the hydrogen-dependent reductive cleavage of 2-(methylthio)ethanesulfonic acid (Gunsalus & Wolfe, 1977), also known as methyl-S-coenzyme M.

In vitro catalysis of the reaction shown in eq 1 requires that

$$CH_3S{\sim}SO_3^- + 2e^- + 2H^+ \longrightarrow CH_4\uparrow + HS{\sim}SO_3^- \quad (1)$$

oxygen by rigorously excluded from reaction mixtures containing either anaerobically prepared cell extract or a minimum of five partially purified components in a reconstituted system (Nagle & Wolfe, 1983). Although the components of the methyl reductase system are not fully defined, they are known to include a nickel-containing hydrogenase(s) (Jacobson et al., 1982), an ATP-binding protein (Rouvierre & Wolfe, 1985), a protein containing a unique nickel tetrahydrocorphinoid cofactor, F430 (structure I) (Ellefson et al., 1982; Pfaltz et al., 1982, 1985), an undefined oxygen-sensitive component(s) (Nagel & Wolfe, 1982), and component B, now known to be (7-mercaptoheptanoyl)threonine phosphate (Noll et al., 1986). The nature of the components required for reconstitution of methanogenesis has remained obscure due to lability of the proteins during purification and storage.

Recent work by Ankel-Fuchs and Thauer (1986) has implicated the F430-containing protein, also known as component C, as being the methyl reductase. In that study, the purified



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F430-containing protein, henceforth designated as methyl reductase, was shown to be catalytically active in the reductive cleavage of methyl-S-coenzyme M to methane in a reaction mixture supplemented only with component B, dithiothreitol, and cyanocobalamin. This important finding focuses attention on the F430 nickel as a possible site of thioether reductive cleavage. The spotlight on F430 is intensified by studies on model nickel compounds (Fassler, 1984) and by EXAFS studies of free and protein-bound F430 (Eidsness et al., 1986) that depict an axially reactive tetrahydrocorphinoid nickel. These insights are suggestive of the methyl reductase acting as a physiological variant of the chemical desulfurization catalyst Raney nickel. However, there are no data at present that define a role for the F430 nickel atom in the reduction of methyl-S-coenzyme M to methane, and in general, mechanistic data on this enzyme are lacking.

One approach to developing insight into the methyl reductase reaction involves determining the effects of substrate modifications on enzyme activity. In the single reported study using methyl-S-coenzyme M substrate analogues, only ethyl-S-coenzyme M was processed to yield a product, in that case ethane (Gunsalus et al., 1978). It was concluded that methyl reductase is highly stringent with regard to its tolerance

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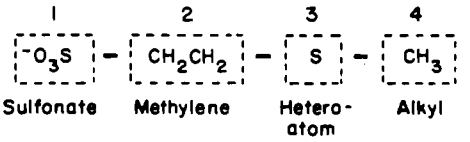
for modifications of the methyl-S-coenzyme M structure. Kinetic parameters were not determined in that study and, in fact, have only been reported with methyl-S-coenzyme M in cell-free extracts of *Methanosarcina barkeri* (Mountfort & Asher, 1984) and *Methanosarcina* sp. strain 227 (Smith, 1983).

Thus, if substrate analogues were to be used as in vitro mechanistic probes, structural changes would have to be extremely subtle and a kinetic system for their analysis would be essential. In this context, four regions of methyl-S-coenzyme M were examined for potential modification in the construction of substrate analogues:



On the basis of the findings of Gunsalus et al. (1978), modifications in the methylene region were deemed unlikely to be productive. Instead, a series of modifications have now been made in the sulfonate, heteroatom, and alkyl regions. The properties of the substrate analogues have been examined with an assay that allows the reproducible measurement of initial reaction velocities that are as low as 20 pmol/min. The steady-state kinetic parameters derived for substrates and inhibitors have been compared in order to begin to gain insight into the nature of the methyl reductase catalytic site.

Materials and Methods

General Materials. Coenzyme M (2-mercaptoethanesulfonic acid) and biochemical reagents used in the preparation of cell extracts and subsequent assay were purchased from Sigma Chemical Co. Other reagents used in organic syntheses were from Aldrich Chemical Co. unless otherwise stated below. All standard gases were purchased from Matheson Gas Products except for difluoromethane, which was obtained from Fairfield Chemical Co. Methyl-, ethyl-, and propyl-S-coenzyme M were prepared as previously described by Gunsalus et al. (1978). 2-(Methylthio)ethanephosphonic acid and 2-[(difluoromethyl)thio]ethanesulfonic acid *S*-oxide were kind gifts from Dr. Spencer Shames, MIT.

2-Methoxyethanesulfonic Acid. To a stirred solution of 2-bromoethyl methyl ether (4.2 g) in 19 mL of ethanol–water (2.8:1) was added 1 equiv of sodium sulfite in 5 mL of water. The solution was heated at 80–85 °C for 7 h, and the free acid was obtained following chromatography on columns of cellulose and Bio-Gel P-2 in 42% yield; mp 214–217 °C; ^{1}H NMR (90 MHz, D_2O) δ 3.50 (s, 3 H), 3.95 (m, 4 H); FABS mass spectrum, m/z 163 ($M^-Na^+H^+$), 185 (M^-2Na^+).

Methyl-seleno-coenzyme M. Sodium selenide was prepared under argon with sodium borohydride (300 mg, 8 mmol) and two consecutive additions of selenium metal (each 300 mg, 3.8 mmol) in 5 mL of anaerobic water. To this was added sodium bromoethanesulfonate (1.56 g, 7.4 mmol) dissolved in 5 mL of anaerobic water, and the mixture was allowed to stir overnight. After solvent evaporation, the solid was taken up in a minimum of water, prepared in a slurry with 10 g of cellulose (Celex-N-1) and acetone, and purified by cellulose column chromatography. The resultant sodium bis(2-selenoethanesulfonate) was dissolved in water under argon and reduced with excess sodium borohydride. The selenol was transferred to an anaerobic vial containing concentrated ammonium hydroxide and methylated with 2.4 equiv of methyl iodide. After reaction in the dark for 16 h at room temperature, the resultant white solid was purified by Bio-Gel P-2 chromatography with water as an eluent in 60% yield; mp 293 °C dec; ^{1}H NMR (90 MHz, D_2O) δ 1.94 (s, 3 H), 3.13 (m, 4 H); FABS mass spectrum, m/z 248 ($M^-Na^+_2$).

Trifluoromethyl-S-coenzyme M. Under an argon atmosphere, 1 g of coenzyme M was dissolved in 20 mL of saturated aqueous ammonia, and 10–20 g of trifluoromethyl iodide was condensed into the flask. Following irradiation by UV light for 70 min, the white powder was purified by chromatography on cellulose and Bio-Gel P-2 to give 24% yield: mp 175–177 °C dec; ^{1}H NMR (60 MHz, D_2O) δ 3.35 (s, 4 H); ^{19}F NMR (90 MHz, D_2O) δ 39.58 (s); FABS mass spectrum, m/z 233 ($M^-Na^+H^+$), 255 ($M^-Na^+_2$).

Trifluoromethyl-seleno-coenzyme M. Sodium bis(2-selenoethanesulfonate) (300 mg, 0.714 mmol) was reduced to the selenol as described above and transferred to 25 mL of liquid ammonia in a dry ice–acetone bath. To this mixture was condensed 30–40 g of trifluoromethyl iodide, and the combination was exposed to ultraviolet irradiation for 90 min. The crude product was purified by cellulose and Bio-Gel P-2 chromatography to give a 36% yield: mp 195 °C dec; ^{1}H NMR (90 MHz, D_2O) δ 3.23 (s, 4 H); ^{19}F NMR (90 MHz, D_2O) δ 33.15 (s); FABS mass spectrum, m/z 303 ($M^-Na^+_2$).

Difluoromethyl-S-coenzyme M. Mercaptoethanesulfonic acid (2 g, 12 mmol) was dissolved in 5 mL of dioxane followed by 1.8 g of NaOH and 10 mL of water. After being warmed to 60 °C, difluorochloromethane (Freon 22, Du Pont Chemical Co.) was bubbled through the mixture for 5 h. The product was purified as previously described to give 56% yield: mp 190 °C dec; ^{1}H NMR (90 MHz, D_2O) δ 3.35 (s, 4 H), 7.10 (t, 1 H, J = 56 Hz); ^{19}F NMR (90 MHz, D_2O) δ 91.53 (d, J = 56 Hz); FABS mass spectrum, m/z 237 ($M^-Na^+_2$).

3-(Methylthio)propionic Acid. Methyl 3-(methylthio)-propionate was hydrolyzed with excess sodium hydroxide. The free acid was prepared with a slight excess of HCl and rotoevaporated to yield a clear oil in 80% yield: ^{1}H NMR (60 MHz, D_2O) δ 2.1 (s, 3 H), 2.7 (t, 4 H); FABS mass spectrum, m/z 143 (MNa^+), 165 ($M^-Na^+_2$).

Allyl-S-coenzyme M. Prepared from allyl bromide and coenzyme M as the sodium salt: ^{1}H NMR (250 MHz, CD_3OD) δ 2.85 (m, 2 H), 3.03 (m, 2 H), 3.18 (d, 1 H, J = 8 Hz), 5.11 (dd, 2 H, J = 19 and 11 Hz), 5.79 (m, 1 H); FABS mass spectrum, m/z 221 ($M^-K^+H^+$).

Cyclopropylmethyl-S-coenzyme M. Prepared from cyclopropylmethyl bromide and coenzyme M as the potassium salt: ^{1}H NMR (250 MHz, D_2O) δ 0.54 (m, 2 H), 0.97 (m, 1 H), 2.48 (d, 2 H, J = 6 Hz), 2.69 (m, 2 H), 2.78 (m, 2 H); FABS mass spectrum, m/z 273 ($M^-K^+_2$).

Cyano-S-coenzyme M. Prepared from cyanogen bromide and coenzyme M as the potassium salt: ^{1}H NMR (250 MHz, D_2O) δ 2.85 (m, 2 H), 3.27 (m, 2 H); FABS mass spectrum, m/z 244 ($M^-K^+_2$).

Anaerobic Cell Extracts. Methanobacterium thermoautotrophicum strain ΔH (ATCC 29096) was grown as previously described (Hausinger et al., 1984). Thereafter, the cells were cooled to room temperature and sparged with CO_2 during anaerobic harvesting with a Pellicon membrane filtration system from Millipore. The cell concentrate was centrifuged anaerobically at 10000*g* for 10 min. Cell pellets were resuspended in 50 mM 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid (TES) buffer, pH 7.0, to a concentration of 1 g of cells/mL of buffer in an anaerobic chamber with a helium atmosphere containing less than 3 ppm oxygen. The suspension was sealed in a glass bottle fitted with a rubber septum, filled to 1.2 atm with hydrogen gas, and stored at −80 °C until used.

The use of thick cell slurries, as described above, yielded concentrated enzyme extracts resulting in higher specific activities and greater stability upon storage. Cell disruption was conducted by one pass at 20000 psi with a continuous French pressure cell fitted with a cannula at the intake and a needle at the exit. The sealed bottle, which had been sparged with hydrogen during collection, was transferred into an anaerobic chamber. Centrifuge tubes that contained rubber O rings were filled, sealed with RTV silicone rubber adhesive sealant (General Electric), transferred out of the glovebox, and centrifuged at 35000*g* for 1 h. The green supernatant fluid was decanted in the glovebox into bottles that were sealed with rubber septa. The helium atmosphere in the bottles was exchanged with hydrogen, and the contents were stored at −20 °C.

The protein content of these anaerobic extracts was 35–45 mg/mL. Nearly identical protein concentrations were obtained with the method of Lowry (1951) using bovine serum albumin as standard and with the Bradford (1976) determination using a 1:1 mixture of carbonic anhydrase–bovine serum albumin as protein standard. Thus, the latter method was used routinely.

Kinetic Assay. Assays were conducted in 5-mL vials fitted with rubber septa. The reaction vials contained, in a final volume of 0.3 mL, 100 mM sodium 1,4-piperazinediethanesulfonate (Na-PIPES), pH 6.3 (pH determined at room temperature), 28 mM $MgCl_2 \cdot 6H_2O$, 3.0 mM Na_2ATP, 24 μM FAD, 1.4 mg of crude extract protein, and the apropriate amount of the substrate of interest. Reaction mixtures lacking enzyme and the coenzyme M substrate were rendered anaerobic by eight cycles of evacuation and gassing with hydrogen to a final pressure of 1.2 atm. These vials were stored at −20 °C and then underwent a second set of purge/evacuation cycles just prior to use. Enzyme was transferred with a hydrogen-scrubbed gas-tight syringe from an anaerobic solution to the reaction vials while the vials were maintained under positive pressure on a gas manifold. The reaction vials were then removed from the gas manifold and incubated at 60 °C for 20 min to deplete endogenous substrates that yielded methane. Thereafter, the vials were stored on ice and purged of methane. These preincubated enzyme samples were found to behave reproducibly in replicate assays, showed no background gas formation, and were observed not to lose activity when stored on ice for at least 8 h. Just prior to being assayed, an enzyme aliquot was warmed to 60 °C, and the reaction was initiated by anaerobic addition of substrate with a gas-tight syringe.

Enzyme activity was monitored with gas chromatography (GC) by sampling the reaction headspace with a hydrogen-scrubbed syringe and withdrawing 50 μL of gas. The appropriate standard gases were used to determine the extent of recovery from the reaction headspace and to calibrate the GC response. Aliquots were taken as needed with a sampling time as short as 10 s.

Analytical Methods. Gas chromatography was performed with a Hach-Carle AGC Series 100 instrument. Gas chromatography–mass spectrometry was used to confirm identities of gaseous products with a Hewlett-Packard 5990 GC/MS. Nuclear magnetic resonance spectroscopy was conducted with a JEOL 90-MHz instrument. Fast atom bombardment mass spectrometry was performed at the MIT Mass Spectrometry Laboratory or at Brock University.

Results and Discussion

General Features of Methyl Reductase Reaction. Methyl-coenzyme M reductase, the F430-containing protein, can be routinely purified from *M. thermoautotrophicum* strain ΔH in 100-mg quantities by the method of Hausinger et al. (1984). However, the functional reconstitution of methanogenic activity from this organism still requires supplementation of methyl reductase with crude material from chromatographic columns plus component B. These undefined protein fractions have proven to support variable levels of methyl reductase dependent activity. Thus, an investigation of the steady-state kinetics of the methyl reductase reaction necessitated the use of the more dependable crude cell-free extract reaction system that has been carefully defined in a series of investigations by Wolfe and colleagues (McBride & Wolfe, 1971; Gunsalus & Wolfe, 1978; Gunsalus et al., 1978). These cell-free extracts contain methyl reductase and F420-reducing hydrogenase at 10% (Ellefson & Wolfe, 1981; Hausinger et al., 1984) and 4% (Fox, 1984) of the total protein, respectively. Thus, the enzym source used in these studies constituted a semidefined system that allowed us to report rate data with respect to the methyl reductase concentration.

Several problems had to be overcome in developing a kinetic system for the study of methyl reductase. First, enzyme activity was observed to increase in a concave upward manner with increasing protein concentration, which may be reflective of protein–protein dissociation constants imposing a rate-limiting effect at low protein concentrations (Orme-Johnson et al., 1977; Yeh et al., 1977). Thus, all kinetic assays described in this paper were conducted with 1.4 mg of protein, a value that is within the range where activity varies with protein in an essentially linear fashion. Second, the enzyme has been demonstrated to undergo an ATP- and Mg^{2+}-dependent activation process (Gunsalus & Wolfe, 1978; Whitman & Wolfe, 1983). Observed rates were maximized by use of saturating concentrations for Mg^{2+} and ATP of 28 and 3 mM, respectively (Nagle & Wolfe, 1983). Third, with substrates that had K_M values below 0.4 mM, we had to derive initial rates from the reaction progress curves at up to 25% of substrate conversion to product. However, inaccuracy in determining these rates was minimized due to the following theoretical and experimental considerations: (i) The equilibrium constant for the hydrogen-coupled thioether cleavage favors complete conversion to product [derived from $\Delta G°$ values from Vogels (1986)]. (ii) The fractional saturation of enzyme with substrate in these reaction mixtures was high. (iii) It was determined that coenzyme M did not show product inhibition at ≤5-fold molar excess with respect to substrate. Furthermore, the experimental reaction curve and the reaction course predicted from use of the integrated Henri–Michaelis–Menton equation closely matched during the initial 25% of the reaction those for methyl-S-coenzyme M and methyl-seleno-coenzyme M at a range of concentrations.

Additionally, several substrates showed extended lag phases in product formation that were shown not to be due to slowness in gaseous product equilibration between gas and liquid phases. Whereas methyl-S-coenzyme M and methyl-seleno-coenzyme M did not show this behavior, difluoromethyl-S-coenzyme M and ethyl-S-coenzyme M, at K_M concentrations, showed moderate lag times of 8.0 and 5.3 min, respectively (Figure 1). No correlation between substrate concentration and lag time was apparent with these latter two substrates. In contrast, 3-(methylthio)propionic acid showed extended lag times that were linearly proportional to the maximal observed rates at each concentration of substrate (Figure 1). With those substrates showing intermediate lag phases, a significant percentage of substrate (15–25%) was consumed before the attainment of linear rates. Rate data, which were corrected for substrate depletion during the lag phase, gave excellent fits

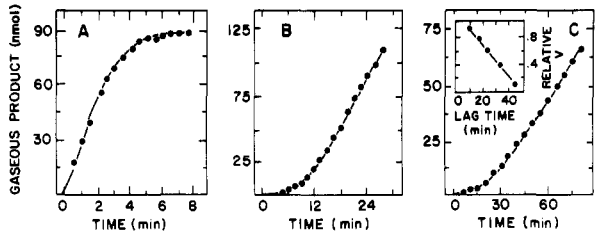


FIGURE 1: Reaction time courses showing differences in lag phases with three substrates at the indicated concentrations: (A) 0.3 mM methyl-Se-coenzyme M; (B) 1.0 mM difluoromethyl-S-coenzyme M; (C) 1.5 mM 3-(methylthio)propanoic acid.

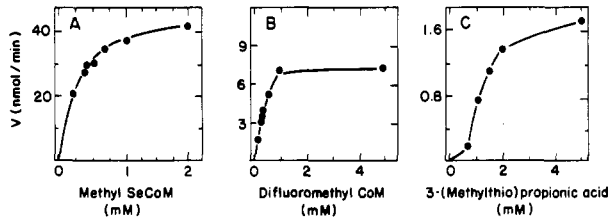


FIGURE 2: Substrate saturation curves determined for (A) methyl-Se-coenzyme M, (B) difluoromethyl-S-coenzyme M, and (C) 3-(methylthio)propanoic acid.

Table I: Kinetic Constants for Substrates of Methyl Reductase

substrate	K_M (mM)	V_{max}[a]	V/K ($\times 10^{-3}$ min^{-1})	k_{cat} (min^{-1})[b]	r[c]
$CH_3SCH_2CH_2SO_3^-$	0.1	11	110	17	0.99
$CH_3SeCH_2CH_2SO_3^-$	0.3	35	117	53	0.99
$CH_3CH_2SCH_2CH_2SO_3^-$	1.3	7.4	5.7	11	0.99
$CF_2HSCH_2CH_2SO_3^-$	2.5	20	8.0	30	0.99
$CH_3SCH_2CH_2CO_2^-$	1.3[d]	1.3	1.0	2.0	

[a] Units of nmol min^{-1} (mg of crude extract protein)$^{-1}$. [b] Based on methyl reductase as 10% of total protein and two active sites per protein (Ellefson & Wolfe, 1981; Ellefson et al. 1982). [c] Correlation coefficient obtained from linear regression analysis of Lineweaver–Burk treatment of initial rate data. [d] $S_{0.5}$ obtained from Hill plot of rate data.

by Lineweaver–Burk treatment (Table I). Kinetic plots with methyl-S-coenzyme M, methyl-seleno-coenzyme M, and ethyl-S-coenzyme M showed hyperbolic saturation of enzyme with substrate (Figure 2A). With difluoromethyl-S-coenzyme M, methyl reductase displayed hyperbolic saturation kinetics up to $0.4K_M$ (1 mM), but the reaction velocity sharply plateaued at greater substrate values (Figure 2B). Further experiments with substrate concentrations of 1–25 mM failed to show evidence for substrate inhibition. The kinetic parameters in Table I are derived from the region showing hyperbolic substrate saturation, which, when analyzed by Lineweaver–Burk treatment, gives a correlation coefficient of 0.995. 3-(Methylthio)propionic acid showed sigmoidal saturation kinetics (Figure 2C). A Hill plot of the data yielded an $S_{0.5}$ of 1.3 mM and an apparent K_{cat} of 2 min^{-1}.

Heteroatom-Substitution Series. Substitution of other group XVI elements for the thioether of methyl-S-coenzyme M provides substrates altered minimally in size but markedly in carbon–heteroatom bond strength and in softness with respect to potential interaction with nickel. A change in heteroatom softness may be anticipated to alter the dissociation constant (K_S) for the substrate if Ni–heteroatom interaction constitutes a major recognition site for enzyme binding of substrate. A substantial change in K_S would likely affect the observed K_M term. A decrease in carbon–heteroatom bond strength would lead to acceleration in K_{cat} if carbon–heteroatom bond cleavage were at least partially rate determining.

The K_M and K_{cat} for the physiological substrate were determined to be 0.1 mM and 17 nmol min^{-1} (mg of protein)$^{-1}$, respectively (Table I). The K_M had not previously been reported in the literature with the enzyme from *M. thermoautotrophicum* strain ΔH, but the observed value is similar to that of 0.21 mM reported with an enzyme extract from *Methanosarcina barkeri* (Mountfort & Asher, 1984). The K_{cat} obtained is consistent with previous reports of specific activities for the enzyme from *M. thermoautotrophicum* strain ΔH determined at saturating concentrations of methyl-coenzyme M (Gunsalus & Wolfe, 1978).

Selenium substitution for the thioether sulfur of methyl-S-coenzyme M resulted in a threefold increase in both K_M and K_{cat}. Thus, K_{cat}/K_M, the apparent second-order rate constant for the reaction, is the same for thio- and selenoether coenzyme M substrates. It should be noted that the covalent radii of sulfur and selenium are similar at 1.02 and 1.16 Å, respectively, but the carbon–selenium bond dissociation energy is weaker by 28 kcal/mol (Weast & Astle, 1980). Note that with some palladium- and nickel–thio/selenoether complexes, dealkylation occurs more readily with the selenium ligand, presumably due to the relative weakness of the C–Se bond (Murray & Hartley, 1981). This may underlie the threefold higher turnover number (53 min^{-1}). Of course, in the methyl reductase catalyzed reaction it is not yet known whether carbon–heteroatom bond cleavage may or may not be a rate-determining step.

The oxygen analogue closely mimics methyl-S-coenzyme M sterically but is characterized by carbon–heteroatom bond energy 21 kcal/mol greater than that of the physiological substrate (Weast & Astle, 1980) and would be expected to chelate less tightly to Ni(I) or Ni(II) species (Murray & Hartley, 1981). Recent work on the reduction of the F430 pentamethyl ester from Ni(II) to Ni(I) (Jaun & Pfaltz, 1986) suggests that this may be the relevant redox transition in enzyme-catalyzed thioether reductive cleavage, in which case substrate would initially bind to enzyme in one of these two redox states. In this study, 2-methoxyethanesulfonic acid was not processed to methane by methyl reductase when present in reaction mixtures at concentrations up to 20 mM. Furthermore, it acted as a substantial inhibitor only at high concentrations relative to the K_M for methyl-coenzyme M. A K_I of 8.3 mM was determined from Lineweaver–Burk plots at several concentrations of methoxyethanesulfonic acid (see Figure 3, inset). Note that the K_I for this inhibitor is 83-fold higher than the K_M for the methyl thioether substrate. From duplicate determinations of points with inhibitor at 3.5 and 10 mM, a noncompetitive inhibition pattern was discernible (Figure 3). This result could be suggestive of two kinetically distinct substrate binding sites on methyl reductase. It is worth noting here that methyl reductase contains two F430 sites per $\alpha_2\beta_2\gamma_2$ holoenzyme of M_r 3×10^5, so the potential for half-site reactivity is present.

Alkyl-Substitution Series. Ethyl- and propyl-S-coenzyme M were previously observed by Gunsalus et al. (1978) to be a substrate and a nonsubstrate, respectively. As shown in Table I, we determined K_M and V_{max} parameters for the former substrate. Propyl-S-coenzyme M did not yield a detectable gaseous product even at substrate concentrations of up to 140 mM, nor did it inhibit appreciably at 10-fold molar excess on admixture with methyl-S-coenzyme M. Vinyl-S-coenzyme M was prepared with the intent of probing the stereochemical outcome of the reductive cleavage of 1,2-*cis*-dideuteriovinyl-S-coenzyme M. However, this analogue was found not to be a substrate for methyl reductase. Cyclopropylmethyl-S-co-

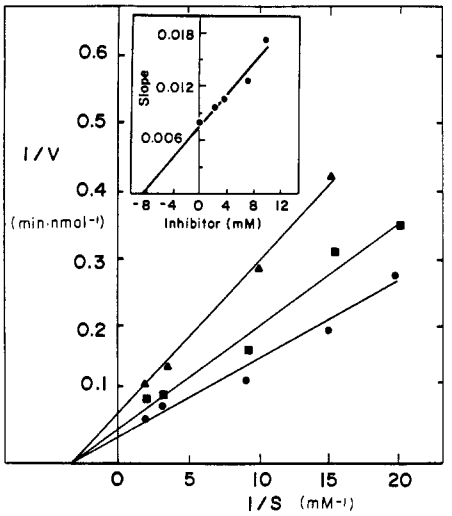


FIGURE 3: Lineweaver–Burk plots for the methyl reductase reaction determined at several concentrations of methyl-S-coenzyme M in the presence of 2-methoxyethanesulfonic acid at concentrations of 0 (●), 3.5 (■), and 10.0 mM (▲).

enzyme M was synthesized to probe a possible homolytic mechanism of C–S bond cleavage as cyclopropylmethyl radical is documented to undergo ring opening to form a butenyl radical with a first-order rate constant of 1.3×10^8 s^{-1} (Maillard et al., 1976). However, incubation of methyl reductase with this substrate analogue failed to yield either methylcyclopropane or butene, and further, inhibition of methane formation from methyl-S-coenzyme M was not observed. Thus, in analogy with its slightly smaller propyl counterpart, cyclopropylmethyl-S-coenzyme M may be sterically excluded from the active site of methyl reductase.

Fluoroalkyl-Substitution Series. Previous studies with model tetrahydrocorphinoids (Fassler et al., 1984; Kratky et al., 1984) and EXAFS studies with free F430 and methyl reductase (Eidsness et al., 1986) indicate that the F430 nickel atom is axially reactive. Thus, one could envisage putative methyl reductase reaction intermediates involving heteroatom chelation, as suggested by the heteroatom-substitution series and/or the formation of a methyl–Ni–F430 species based on the precedent for methyl-B_{12} (Taylor & Weissbach, 1973). Furthermore, it should be noted that tetraaza- and diaza–Ni–methyl model compounds have been described that undergo acid- or alcohol-mediated protonolysis, decomposing to yield methane (Wilke & Herrman, 1966; D'Aniello & Barefield, 1976; Ram et al., 1986). These considerations prompted us to examine, in the context of the methyl reductase reaction, the well-precedented stabilization of alkylnickel species by the substitution of electron-withdrawing fluorine for hydrogen (Jolly & Wilke, 1974). Difluoromethyl- and trifluoromethyl-S-coenzyme M were prepared with the intent of stabilizing a putative Ni–alkyl intermediate that could arise during enzyme catalysis and to allow its possible spectroscopic detection if the (difluoromethyl)- or (trifluoromethyl)nickel species would accumulate. It should be noted here that fluorine has a relatively small van der Waals radius of 1.35 Å (Walsh, 1983) and so should not preclude access to the active site of the highly selective methyl reductase.

As previously shown in Figure 1B, Figure 2B, and Table I, difluoromethyl-coenzyme M served as a substrate for methyl reductase. Difluoromethane was the exclusive gaseous product as determined by GC analysis and by GC/MS. The turnover number of 29 min^{-1} for difluoromethane production was 1.8-fold higher than the turnover number for methane production from methyl-S-coenzyme M. In a previous study (Penley et al., 1970) in which (difluoromethyl)cobalamin was added to cell extracts from *Methanobacterium* M.O.H. (now *M. bryantii*), the major product was methane, and some fluoromethane was detected. In light of the observations reported here, it is likely that, in the previous work, the fluorines were not lost at the methyl reductase stage but may have been lost via carbene formation and then trapping by B_{12} or, alternatively and probably less likely, during methyl group transfer to coenzyme M. Furthermore, the failure to observe methane or fluoromethane in the present study argued against such a difluoromethyl-B_{12} intermediate participating in the methyl reductase reaction. This observation is consistent with the data of Ankel-Fuchs and Thauer (1986), which indicated that, in a reaction mixture with dithiothreitol and purified methyl reductase, B_{12} may serve in electron input into methyl reductase. The detection of difluoromethane as the sole product from difluoromethyl-coenzyme M may mitigate against a C–S cleavage transition state with carbanionic character as one might have anticipated F^- loss to form monofluorocarbene leading to alternate products not containing both fluorine substituents. The higher turnover, on the other hand, is permissible if CF_2H– radical species are involved in C–S cleavage and, perhaps, on the way to a subsequently protonolyzable HF_2C–F430 intermediate.

A dramatic difference was observed when trifluoromethyl-S-coenzyme M was examined as a potential substrate. Gaseous products were not detected with substrate concentrations as high as 20 mM, ruling out trifluoromethane, difluoromethane, fluoromethane, and methane as products. In admixture with methyl-S-coenzyme M, trifluoromethyl-S-coenzyme M inhibited methane formation with an I_{50} concentration determined to be 7.0 mM. This value was intermediate to the K_M of 2.5 mM for difluoromethyl-S-coenzyme M and the K_I of 8.3 mM for 2-methoxyethanesulfonic acid but much different than the K_M of 0.1 mM for the physiological substrate. These observations were consistent with Ni–heteroatom interaction being significant in catalysis as increasing fluorine substitution at the C_1 fragment decreases electron density on sulfur, making it more like the ether oxygen in 2-methoxyethanesulfonic acid.

It is not currently clear why difluoromethyl-S-coenzyme M served as a substrate but trifluoromethyl-S-coenzyme M did not. One possible explanation rested on the previously determined increase in carbon–sulfur bond energy with fluorine substitution on carbon. Note the CF_3–S bond energy of trifluoromethyl methyl sulfide is 96 kcal/mol and the CH_3–S bond energy of dimethyl sulfide is 80 kcal/mol (Cullen et al., 1970). Perhaps in going from two to three fluorines the carbon–sulfur bond cleavage barrier had been titrated out. In order to test this, trifluoromethyl-seleno-coenzyme M was prepared. On the basis of comparative carbon–sulfur and carbon–selenium bond strengths and the additive effect of fluorine substituents, the trifluoromethyl–selenium bond energy was projected as being less than the carbon–sulfur bond energy of methyl-S-coenzyme M. However, trifluoromethyl-seleno-coenzyme M was not a substrate for methyl reductase but did show properties as an inhibitor comparable to trifluoromethyl-S-coenzyme M (Table II). Thus, the failure of methyl reductase to process trifluoromethyl-S-coenzyme M is likely not due to a bond energy barrier. Initial UV–visible spectroscopic studies have not provided evidence for a (fluoroalkyl)nickel species on interaction of methyl reductase with any of the fluorinated substrates or inhibitors.

Inhibitors. Although methyl reductase is inert to propyl-S-coenzyme M, allyl-S-coenzyme M was found to be a potent

Table II: Inhibition of Methyl Reductase by Substrate Analogues

substrate analogue	I_{50} (mM)[a]
$CH_2{=}CHCH_2SCH_2CH_2SO_3^-$	0.023 ± 0.001[b]
$N{\equiv}CSCH_2CH_2SO_3^-$	0.032 ± 0.003[b]
$CF_2HS(O)CH_2CH_2SO_3^-$	0.020 ± 0.003[b]
$CF_3SeCH_2CH_2SO_3^-$	3.4 ± 0.3[c]
$CF_3SCH_2CH_2SO_3^-$	7.0 ± 0.3[d]

[a] Defined as the concentration that gives 50% of the uninhibited rate in a mixed incubation with methyl-S-coenzyme M at 0.1 mM (K_M). [b] Derived from linear regression analysis of a plot of $1/V$ vs. [I]. [c] Derived from linear regression analysis of a plot of $1/V$ vs. $[I]^2$. [d] Derived from linear regression analysis of a plot of $1/V$ vs. $[I]^3$.

inhibitor (Table II). The marked difference in apparent enzyme recognition could be indicative of an allyl–Ni interaction, and so this substrate was further investigated. Allyl-S-coenzyme M is a tight-binding inhibitor of methyl reductase, with an I_{50} of 23 μM. Preincubation of enzyme and inhibitor did not increase the extent of inhibition, suggesting that it did not inactivate the enzyme by covalent modification, nor was it a slow release inhibitor. Spectral changes at visible wavelengths were not observed upon incubation of enzyme and inhibitor under turnover conditions. The issue of whether allyl-S-coenzyme M generates a symmetrical allylnickel intermediate has yet to be resolved. Another substrate analogue designed to interact with the putative F430–Ni active center was cyano-S-coenzyme M. This probe, likewise, strongly inhibited the methyl reductase reaction with an I_{50} of 32 μM. However, with this substrate analogue also, spectral perturbations of the enzyme-bound F430 chromophore were not observed. Given the low in vitro turnover number of crude extracts compared to intact cells [11 vs. 7000 nmol min^{-1} (mg of protein)$^{-1}$; for discussion, see Daniels et al. (1984)], it is unclear what fraction of the methyl reductase molecules are active and accessible to stoichiometric spectroscopic probes. An effective mechanism-based enzyme inactivator should help resolve that issue.

Conclusions. Following the initial report of methyl reductase purification in 1980 (Gunsalus & Wolfe), reconstruction experiments have been plagued by protein instability and low in vitro rates. Because of this, mechanistic studies of methyl reductase have not appeared in the literature. We have sought to fill this void by preparing mechanistically useful substrate analogues and developing a kinetic system for their analysis. These methods will serve as a prelude for further studies on the stereochemical outcome of substrate processing, for probing electron transfer into methyl reductase following identification of the physiological electron donor, and for attempts to spectroscopically determine possible changes in redox state or axial ligation of the F430 nickel atom during catalysis with highly active preparations of methyl reductase.

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Registry No. $MeS(CH_2)_2SO_3H$, 53501-90-9; $EtS(CH_2)_2SO_3H$, 69536-68-1; $MeSe(CH_2)_2SO_3H$, 109765-31-3; $F_2CHS(CH_2)_2SO_3H$, 109765-32-4; $MeS(CH_2)_2CO_2H$, 646-01-5; $CH_2{=}CHCH_2S(CH_2)_2SO_3H$, 51778-82-6; $NCS(CH_2)_2SO_3H$, 109786-29-0; $F_3CS(CH_2)_2SO_3H$, 109765-33-5; $F_3CSe(CH_2)_2SO_3H$, 109765-34-6; $F_2CHS(O)(CH_2)_2SO_3H$, 109765-35-7; $MeO(CH_2)_2SO_3H$, 109765-37-9; $Br(CH_2)_2OMe$, 6482-24-2; Na_2SO_3, 7757-83-7; $Br(CH_2)_2SO_3Na$, 4263-52-9; Na_2Se, 1313-85-5; $Se[(CH_2)_2SO_3Na]_2$, 109765-38-0; $HS(CH_2)_2SO_3H$, 45127-11-5; CF_3I, 2314-97-8; CHF_2Cl, 75-45-6; $MeS(CH_2)_2CO_2Me$, 13532-18-8; $CH_2{=}CHCH_2Br$, 106-95-6; BrCN, 506-68-3; methyl-S-coenzyme M reductase, 53060-41-6; cyclopropylmethyl-S-coenzyme M, 109765-36-8; cyclopropylmethyl bromide, 7051-34-5.

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Partial Purification and Functional Properties of an Endoprotease from Bovine Neurosecretory Granules Cleaving Proocytocin/Neurophysin Peptides at the Basic Amino Acid Doublet†

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ABSTRACT: An enriched preparation of neurosecretory granules from bovine pituitary neural lobes was used as a source of processing enzymes possibly involved in the cleavage of the proocytocin/neurophysin precursor. A synthetic eicosapeptide reproducing the entire (1–20) sequence of the NH_2-terminal domain of the bovine ocytocin/neurophysin precursor was used as a substrate to monitor an endoprotease activity cleaving at the Lys^{11}–Arg^{12} doublet. The 58-kDa endoprotease detected in the lysate of neurohypophyseal granules produced a single cleavage, after the doublet, at the Arg^{12}–Ala^{13} peptide bond. This endoprotease with pH_i 6.9 and 7.2 exhibits maximal activity at pH around neutrality (7.0) and was strongly inhibited by divalent cation chelating agents [ethylenediaminetetraacetic acid and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,-*N*′-tetraacetic acid] and to some extent by *p*-(chloromercuri)benzoate and *p*-(chloromercuri)benzenesulfonic acid, while phenylmethanesulfonyl fluoride and pepstatin were not active. This endoprotease action was sensitive to any modification of the substrate at either basic amino acid of the doublet since replacement of either L-Lys^{11} or L-Arg^{12} by D-Lys or D-Arg and by L-Nle abolished the cleavage reaction. In contrast, reversal of the polarity of the doublet in [Arg^{11},Lys^{12}]proocytocin/neurophysin(1–20) had no effect on the mode of endoproteolytic cleavage as well as modifications of Gly^{10} (replaced by Ala^{10}). It is concluded that the selectivity of this endoprotease, which may be involved in the primary event occurring in proocytocin/neurophysin processing, is strictly dependent upon the integrity of the basic doublet but that other parameters determined by the amino acid sequence around this doublet may play an important role.

It is now well established that all known neuropeptides possess larger molecular weight biosynthetic precursors. Proteolytic cleavage of these pro forms occurs at selective loci constituted by basic amino acids often arranged as pairs, allowing the release of the corresponding active forms of the peptide hormones or neurotransmitters. In the hypothalamo–neurohypophyseal tract, as well as the ovarian corpus luteum, ocytocin (OT) is associated within secretory granules with a small (10 kDa) high disulfide containing protein called neurophysin (Np) [for reviews, see Breslow et al. (1979), Cohen et al. (1979), and Ivell et al. (1986)]. Both components derive from a common biosynthetic precursor (pro-OT/Np; 11 kDa) in which the nonapeptide ocytocin sequence, situated next to the leader peptide of the prepro form, occupies the NH_2-terminal sequence of the pro form (Land et al., 1983). It is separated from the neurophysin domain by a processing sequence Gly^{10}–Lys^{11}–Arg^{12}, which is excised during the maturation and which is probably involved in recognition by both proteolytic and amidating enzymes (Clamagirand et al., 1986; Kanmera & Chaiken, 1985). Examination of the proocytocin gene structure (Ivell & Richter, 1984a,b) indicates that the 1–21 NH_2-terminal domain of pro-OT/Np is encoded by the first (exon A) of a three exon containing 0.85 kba DNA. Prediction of the secondary structure for this domain indicates the conservation of a highly ordered structure constituted by a β-sheet/two β-turn/α-helix arrangement (Rholam et al., 1986) on which modification of amino acids at positions 2, 4, 6, 7, and 9 of the neurophysin sequence, observed in various animal species, has no influence. Therefore, we have used synthetic

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