Methanofuran (Carbon Dioxide Reduction Factor), a Formyl Carrier in Methane Production from Carbon Dioxide in *Methanobacterium*†

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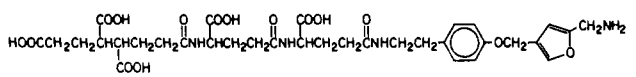
ABSTRACT: Methanofuran (carbon dioxide reduction factor) became labeled when incubated in cell extracts of *Methanobacterium* under hydrogen and $^{14}CO_2$ in the absence of methanopterin. Proton NMR spectroscopy revealed that a formyl group was bound to the primary amine of methanofuran. [^{14}C]Formylmethanofuran was enzymically converted to $^{14}CH_4$ in the presence of CH_3-S-CoM [2-(methylthio)ethanesulfonic acid], hydrogen, and methanopterin, establishing the formyl moiety as an intermediate in methanogenesis. In the absence of methanopterin, a substantial portion of the formyl label was oxidized to $^{14}CO_2$ rather than reduced to $^{14}CH_4$, consistent with a model in which the C_1 intermediate is first bound to methanofuran and then to methanopterin, during its reduction. When CH_3-S-CoM was replaced by HS-CoM (2-mercaptoethanesulfonic acid), most of the formyl label was oxidized to $^{14}CO_2$, indicating that methyl group reduction by the CH_3-S-CoM methylreductase is required for the conversion of formylmethanofuran to methane.

Carbon dioxide activation and reduction to methane by methanogenic bacteria involves a complex oxygen-sensitive multienzyme system; so far, the complete reduction can be studied only in crude extracts. When cell extracts of *Methanobacterium thermoautotrophicum* were passed through an anoxic Sephadex G-25 column, they were resolved for an unknown factor, named the carbon dioxide reduction factor (CDR factor), that was required for the conversion of CO_2 to CH_4 (Romesser & Wolfe, 1982a). This fraction was studied in detail (Leigh & Wolfe, 1983) and was separated into two fractions, both of which were required for CO_2 reduction to CH_4. One fraction was shown to be methanopterin, a newly identified pterin unique to methanogens (Keltjens et al., 1983a,b), and for the other fraction, the name CDR factor was retained. A structure for methanopterin has been reported (van Beelen et al., 1984a). The active form of methanopterin has been shown to be tetrahydromethanopterin, a carrier of C_1 moieties at the methenyl, methylene, and methyl levels of oxidation (van Beelen et al., 1984b; Escalante-Semerena et al., 1984a,b). The CDR factor has been determined to be 4-[[*p*-[[[[*N*-(4,5,7-tricarboxyheptanoyl)-L-γ-glutamyl]-L-γ-glutamyl]amino]ethyl]phenoxy]methyl]-2-(aminomethyl)furan (Leigh et al., 1984), and its structure is



methanofuran

Since a furan moiety is unusual in biochemistry, we have proposed the trivial name methanofuran for the new cofactor (Leigh et al., 1984). The role of methanofuran in CO_2 reduction remained obscure when both methanofuran and methanopterin were added to resolved Sephadex G-25 treated extracts. When methanopterin was not added, a C_1 intermediate accumulated; we report here that the first stable intermediate in this novel system of CO_2 fixation is formylmethanofuran.

CO_2 activation and reduction is not a simple enzymic reaction. In extracts of methanogenic bacteria, this process is dependent on and coupled to the terminal CH_3-S-CoM[1] methylreductase reaction; ratios (moles of CO_2 reduced to CH_4 per moles of CH_3-S-CoM added) as high as 11 have been observed (Gunsalus & Wolfe, 1977). This phenomenon has been termed the RPG effect after its discoverer R. P. Gunsalus. The terminal methylreductase reaction is a poorly understood system composed of four proteins (only one of which has been purified to homogeneity) and requires H_2, ATP, Mg^{2+}, FAD, CH_3-S-CoM, and component B, a coenzyme of unknown structure (Gunsalus & Wolfe, 1980; Nagle & Wolfe, 1983). The deazaflavin F_{420} (Eirich et al., 1978) and the nickel-containing tetrapyrrole F_{430} (Ellefson et al., 1982; Pfaltz et al., 1982; Livingston et al., 1984) are believed to be involved, although the hydrogen-driven system has not yet been resolved for these two components. Thus, activation of CO_2 and reduction to formylmethanofuran is part of a complex system in which some of the energy from hydrogen oxidation and methyl group reduction appears to be conserved in an unknown manner to drive CO_2 fixation. Our purpose here is to present evidence indicating that formylmethanofuran is the first stable product of CO_2 fixation and that the formyl moiety is an intermediate in methanogenesis.

Experimental Procedures

Preparation of ^{14}C-Labeled C_1 Intermediate. A methanogenic reaction as described previously (Leigh & Wolfe, 1983) was scaled up 10-fold and carried out in a 50-mL serum bottle sealed with a black butyl rubber stopper. CH_3-S-CoM was prepared according to Romesser & Balch (1980), and methanofuran, methanopterin, G-25 enzyme mixture, and com-

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[1] Abbreviations: CH_3-S-CoM, methyl coenzyme M or 2-(methylthio)ethanesulfonic acid; HS-CoM, coenzyme M or 2-mercaptoethanesulfonic acid; RPG effect, CH_3-S-CoM stimulation of CO_2 reduction to CH_4; component B, a coenzyme of unknown structure required for the CH_3-S-CoM methylreductase reaction; PIPES, 1,4-piperazinediethanesulfonic acid; G-25 enzyme mixture, whole extract freed of unbound low molecular weight factors by Sephadex G-25 chromatography; HPLC, high-performance liquid chromatography; PPO, 2,5-diphenyloxazole.

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ponent B were purified from extracts of *Methanobacterium thermoautotrophicaum* strain ΔH according to Leigh & Wolfe (1983). A standard reaction mixture contained unless otherwise noted 150 μmol of NaPIPES buffer at pH 6.9 (room temperature), 2 μmol of ATP, 2 μmol of CH_3-S-CoM, 100 μg of methanofuran, 1 mg of methanopterin, 750 μL (10.5 mg of protein) of G-25 enzyme mixture (whole extract freed of unbound low molecular weight factors by Sephadex G-25 chromatography) prepared with Mg^{2+}, and 50 μL of partially purified component B, in a final volume of 2 mL. After the addition of the above components the bottle was placed in an ice bath and flushed with a mixture of H_2 and CO_2 (80:20) for 5 min by means of inlet and outlet needles inserted through the stopper; the flow rate at the outlet was 100 mL/min. After the needles were removed, $Na_2{}^{14}CO_3$ (1 mCi) was added anaerobically by syringe, and CO_2 and $Na_2{}^{14}CO_3$ were allowed to equilibrate for 5 min. The reaction was initiated by transferring the bottle from the ice bath to a shaking water bath at 60 °C. The reaction was terminated by injecting 4 mL of anoxic methanol containing 0.75% (v/v) of formic acid, 0.35% (w/v) of ammonium formate, and 10 mmol of 2-mercaptoethanol per L. With appropriate precautions, the bottle was unstoppered, and the contents were adjusted to pH 3 with HCl and swirled under a stream of N_2 for 10 min to remove $^{14}CO_2$. Thereafter, the material was treated without anaerobic precautions. The mixture was sealed into a stainless steel centrifuge tube and centrifuged at 12000*g* for 10 min. Nearly 100% of the label in the centrifuge tube remained in the supernatant solution, which was decanted and evaporated under a stream of N_2 in the fume hood. The yellow syrup that remained was dissolved in 0.5 mL of high-performance liquid chromatography (HPLC) buffer (1% formic acid adjusted to pH 3 with NH_4OH), cleared of insoluble material by centrifugation in an Eppendorf centrifuge for 1 min, and chromatographed by HPLC with a Waters μBondapak C_{18} column (3.9 mm × 30 cm, 10 μm) preceded by a guard column. The HPLC equipment consisted of Waters Model 6000A pumps, a Kratos Model SF740 multiwavelength UV detector (Schoeffel Instrument Division, Westwood, NJ), and a Waters Model 660 gradient programmer. The flow rate was 2 mL/min. Fractions were eluted with HPLC buffer for 5 min, followed by a 1-h linear gradient (0–70% methanol in 1% formic acid, adjusted to pH 3 with NH_4OH). Fractions were dried and desalted by lyophilization.

Assay for Production of $^{14}CH_4$ or $^{14}CO_2$ from [^{14}C]-Formylmethanofuran. The assay was prepared as described for CH_4 production from CH_3-S-CoM and CO_2 (Leigh & Wolfe, 1983), except that [^{14}C]forymylmethanofuran was added anaerobically after flushing the assay vial with H_2. The reaction was carried out at 60 °C and was terminated by removing the vial from the temperature block. HCl (1 M, 25 μL) was added through the stopper by syringe, and a portion (0.5 mL) of the gas phase and a portion (10 μL) of the liquid phase were removed for measurement of radioactivity. KOH (1 M, 280 μL) was added, and another set of measurements was made. The radioactive gas was assayed in a proportional counter as described previously (Leigh & Wolfe, 1983). The liquid was added to 3 mL of scintillation cocktail (280 mL of toluene, 950 mL of Triton X-100, and 2.8 g of PPO) in a 5-mL miniature vial (Research Products International Corp., Mount Prospect, IL). The vial was inserted into a scintillation vial, and the disintegrations were counted as described previously (Leigh & Wolfe, 1983). For assay of the liquid after base addition, phenethylamine was added (0.25 mL to 3 mL of cocktail).

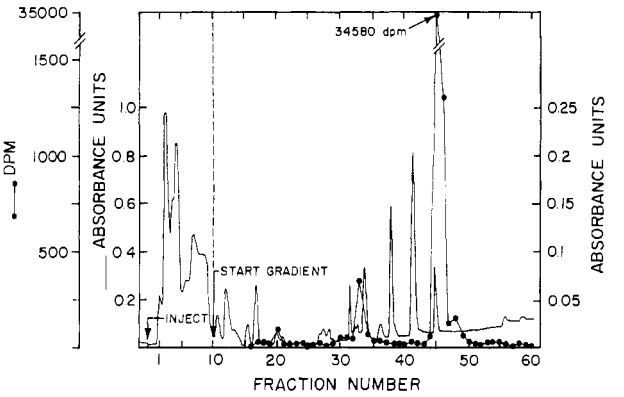


FIGURE 1: Fractionation by HPLC of potential intermediates in CH_4 production from CO_2. Compounds in the eluate were detected by UV absorbance at 215 nm. Radioactivity (dpm) represents the value obtained for a 100-μL portion of each 1-mL fraction. Absorbance units (continuous trace) to the left of the dashed line are measured on the left-hand scale and to the right on the right-hand scale.

^1H NMR Spectroscopy of Formylmethanofuran. The sample was desalted by HPLC as described previously (Leigh et al., 1984), and the spectrum was obtained on a Nicolet 360-MHz Fourier-transform spectrometer. The observed frequency was 360.061 MHz, the sweep width was 2000 Hz, the pulse width was 3 μs, and the postacquisition delay was 250 μs. A microcell sample bulb (Kontes Glass Co., Vineland, NJ) containing the sample was inserted into an NMR tube containing CCl_4 or $CDCl_3$.

RESULTS

Isolation of a New Intermediate in CH_4 Production from CO_2. In an effort to find new C_1 intermediates, a large-scale methanogenic reaction was carried out in the presence of $Na_2{}^{14}CO_3$, and a methanol extract was obtained and chromatographed by HPLC, as described under Experimental Procedures. All components required for CH_4 production from CO_2 were present (G-25 enzyme mixture, methanofuran, component B, ATP, Mg^{2+}, and CH_3-S-CoM), except methanopterin, which was omitted in order to detect an accumulation of any intermediate prior to the C_1 methanopterin derivatives. The reaction was terminated just after a maximal rate of CH_4 production was obtained (5 min; 500 nmol of CH_4 produced). A large accumulation of radioactivity was obtained in fraction 44 of the HPLC eluate (Figure 1). Fraction 44 served as a substrate for methanogenesis and also contained methanofuran activity in the methanogenic assay of Leigh & Wolfe (1983). In a separate experiment in which methanopterin was added, the fraction 44 peak was not present; in this case, the only intermediate found comigrated with CH_3-S-CoM (6.6 mL after injection). These results indicated that methanofuran was a carrier of a C_1 intermediate prior to methanopterin. A peak corresponding to fraction 44 was not detected when CH_3-S-CoM and methanopterin were both omitted; this observation was consistent with the previously proposed dependence of CO_2 activation on the CH_3-S-CoM methylreductase reaction (RPG effect; Gunsalus & Wolfe, 1977).

Structural Identification of the Intermediate. Fraction 44 was further characterized by ^{1}H NMR spectroscopy (Figure 2). Because of the small sample size, a microcell was used. The peak at 4.66 ppm and the signals between 0.8 and 1.6 ppm appeared to be contaminants present in a control sample (top) containing just D_2O in a microcell. A contribution to the peak at 4.66 ppm could also arise as a spinning sideband of HDO. In a spectrum (not shown) of fraction 44 in a microcell inserted into an NMR tube containing $CDCl_3$ (instead of CCl_4), the

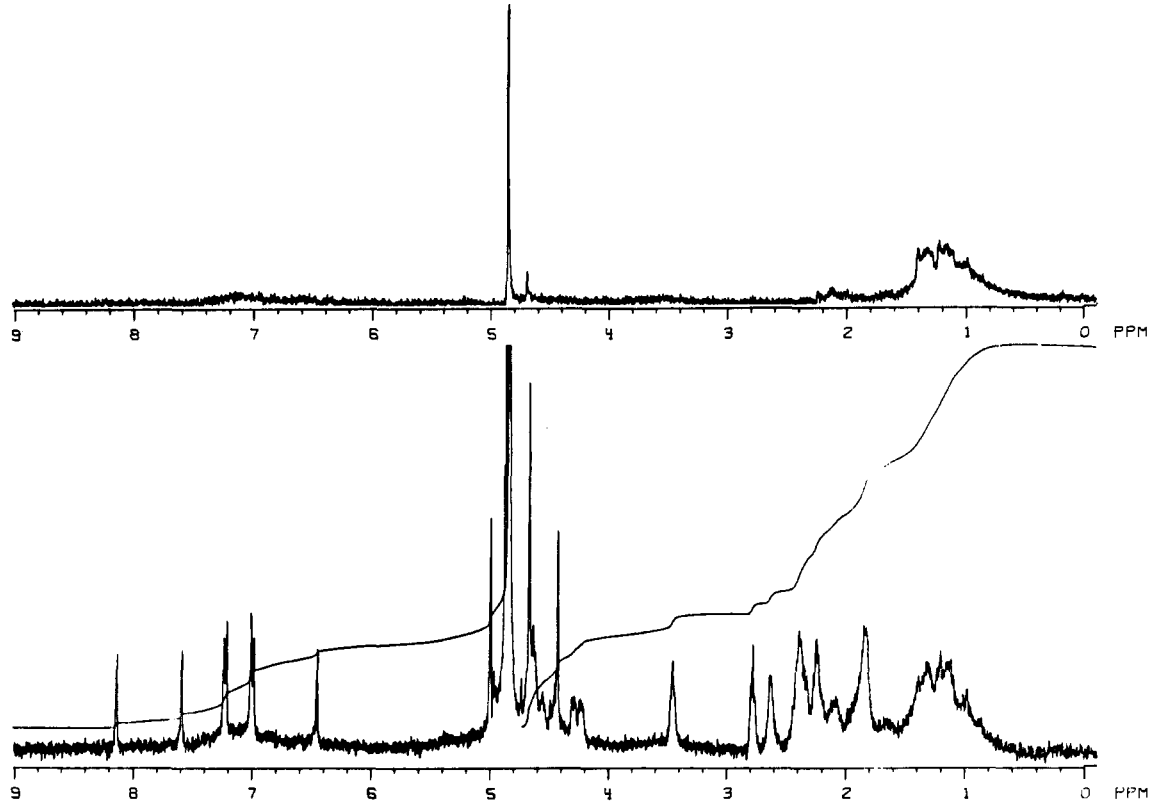
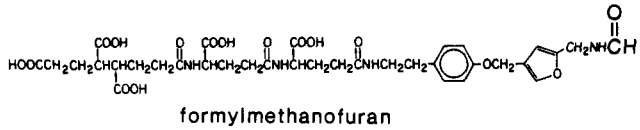


FIGURE 2: ^1H NMR spectrum of fraction 44 at pH 3. The solvent was D_2O. (Bottom) Fraction 44; (top) D_2O control.

peak at 4.66 ppm was not present. The only substantive differences between the spectrum of fraction 44 (pH 3) shown here and that of methanofuran at pH 2 (Leigh et al., 1984) were the presence of an additional signal at 8.13 ppm in fraction 44 and changes in the chemical shifts of certain signals noted below. The signal at 8.13 ppm was a one-proton singlet, indicating a formyl group. The formyl group was shown to be bound to the 2-(aminomethyl) group on the furan, since the methylene protons of the 2-(aminomethyl) group (4.42 ppm) were shifted downfield 0.21 ppm relative to the corresponding signal in methanofuran at pH 2. In addition, the signals of the furan protons in the 3 and 5 positions (6.46 and 7.59 ppm, respectively) were shifted upfield 0.21 and 0.08 ppm, respectively, relative to the corresponding signals in methanofuran at pH 2. The position of the formyl signal (8.13 ppm) agreed well with that of dimethylformamide (8.02 ppm; Bhacca et al., 1962) and, thus, was consistent with the structure



formylmethanofuran

The remaining signals in the spectrum agreed well with the spectrum of methanofuran at pH 2 and were assigned as follows (Leigh et al., 1984): The doublets at 7.23 and 6.99 ppm were H-3 and H-2, respectively, of the phenolic group. The signal at 4.99 ppm represented the oxymethyl protons of the (oxymethyl)furan. The signals around 4.25 ppm were the methine protons of both glutamic acid residues. The signals at 3.45 and 2.78 ppm were the H-2′ and H-1′ protons, respectively, of the (aminoethyl)phenol. The signal at 2.63 ppm represented both methine protons of the 4,5-dicarboxyoctanoic acid group. The remaining signals in the complex region between 2.5 and 1.7 ppm represented the methylene protons of both glutamic acid residues and the 4,5-dicarboxyoctanoic acid group. We concluded that fraction 44 consisted of formylmethanofuran.

Table I: Production of $^{14}CH_4$ and $^{14}CO_2$ from [^{14}C]Formylmethanofuran[a]

component omitted	after acid addition		after base addition		
	gas phase	liquid phase	gas phase	liquid phase	increased dpm in liquid phase
none	5390	1010	5190	1410	400
methanopterin	4510	2290	3190	4390	2100
CH_3-S-CoM	4695	1410	200	5290	3880

[a] Each reaction mixture contained (unless omitted) 15 μmol of Na-PIPES buffer at pH 6.9 (room temperature), 0.2 μmol of ATP, 75 μL (1.0 mg of protein) of G-25 enzyme mixture with Mg^{2+}, 200 nmol of CH_3-S-CoM, 200 nmol of HS-CoM if CH_3-S-CoM omitted, 2 μg of methanofuran, 30 μg of methanopterin, and 5 μL of component B in a total volume of 240 μL. After the mixture was flushed with 100% H_2, [^{14}C]formylmethanofuran was added anaerobically by syringe. In calculating the radioactivity present, adjustments were made for sample removed for previous measurements.

Conversion of [^{14}C]Formylmethanofuran to $^{14}CH_4$ or $^{14}CO_2$. In the methanogenic assay under H_2, [^{14}C]formylmethanofuran was converted to $^{14}CH_4$ or $^{14}CO_2$, depending on the conditions (Table I). The two gases comigrated in the gas chromatography system used, and were distinguished by the absorption of $^{14}CO_2$ into base. The CH_3-S-CoM added was about one-third converted to CH_4 10 min after the reaction was initiated, and the reaction was terminated after 2 h. The liquid contents were acidified, and the total radioactive gas produced ($^{14}CH_4$ + $^{14}CO_2$) was measured in the gas phase of the assay vial. Counts remaining in the liquid represented formylmethanofuran and C_1 derivatives not converted to gas. Then, base was added, and $^{14}CH_4$ was measured in the gas phase. $^{14}CO_2$ was estimated as the increase in radioactivity in the liquid following the addition of base. Results of controls

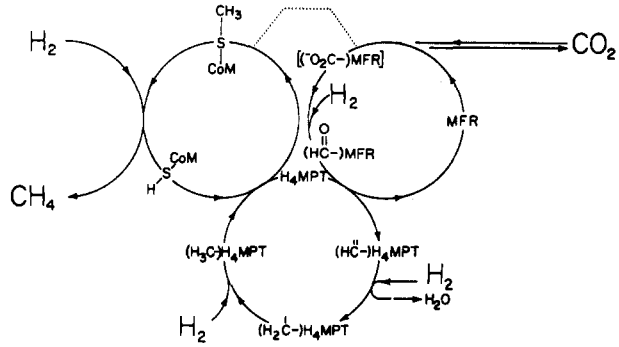


FIGURE 3: Proposed pathway of CH_4 production from CO_2. Abbreviations: MFR, methanofuran; H_4MPT, tetrahydromethanopterin. The dotted line indicates coupling between the terminal CH_3-S-CoM methylreductase reaction and the early steps in the pathway. Adapted from Escalante-Semerena (1983).

with standard $^{14}CO_2$ showed that CO_2 was completely absorbed into the liquid after base addition. As shown in Table I, when all components required for CH_4 production from CO_2 were present, about three-fourths of the label was converted to $^{14}CH_4$, and little to $^{14}CO_2$. When methanopterin was omitted, only two-thirds of the label was converted to gas; about equal amounts of $^{14}CH_4$ and $^{14}CO_2$ were produced, indicating that the formyl moiety of formylmethanofuran can be oxidized to CO_2 when the C_1 carrier methanopterin is limiting. When CH_3-S-CoM was omitted (replaced by HS-CoM), most of the formyl moiety was oxidized to $^{14}CO_2$, and very little was reduced to $^{14}CH_4$. This result indicates that an active CH_3-S-CoM methylreductase reaction is required to mobilize the formyl moiety in the methanogenic pathway; the RPG effect appears to function in the conversion of formylmethanofuran to CH_4 as well as in the conversion of CO_2. When G-25 enzyme mixture was omitted, no label was detected in the gas phase after addition of base and no increase was found in the liquid phase, indicating that the reactions responsible for the above conversions were enzymic. Similar to the above results, [^{14}C]formylmethanofuran was readily converted to $^{14}CH_4$ under an atmosphere of H_2 and CO_2 (80:20) (data not shown); since a large pool of nonradioactive CO_2 was present, this result showed that [^{14}C]formylmethanofuran was not converted first to $^{14}CO_2$ and then to $^{14}CH_4$.

Attempt To Isolate Carboxymethanofuran. In an attempt to show that methanofuran was a C_1 carrier at the carboxyl level as well as the formyl level, a large-scale incubation was performed as described above, under N_2 $^{14}CO_2$ (80:20) instead of H_2 $^{14}CO_2$ in the hope of obtaining carboxymethanofuran. A methanol extract was obtained and chromatographed by HPLC as described above. [^{14}C]Formylmethanofuran was produced, presumably due to endogenous reducing equivalents in the extract, but no other accumulation of radioactivity was detected. The experiment was repeated with a 25-min preincubation to purge the extract of endogenous reducing equivalents before adding anoxic methanofuran. No radioactive compounds were detected, indicating that neither carboxymethanofuran nor formylmethanofuran were produced. However, if the site of binding of the carboxyl group were the primary amine of methanofuran (i.e., the same as the formyl binding site), the unstable nature of such a carbamate would render carboxymethanofuran nonisolable.

Discussion

A previous report (Leigh & Wolfe, 1983) has shown that $^{14}CO_2$ is quantitatively converted to $^{14}CH_4$ by extracts of *M. thermoautotrophicum*. Thus, a compound that acquires label from $^{14}CO_2$ and donates this label for $^{14}CH_4$ formation can be considered to be a carrier of an intermediate in CH_4 production from CO_2. Intermediates identified so far are the methyl moiety of CH_3-S-CoM (Taylor & Wolfe, 1974a,b; Romesser & Wolfe, 1982a) and C_1 units attached to tetrahydromethanopterin (van Beelen et al., 1984b; Escalante-Semerena et al., 1984b). Here we have shown that methanofuran functions as a formyl carrier in methanogenesis and have identified the site of binding of the formyl group as the furfuryl amine of methanofuran. Although carboxymethanofuran proved nonisolable, it seems likely that methanofuran is the carrier at the carboxyl level as well, since no additional fractions were required for the formation of formylmethanofuran from CO_2 or the oxidation of the formyl moiety to CO_2. The formation of a carbamate between methanofuran and CO_2 would be analogous to the activation of ribulose-1,5-bisphosphate carboxylase, where the formation of an unstable carbamate occurs between a lysyl ϵ-amino group and CO_2 (Donnelly et al., 1983). Methanofuran functions prior to methanopterin in the pathway, since the absence of methanopterin elicits an accumulation of formylmethanofuran, and the conversion of formylmethanofuran to CH_4 is stimulated by methanopterin. The conversion of formylmethanofuran to CH_4 is stimulated by CH_3-S-CoM, indicating that the methylreductase is coupled to the reduction of formylmethanofuran in a manner similar to the RPG effect previously demonstrated for the conversion of CO_2 to CH_4. The proposed role of methanofuran and methanopterin are illustrated in Figure 3. CO_2 binds to methanofuran and is reduced to the formyl level. The formyl moiety is transfered to tetrahydromethanopterin as a methenyl group and is reduced through the methylene to the methyl level. The methyl moiety is transfered to HS-CoM and reduced to CH_4. The terminal CH_3-S-CoM methylreductase reaction stimulates the early steps in the pathway, and the cycle continues. The electron carriers involved in methanogenesis from H_2 and CO_2 are evidently present in G-25 enzyme mixture and have not been identified. However, the deazaflavin F_{420} (Eirich et al., 1978), which is present in extracts of *M. thermoautotrophicum*, is a likely candidate. The early steps in CO_2 reduction to CH_4 have been difficult to study in detail. The identification of formymethanofuran as the first stable intermediate in CO_2 reduction should help to open the way for further enzymological studies of this interesting pathway.

Registry No. CH_3-S-CoM, 53501-90-9; CH_3-S-CoM reductase, 53060-41-6; methanofuran, 89873-36-9; formylmethanofuran, 94483-60-0; methanopterin, 79484-89-2; hydrogen, 1333-74-0; carbon dioxide, 124-38-9; methane, 74-82-8.

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Affinity-Dependent Cross-Linking to Neurotoxin Sites of the Acetylcholine Receptor Mediated by Catechol Oxidation†

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ABSTRACT: The choline homologue 3-[(trimethylammonio)methyl]catechol (TMC) has been synthesized, and the controllable features of its complex oxidation have been examined spectroscopically and correlated with its toxin binding inactivating reactions with the acetylcholine receptor (AcChR) from *Torpedo californica* electroplax. Affinity-dependent reactions of early intermediates in the oxidation of TMC are suggested to intercede covalently in this inactivation. At pH 7.4, where the oxidative polymerization of catechols proceeds spontaneously, pyrocatechol produced no effect on the toxin binding function of AcChR, whereas comparable concentrations of TMC led to inactivation of half of all available sites. Lower concentrations of TMC converted via oxidation with ceric salts to an in situ mixture of monohydroxylated catechols were shown to be effective in short-term incubations in inactivating approximately half of the toxin binding sites by covalent labeling of the receptor. Mixtures of dihydroxycatechol intermediates, hydroxy-*p*-quinones, and polymeric products led to nonspecific toxin binding site inactivation of AcChR in excess of half of all available sites. Collectively, the results suggest that both covalent labeling and oxygen reduction product inactivating mechanisms are operative in these model macromolecular site reactions and that catechol-containing affinity reagents may be useful in elucidating the molecular features of sites to which they are directed.

Impressive control mechanisms have evolved over the reactions associated with the biological oxidation of catechols. Their oxidation occurs spontaneously at physiological pH with half-lives for this autocatalytic, complex reaction ranging from minutes to hours depending on the structure of the catechol. It is generally believed that cells that contain high concentrations of catechols also possess the means of blocking or controlling their spontaneous oxidation [cf. studies on chromaffin granules by Njus et al. (1982, 1983)]. Both reduced molecular oxygen products and reactive, electrophilic quinones are formed in the course of spontaneous catechol oxidation, and there is evidence that both classes of products are toxic to cells (Jonsson, 1980).

Despite the toxicity of the reaction products, spontaneous or enzyme-catalyzed oxidation of catechols mediates a number of important biological processes. Cross-linking reactions between proteins and the reactive quinones generated are known or are suspected to underlie most of these phenomena, which represent either compartmentalized or extracellular polymerizations or limited cytotoxic reactions. The formation of the pigment polymers of melanin is perhaps the most well-known such example (Pawelek & Korner, 1982) while the reactions accompanying the hardening of barnacle and other arthropod secretions (Pryor, 1940; Lindler et al., 1973), the cell-mediated immune responses to the plant wax catechols derived from poison ivy, oak, or sumac (Symes & Dawson, 1954; Mayer, 1955; Liberato et al., 1981), and the defensive reactions of some plants toward an attacking virus (Mink,

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