Targeting Methanopterin Biosynthesis To Inhibit Methanogenesis

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This paper describes the design, synthesis, and successful employment of inhibitors of 4-(β-D-ribofuranosyl)aminobenzene-5'-phosphate (RFA-P) synthase, which catalyzes the first committed step in the biosynthesis of methanopterin, to specifically halt the growth of methane-producing microbes. RFA-P synthase catalyzes the first step in the synthesis of tetrahydromethanopterin, a key cofactor required for methane formation and for one-carbon transformations in methanogens. A number of inhibitors, which are N-substituted derivatives of *p*-aminobenzoic acid (pABA), have been synthesized and their inhibition constants with RFA-P synthase have been determined. Based on comparisons of the inhibition constants among various inhibitors, we propose that the pABA binding site in RFA-P synthase has a relatively large hydrophobic pocket near the amino group. These enzyme-targeted inhibitors arrest the methanogenesis and growth of pure cultures of methanogens. Supplying pABA to the culture relieves the inhibition, indicating a competitive interaction between pABA and the inhibitor at the cellular target, which is most likely RFA-P synthase. The inhibitors do not adversely affect the growth of pure cultures of the bacteria (acetogens) that play a beneficial role in the rumen. Inhibitors added to dense ruminal fluid cultures (artificial rumena) halt methanogenesis; however, they do not inhibit volatile fatty acid (VFA) production and, in some cases, VFA levels are slightly elevated in the methanogenesis-inhibited cultures. We suggest that inhibiting methanopterin biosynthesis could be considered in strategies to decrease anthropogenic methane emissions, which could have an environmental benefit since methane is a potent greenhouse gas.

Biological methane formation is a microbial process catalyzed by methanogens, which are members of the *Archaea* domain, the third kingdom of life (23). Methanogens are found in most anaerobic environments, including the rumen of domesticated livestock (24). The beneficial effects of methanogenesis include the removal of H₂ formed during the oxidative metabolism of biomass, thus enhancing the biodegradation process (equations 1 and 2). However, there are several negative aspects of ruminant methanogenesis. Since methane production in the rumen results in a loss of between 3 and 12% of feed gross energy, inhibition of methanogenesis has long been considered as a strategy to improve agricultural productivity (25). Inhibition of ruminal methanogenesis can enhance production of the volatile fatty acids (VFAs) that are useful to the host (10). Furthermore, methane is a potent greenhouse gas and thus contributes to the problem of global warming (4). Ruminal methanogenesis produces about 80 million tons of methane per year (11), second only to the mining, processing, and use of coal, oil, and natural gas (100 million tons).

The objective of the research described here is to specifically inhibit a key methanogenic enzyme that is not present in the animal or in ruminal bacteria. We have targeted a biosynthetic

enzyme, 4-(β-D-ribofuranosyl)aminobenzene-5'-phosphate (RFA-P) synthase, which catalyzes the first step in methanopterin biosynthesis. The reduced form of methanopterin, tetrahydromethanopterin, is involved in multiple steps in methanogenesis; it also replaces the functions of tetrahydrofolic acid, the predominant one-carbon carrier in eukaryotes and bacteria. Given the importance of tetrahydromethanopterin in growth and in energy production by methanogens, the inhibition of RFA-P synthase should specifically halt methanopterin biosynthesis and thereby preclude methanogenesis without adversely affecting the metabolism of ruminal bacteria or the animal. The results described herein support this expectation.

In the first dedicated step of methanopterin biosynthesis, RFA-P synthase catalyzes the conversion of phosphoribosylpyrophosphate (PRPP) and *p*-aminobenzoate (pABA) to CO₂, inorganic pyrophosphate, and β-RFA-P (Fig. 1). Rasche and White have partially purified and characterized the methanogenic RFA-P synthase (17), and the enzyme from *Archaeoglobus fulgidus* has recently been purified to homogeneity and cloned and heterologously overexpressed (20). The reaction is thought to proceed via the oxycarbenium intermediate and its adduct with pABA (Fig. 1, structures 4 and 5, respectively). We have focused on designing competitive inhibitors that are structural analogs of pABA (Fig. 2). Analogs of pABA that inhibit RFA-P synthase are expected to be highly selective because the amino group is the nucleophile in most pABA-dependent reactions while the ring carbon 4 is the nucleophile in the RFA-P synthase-catalyzed reaction.

The inhibitors described herein both impair RFA-P synthase activity and arrest methanogenesis in pure cultures of methanogens (some in the submicromolar range) and in dense ru-

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FIG. 1. The reaction catalyzed by RFA-P synthase.

minimal fluid cultures (artificial rumena). Supplying an excess of the natural substrate pABA to the culture relieves the inhibition, suggesting that RFA-P synthase is the cellular target. The inhibitors do not adversely affect the growth of acetogenic bacteria, which play a beneficial role in the rumen. Our results also indicate that ruminal bacterial metabolism and ruminal dynamics in general are not adversely affected since there is no inhibition and, under some conditions, a slight elevation of VFA production in the methanogenesis-inhibited artificial rumen system. Based on comparisons of the inhibition constants among various inhibitors, we propose that the pABA binding site in RFA-P synthase has a relatively large hydrophobic pocket near the amino group.

MATERIALS AND METHODS

Materials. Sodium sulfide was purchased from Sigma-Aldrich. Monobromobimane (Thiolite) was purchased from Novabiochem. All buffers, media ingredients, and other reagents were acquired from Sigma-Aldrich. Solutions were prepared with nanopure deionized water. N₂ (99.98%), N₂-H₂ (90:10 [vol/vol]), Ar (99.8%), H₂-CO₂ (80:20 [vol/vol]), and CH₄-N₂ (0.2:99.8 [vol/vol]) were obtained from Linweld (Lincoln, Nebr.).

Growth of organisms. *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain Marburg) (strain OCM82) was obtained from the Oregon Collection of Methanogens and was cultured on H₂-CO₂-H₂S (80:20:0.1 [vol/vol/vol]) at 65°C in 15-ml Hungate tubes. Growth was measured by the optical density at 580 nm (OD₅₈₀). *Moorella thermoacetica* (formerly *Clostridium thermoacetatum*) (strain ATCC 39073) was grown at 55°C as previously described (1). *Methanobrevibacter smithii* (ATCC 35061) was grown at 37°C in 20-ml Hungate tubes containing 5 ml of media that included 12.5 g each of cysteine HCl and Na₂S per liter as reducing agents and 1.1 mM vancomycin with shaking at 200 rpm (16). The culture tubes were pressurized initially

and at 30-h intervals in H₂-CO₂ (80:20 [vol/vol]) to 190 kPa, and growth was assessed by measuring the OD₅₈₀.

Ruminal organisms were cultured in a shaking water bath (45 rpm) by a batch method (5) that used a bicarbonate- and phosphate-based buffer with added macro- and microminerals, cellobiose (2 g/liter), Trypticase (2 g/liter), and 12.8 mM Na₂S as a reductant. Five-milliliter cultures were incubated in 9.4-ml glass vials that were sealed and crimped with gas-tight septa. Fresh ruminal fluid was obtained from the rumena of two fistulated steers maintained on a diet of 70% forage and 30% grain, strained through four layers of cheesecloth, and added to buffer at 20% of final volume. The culture vials (5.4-ml headspace) were pressurized initially and after 12 h of incubation at 37°C in H₂-CO₂ (80:20 [vol/vol]) to 190 kPa. Candidate inhibitors were added to triplicate cultures in logarithmically spaced concentrations between 10 and 0.01 mM. Each experiment included cultures containing a known inhibitor of methanogenesis, 2-bromoethanesulfonate, as a positive control. After 30 h of incubation, the vials were cooled to 22°C, the headspace pressure was measured with a manometer, and 500 µl of the gas phase was assayed for methane and hydrogen by gas chromatography with a silica gel column equipped with a thermal conductivity detector.

Purification and assays. RFA-P synthase was partially purified as previously described (17). The enzymatic assay was performed in a reaction mixture with a total volume of 0.25 ml containing 3 mM [¹⁴C]carboxyl-labeled pABA, 10 mM PRPP, 25 mM MgCl₂, and 100 mM TES [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid] buffer, pH 4.8, and was initiated by adding enzyme (typically, 0.04 mg). The ¹⁴C label is eliminated as ¹⁴CO₂ during the reaction (Fig. 1). The reaction mixture was quenched with 100 µl of 1 M citric acid, pH 3.5, and the residual radioactivity in the reaction mixture was determined by liquid scintillation counting. (For further details, see the figure legends.)

The VFA concentration in the liquid phase of the ruminal batch cultures was assayed after centrifuging the cells and precipitating the proteins by adding one-fourth volume of 20% metaphosphoric acid. The VFA concentration in the supernatant was determined by gas chromatography with a Chromasorb WAW column and a flame ionization detector. The VFA concentration was also determined in parallel cultures in which ground brome hay replaced cellobiose, and headspace was pressurized with only CO₂ at inoculation of the cultures.

Synthesis of 4-(alkylamino)benzoic acid derivatives. Na(CN)BH₃ (1.4 molar equivalents) was carefully added to a nitrogen-blanketed mixture of 95% ethanol and acetic acid (90:10, vol/vol) containing 0.2 M pABA (1 molar equivalent) and the requisite aldehyde (1.3 molar equivalents). The resulting mixture was stirred at room temperature for approximately 24 to 36 h. Afterwards, the reaction mixture was diluted with water (ca. four times the volume of ethanol) and extracted three times with ethyl acetate. The combined organic layers were dried with anhydrous Na₂SO₄ and concentrated. The residue was purified by chromatography on silica with a mixture of hexanes and ethyl acetate as eluent or by crystallization from ethyl acetate. All compounds gave satisfactory spectral analysis and elemental composition. The *N*-alkyl-pABA derivatives are very stable compounds that are even resistant to autoclaving. The same resistance results are obtained whether the compounds are autoclaved or filter sterilized before addition to the medium. The pABA derivatives were dissolved as a stock solution in water before being added to the culture medium.

FIG. 2. A series of analogs of pABA (structure 6), wherein R¹ is a nonpolar or polar group of varying steric demand, was synthesized via the reductive amination of pABA.

FIG. 3. pABA analogs evaluated for RFA-P synthase inhibition and methanogen growth inhibition. K_i values for RFA-P synthase inhibition [μM ($\pm$ standard deviation)] are given in bold. Methanogen growth inhibition data are given in brackets for complete inhibition or delayed growth (growth lag in hours). NI, no inhibition at 1 mM concentration.

RESULTS AND DISCUSSION

The goal of the work described here was to test the concept that a specific inhibitor of methanogenesis could be developed based on the inhibition of an enzyme involved in the biosynthesis of a key cofactor, methanopterin. As described here, we were successful in developing inhibitors that target the first committed step in methanopterin biosynthesis and halt the growth of methanogens without adversely affecting bacteria involved in VFA production, as tested in pure culture and in a rumen model system.

A previously identified inhibitor, 4-(methylamino)benzoic acid (17) (Fig. 2, structure 6, $R^1 = \text{Me}$), was reexamined and found to have an inhibition constant (K_i) of 145 μM (Fig. 3). A number of pABA derivatives (Fig. 3) were tested for their ability to inhibit the RFA-P synthase reaction with the substrates (pABA and PRPP) at saturating concentrations. First, each compound was tested at a concentration of 1 mM, and if inhibition was observed, its concentration was varied to obtain a complete inhibition curve. Figure 4 shows representative results with 4-(isopropylamino)benzoic acid. The data for all inhibitors fit well to a competitive inhibition equation. Figure 3 shows the inhibition constants and the standard deviations for the pABA derivatives that were tested. Several of the new inhibitors have K_i values below 20 μM . pABA derivatives bear-

ing *n*-propyl, isopropyl, and isobutyl nitrogen substituents strongly inhibit the enzyme. These results suggest that the pABA binding site in RFA-P synthase has a relatively large hydrophobic pocket near the amino group. It is not clear why complete inhibition of the enzyme is not achieved; the final percentage of inhibition varied from 60 to 85%. The 2-hydroxyethyl and several aromatic derivatives, e.g., the furanyl-, thiophenyl-, phenyl-, and 2-pyridylmethyl derivatives, are particularly effective inhibitors. With the exception of the isobutyl derivative, branched, unbranched, and cyclic alkyl derivatives of four or more carbon atoms are ineffective. The *N,N*-dimethylamino analog of pABA is neither a substrate nor an inhibitor of the enzyme.

Each of the pABA analogs shown in Fig. 3 was then tested for its ability to inhibit methanogenesis and the growth of the methanogen *M. marburgensis* (Fig. 3 and 5A; Table 1). As in the enzyme inhibition experiments, the cultures were first grown in the presence of 1 mM analog. The cultures that did not alter growth were not pursued further and, as shown in Fig. 3, were scored as noninhibitory. Analogs that inhibited growth at 1 mM were studied further in various concentrations to obtain complete inhibition curves. The concentrations at which growth was completely inhibited are shown in Fig. 3, and the results of a representative experiment with isopropylamino-

FIG. 4. Inhibition of RFA-P synthase by pABA analog. Partially purified RFA-P synthase (17) was reacted with [^{14}C]carboxyl-labeled pABA and PRPP in the presence and absence of 4-(isopropylamino) benzoic acid. Elimination of $^{14}\text{CO}_2$ associated with RFA-P formation followed. The solid line shows the fit of the data to a competitive inhibition equation.

benzoate are shown in Fig. 5A. These presumed active-site-directed inhibitors extend the lag phase and decrease the final cell density in a dose-dependent manner. Methanogens are known to produce methane even in stationary phase; however, these derivatives inhibit methanogenesis in parallel with cell growth (Table 1). Insignificant amounts of methane were measured in the headspace of *M. marburgensis* cultures whose growth was completely inhibited. Several N-substituted pABA derivatives that inhibit growth at high concentrations (0.5 to 1 mM) do not inhibit the synthase reaction. These compounds probably exert their effect by a mechanism unrelated to the RFA-P synthase and have not been further studied. Figure 3 shows that the best inhibitors of RFA-P synthase also are the most potent inhibitors of *M. marburgensis*. At 100 nM, the most potent inhibitor currently, 4-[(2-pyridylmethyl)amino]benzoic acid, completely arrests the growth of and methane formation by *M. marburgensis*. Inhibition is fully reversed by supplementing the medium with pABA, indicating a competitive interaction between pABA and the inhibitor at the cellular target, which is most likely RFAP synthase. When the cells were

FIG. 5. (A) Inhibition of methanogen growth by pABA analog. *M. marburgensis* was cultured at 65°C as described elsewhere (6, 19) in the presence of 0 (●), 15 (□), 25 (▲), 75 (■), and 90 (◇) μM 4-(isopropylamino)benzoic acid. Growth was followed by measuring absorbance at 580 nm. (B) Growth of acetogen in the presence of pABA analogs. *M. thermoacetica* was grown in 155-ml vials with glucose as the carbon source at 55°C (1) and growth was followed by measuring absorbance at 600 nm in the presence (■) and absence (●) of 1 mM 4-(isopropylamino)benzoic acid. A, absorbance.

TABLE 1. Inhibition of growth and methanogenesis of *M. marburgensis*

β -RFA-P synthase inhibitor	Methane produced ^a (μmol)	Inhibition of growth ^b (μM)
4-Propylaminobenzoic acid	0.050 $\pm$ 0.002	600
4-Isopropylaminobenzoic acid	0.010 $\pm$ 0.001	100
4-Isobutylaminobenzoic acid	0.020 $\pm$ 0.001	500
4[(Pyridin-2-ylmethyl)amino]benzoic acid	0.078 $\pm$ 0.002	0.1
4-(2-Hydroxyethylamino)benzoic acid	0.072 $\pm$ 0.002	300
4-[(Furan-3-ylmethyl)amino]benzoic acid	0.059 $\pm$ 0.001	100
Control experiment	598 $\pm$ 2	NA

^a Total amount of methane produced $\pm$ standard deviation after incubation for 48 h.

^b Minimum inhibitory concentration. NA, not applicable.

grown in the presence of inhibitor for 5 days (instead of the standard incubation for 2 days), the OD continued to decrease to zero.

One might wonder why we used this thermophilic methanogen instead of a ruminal microbe. These studies, which involve hundreds of biological assays, require the use of a rapidly growing and robust methanogen like *M. marburgensis* that can be cultured to fairly high cell density. In our experience, the ruminal methanogen *Methanobrevibacter ruminantium* does not meet these requirements. To rule out the hypothesis that inhibition is exclusive to this one strain, we performed two types of experiments. First, although our studies of other strains are not as extensive, the best inhibitors for *M. marburgensis* also inhibit the methanogenesis and growth of *M. smithii*, a mesophilic human intestinal methanogen (data not shown). Second, as described below, we did observe the inhibition of methanogenesis in an artificial rumen system (containing all

the naturally occurring ruminal microbes) with the same inhibitors that prevent growth of *M. marburgensis*.

Acetogenesis is an anaerobic and hydrogenotrophic bacterial process (equation 3) that competes with methanogenesis in many anaerobic habitats, including the rumen (13, 14). Acetogenic bacteria are beneficial since ruminant animals can use acetate as a nutrient. Each of the inhibitors was tested for its effect on the growth of the acetogenic bacterium *M. thermoacetica*. Methanopterin is not required for survival of bacteria; accordingly, none of the RFA-P synthase inhibitors described here affect the growth of *M. thermoacetica* at concentrations as high as 1 mM (Fig. 5B). Acetogenic bacteria, which use the Wood-Ljungdahl pathway, demand high levels of folate since they contain 1,000-fold higher amounts of tetrahydrofolate enzymes than most other organisms. Folic acid is not added to the medium beyond the amount present in yeast extract. That these compounds do not adversely affect the growth of acetogens at concentrations of over 100-fold higher than those required to inhibit methanogens suggests that these pABA derivatives do not inhibit folate biosynthesis. Although acetogens are the only class of bacteria that have been specifically tested in pure culture with the RFA-P synthase inhibitors, results with the artificial rumen indicate that bacterial metabolism in general is not adversely affected (see below).

We tested the effect of the inhibitors on methane formation and VFA production in an artificial rumen. Ruminal fluid, obtained from fistulated steers, was cultured in the presence of inhibitors of the RFA-P synthase or the cultured methanogen. Ruminal fluid is a complex medium containing more than 60 species of bacteria at a density exceeding 10^{11} cells/g plus numerous species of archaea, protozoa, and fungi. Remarkably, at least three of the active pABA derivatives inhibit ($P < 0.01$) methanogenesis in the artificial rumen. Methane production is completely inhibited by 5 mM 4-(ethylamino)benzoate or 9 mM 4-(isopropylamino)benzoate, and 5 mM 4-(2-hydroxyethylamino)benzoate inhibited methane production to 2.5% of the control level. As a control, 1 mM bromoethanesulfonate, an inhibitor of methyl-coenzyme M reductase, completely inhibited ($P < 0.01$) methane production in all experiments. We suspect that a higher concentration of the pABA analog than of the enzyme is required to inhibit growing cultures because of competition with pABA produced by the cells.

We determined the effect of some of the effective inhibitors on VFA production in the ruminal fluid culture (Fig. 6). VFA production by ruminal organisms is not depressed by adding an RFAP synthase inhibitor at concentrations that completely block methanogenesis. For example, when 7 mM 4-ethylaminobenzoate was added to the artificial rumen system, acetate ($P < 0.05$) and propionate ($P < 0.10$) levels were elevated relative to the controls unexposed to the inhibitors. These results are consistent with the studies with pure cultures of acetogenic bacteria and indicate that the inhibitors do not adversely affect other ruminal bacteria or ruminal dynamics. These experiments are important because a strategy for reducing methane emissions from ruminal livestock will only be practical if it does not adversely affect ruminal dynamics or the health of the host. This requirement was a key factor in the

FIG. 6. Effect of a pABA-based RFAP synthase inhibitor on VFA production in a mixed culture of ruminal organisms. Fresh, strained ruminal contents (0.8 ml) were incubated at 37°C for 30 h in 10-ml vials ($n = 4$ for 0 dose; $n = 2$ for all other doses) with 3.2 ml of McDougal's buffer containing Ti citrate, Trypticase, and bromegrass hay ground to pass through a 1-mm-pore-size screen. Headspace was pressurized to 10 kPa with CO_2 at time zero and again after 18 h. VFA concentration was determined by gas chromatography and flame ionization detection. Dose effects were analyzed by analysis of variance (overall F-test, $P < 0.13$), and a least significant difference test was used for mean separation. Error bars represent standard errors of the means. a, increased concentration of acetate versus the level of the control ($P < 0.05$); b, increased concentration of propionate versus the level of the control ($P < 0.10$).

strategy of targeting RFAP synthase, which should be specific to methanogens. The slight increases in acetate and propionate are consistent with the expectation that inhibition of ruminal methanogenesis will enhance the conversion of fibrous feedstuffs into metabolites that are useful to the host rather than lost to the environment. VFAs produced by ruminal bacteria constitute the ruminant animal's primary energy source.

During the past 30 years, various strategies for inhibiting methanogenesis have been considered. Davies et al. (3) successfully inhibited methanogenesis with a series of 2,4-bis (trichloromethyl)benzo[1,3] dioxins in mixed ruminal cultures, in acute intraruminal models, and in chronic (4-week) experiments with sheep and cattle. Concurrent with significantly less methanogenesis, VFA production was in some cases unchanged, and in other cases it was increased. Feed efficiency during 14 weeks of treatment was improved in cattle fed the methanogenesis inhibitors. Eli Lilly markets monensin, an ionophore and gram-positive antibiotic that decreases methanogenesis (21), which is fed to nearly all feedlot cattle in the United States. An increase in VFA production was observed when methanogenesis was inhibited by the ionophore monensin (2, 15, 21). The increased feed efficiency achieved by feeding monensin to cattle was interpreted as a result, at least in part, of decreased methanogenesis (21). However, monensin, unlike the RFA-P synthase inhibitors, impairs all H_2 -forming microbial processes, which negatively impacts beneficial processes like acetogenesis as well as methanogenesis. Other non-specific inhibitors of methanogenesis include nitrogen oxides (nitrate, nitrite, NO, and N_2O), which have been studied in rice field soils (12, 18). Bromoethanesulfonate, a specific inhibitor of a key enzyme in methane formation (methyl-coenzyme M

reductase) (7), has been tested in an infused sheep rumen; however, resistance develops within a few days (10). Future studies will be required to determine if methanogens develop resistance to these RFA-P synthase inhibitors.

Our studies are based on (and so far are consistent with) the hypothesis that treating animals with a specific inhibitor of methanogenesis will have a beneficial effect on the animal by increasing the levels of VFAs in the rumen. Hackstein et al. have proposed that methanogens form a symbiotic relationship with mammals, birds, and reptiles and that the development of a gastrointestinal system that can house methanogens is evolutionarily advantageous (8, 9). The ability to specifically inhibit methanogenesis would allow long-term monitoring of an animal's growth rate, feed efficiency, ruminal function, and overall health and offer a test of Hackstein's hypothesis.

The global atmospheric methane burden has doubled over the past 200 years to reach its present value of 1.75 ppm. The continuing rise in methane levels is due predominantly to greenhouse gas emissions from human activities and contributes to climate change. It has been noted by the U.S. Environmental Protective Agency that, unlike other methane emission sources for which there are technologies or practices aimed specifically at reducing emissions, no control options are currently available for reducing enteric fermentation (22). Based upon the results discussed above, these inhibitors of RFA-P synthase hold promise for use as antimicrobial agents in ruminant livestock to reduce methane emissions.

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