Elucidation of methanogenic coenzyme biosyntheses: from spectroscopy to genomics

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Methanogenesis, the anaerobic production of methane from CO_2 or simple carbon compounds, requires seven organic coenzymes. This review describes pathways for the biosynthesis of methanofuran, 5,6,7,8-tetrahydromethanopterin, coenzyme F_{420}, coenzyme M (2-mercaptoethanesulfonic acid) and coenzyme B (7-mercaptoheptanoyl-L-threonine phosphate). Spectroscopic evidence for the pathways is reviewed and recent efforts are described to identify and characterize the biosynthetic enzymes from methanogenic archaea. The literature from 1971 to September 2001 is reviewed, and 169 references are cited.

1 Introduction
2 Methanofuran
3 Methanopterin
4 Coenzyme F_{420}
5 Coenzyme M
6 Coenzyme B
7 Discussion
8 Acknowledgements
9 References

1 Introduction

Each year methanogenic microbes produce more than 400 million tons of methane.[1] Unlike most metabolic processes, methanogenesis is performed by only one group of organisms, the Archaea. Archaea are prokaryotic: their morphologies, ecologies and physiologies are similar to those of bacteria.[2] However analyses of archaeal small-subunit ribosomal RNA sequences showed that they are evolutionarily distinct from bacteria.[3] In fact, phylogenetic analysis of critical genes that comprise their information processing systems (DNA replication, transcription and translation) shows the archaea to be more similar to Eucarya than to Bacteria.[4]

In addition to their divergent evolutionary history, many archaea live in extreme environments (temperature, salinity, pH, *etc.*).[5] Studies of archaea and their physiologies are motivated by interest in life under these extreme conditions and by

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Robert White was born in 1946 in the United States and received his BS in Chemistry from Indiana University, Bloomington, in 1968. While serving for two years in the Army he did research in the laboratory of Professor Roger Cramer at the University of Hawaii. He obtained his PhD in Biochemistry from the University of Illinois, Urbana-Champaign, in 1974. After one year postdoctoral positions with Professor Stanley L. Miller and then Professor Trevor McMorris at the University of California San Diego he moved to Rice University as a lecturer-spectroscopist. In 1980 he joined the Department of Biochemistry at Virginia Tech, Blacksburg. He has had a long interest in the biosynthesis of the coenzymes and how their biosyntheses are related to the origin of metabolism and life.

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questions about the evolution of the modern lineages. However, these organisms are also ecologically important in many environments. Recent studies of archaeal metabolic processes such as methanogenesis and cofactor biosyntheses have unexpectedly contributed to our knowledge of some analogous reactions in eukaryotic and bacterial organisms.

The methanogenic archaea produce CH_4 from CO_2 (using electrons from H_2 or formate), from methyl group-containing compounds such as methanol or methylamine, or from acetate by a fermentative process.[6] Many methanogens such as *Methanococcus* spp. are specialists; they can produce methane from only a few substrates. Others, such as *Methanosarcina* spp., can metabolize a number of different carbon substrates, including CO_2, formate, acetate or pyruvate.[7] All of the methanogens are anaerobes. Traces of oxygen either inhibit methanogenesis temporarily or kill the cells altogether.[8] Methanogens thrive in anaerobic environments, including wetlands, animal rumens, termite guts, sludge digesters and hydrothermal vents where there are significant pools of reduced compounds but few high potential electron acceptors.[6] Methanogenesis is an impecunious lifestyle—cells derive less than one ATP equivalent from each molecule of methane produced.[9] Yet many methanogens are autotrophs (CO_2 is their only source of carbon) and can grow with doubling times less than one hour.

Modern studies of the methanogenic CO_2 reduction pathway began in the laboratory of Ralph Wolfe in the early 1970's.[10,11] Although many questions about the methanogenic enzymes remain unanswered, the pathway was elucidated by the early 1990's. During this twenty year interval, six new methanogenic coenzymes were discovered in Wolfe's lab.[12] Coenzymes are "nonprotein molecules that function as essential parts of enzymes."[13,14] In this review we describe the proposed biosynthetic pathways for five of these coenzymes and summarize recent work discovering the enzymes that assemble these coenzymes.

The methanogenic reduction of CO_2 requires eight enzymes and six coenzymes (Fig. 1).[15–18] It begins with the carboxylation of the amino group of methanofuran **1** to form an *N*-carboxymethanofuran.[19] A molybdenum or tungsten metalloenzyme, formylmethanofuran dehydrogenase (EC 1.2.99.5), reduces this carbamate to formylmethanofuran **2**.[20,21] The formylmethanofuran:tetrahydromethanopterin *N*-formyltransferase (EC 2.3.1.101) moves the formyl group to N-5 of 5,6,7,8-tetrahydromethanopterin **3** (H_4MPT).[22] Methenyl-H_4MPT cyclohydrolase (EC 3.5.4.27) cyclizes the N^5-formylmethanopterin **4** producing the N^5,N^{10}-methenyl-H_4MPT **5** intermediate.[23] Reduction of the cyclic intermediate is catalyzed by either an F_{420}-dependent methylene-H_4MPT dehydrogenase (EC 1.5.99.9)[24] or a H_2-dependent methylene-H_4MPT dehydrogenase (EC 1.5.99.11).[25] A second reductive step is catalyzed by F_{420}-dependent methylene-H_4MPT reductase.[26,27] Both coenzyme F_{420}-dependent oxidoreductases use the reduced form of deazariboflavin coenzyme F_{420} **6**. The resulting N^5-methyl-H_4MPT **7** is a substrate for methyl-H_4MPT:coenzyme M methyltransferase (EC 2.1.1.86).[28] This corrinoid-containing enzyme methylates the thiol of coenzyme M **8** (CoM; 2-mercaptoethanesulfonic acid) using methyl-H_4MPT. Methyl-coenzyme M reductase releases methane and forms the heterodisulfide of CoM with coenzyme B **9** (CoB; 7-mercaptoheptanoyl-L-threonine phosphate).[29] This oxidoreductase contains a nickel-tetrapyrrole cofactor (F_{430}).[30] Finally, heterodisulfide reductase (EC 1.12.99.2) reduces the CoM–S–S–CoB disulfide bond, regenerating the CoM and CoB thiols.[31]

A few model organisms have been used in most studies of methanogenesis and its requisite cofactors. All of the archaea known to produce methane are members of the Euryarchaeota, although not all euryarchaea are methanogens.[32,33] *Methanobacterium thermoautotrophicum* strains Marburg and ΔH[34] have been studied extensively—they are ecologically important, readily cultured in large reactors and the complete genome sequence of strain ΔH is now available.[35] However these organisms have a rigid pseudomurein-containing cell wall that hinders genetic manipulation. *Methanococcus jannaschii* JAL-1 is a hyperthermophilic marine methanogen[36] and its DNA sequence was the first complete archaeal genome sequence published.[37] *M. jannaschii* can be grown to high cell densities in a reactor [38] and genetic systems have been developed for mesophilic *Methanococcus* spp.[39] Rumen isolate 10–16B is a methanogenic coccobacillus that grows autotrophically but readily assimilates exogenous compounds.[40] That strain has proved useful for isotope incorporation studies. Finally *Methanosarcina* spp. are ecologically abundant organisms that are model systems for studying methanogenesis from diverse carbon substrates.[6] These cells are readily grown in culture, several partial and complete genome sequences are available and sophisticated genetic systems are available.[41] Although all of these organisms carry out methanogenesis using a similar set of enzymes and cofactors,[16,42] it has become clear from genome sequences that they differ in their specific biosynthetic pathways and enzymology. Therefore proposed biosynthetic schemes must be tested in organisms from each lineage.

A classical method of elucidating biochemical pathways is to feed an organism isotopically labeled substrates and then measure the label incorporated into product. Despite the diversity of known metabolic pathways and reactions, almost all biomolecules are derived from a small set of organic molecules: glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, erythrose-4-phosphate, triose phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, acetyl-CoA, 2-oxoglutarate, succinyl-CoA and oxaloacetate that are made from a handful of inorganic compounds (H_2O, H_2, CO_2, formate, O_2, S (various oxidation states), PO_4^{3-} and NH_4^+).[43,44] From measurements of the extent and distribution of label incorporated into nucleotides, sugars or amino acids, one can deduce the primary precursors of the compound being studied. This retrobiosynthetic strategy is frequently applied using either NMR or mass spectrometry.[45] NMR spectroscopy, using ^{13}C, ^{15}N and ^{18}O isotopes, gives considerable structural information about the position of label incorporation, even in complex mixtures. Mass spectrometry is highly sensitive; fragmentation patterns can give insight into structure and deuterium exchange often chronicles unseen intermediary reactions. Radioisotopically labeled compounds are easy to detect during purification and characterization, but specific incorporation and the atomic position usually cannot be directly measured.

NMR and MS techniques are complementary and have been used by the Bacher and White lab groups to study the biosyntheses of methanogenic coenzymes.[14,45] Similar experiments have been used to survey general biosynthetic pathways in the methanogen *Methanospirillum hungatei*,[46] amino acid biosynthesis in the halophilic euryarchaeon *Haloarcula hispanica*,[47] and amino acid biosynthesis in *Methanococcus voltae* and *Methanococcus maripaludis*.[48,49]

Once the origin of each atom in the novel compound is known, a biosynthetic scheme must be proposed. As in all other syntheses, familiar, analogous reactions are preferred over novel ones. Enzymology often vindicates such predictions, as genes are frequently recruited to new pathways. Applying Ockham's razor, the most parsimonious scheme is usually the best starting point for *in vitro* experiments. By studying cell-extract catalyzed conversion of putative substrates into intermediates and products, one can test the plausibility of the proposed schemes. Synthetic intermediates, when available, are invaluable for confirming the identity of isolated intermediates as well as for testing their conversion into product. Unfortunately, a number of key intermediates in coenzyme biosynthesis are unstable. Others are difficult to synthesize with necessary stereochemical purity. Such intermediates are usually best synthesized enzymatically, sometimes through coupled reactions. No proposed

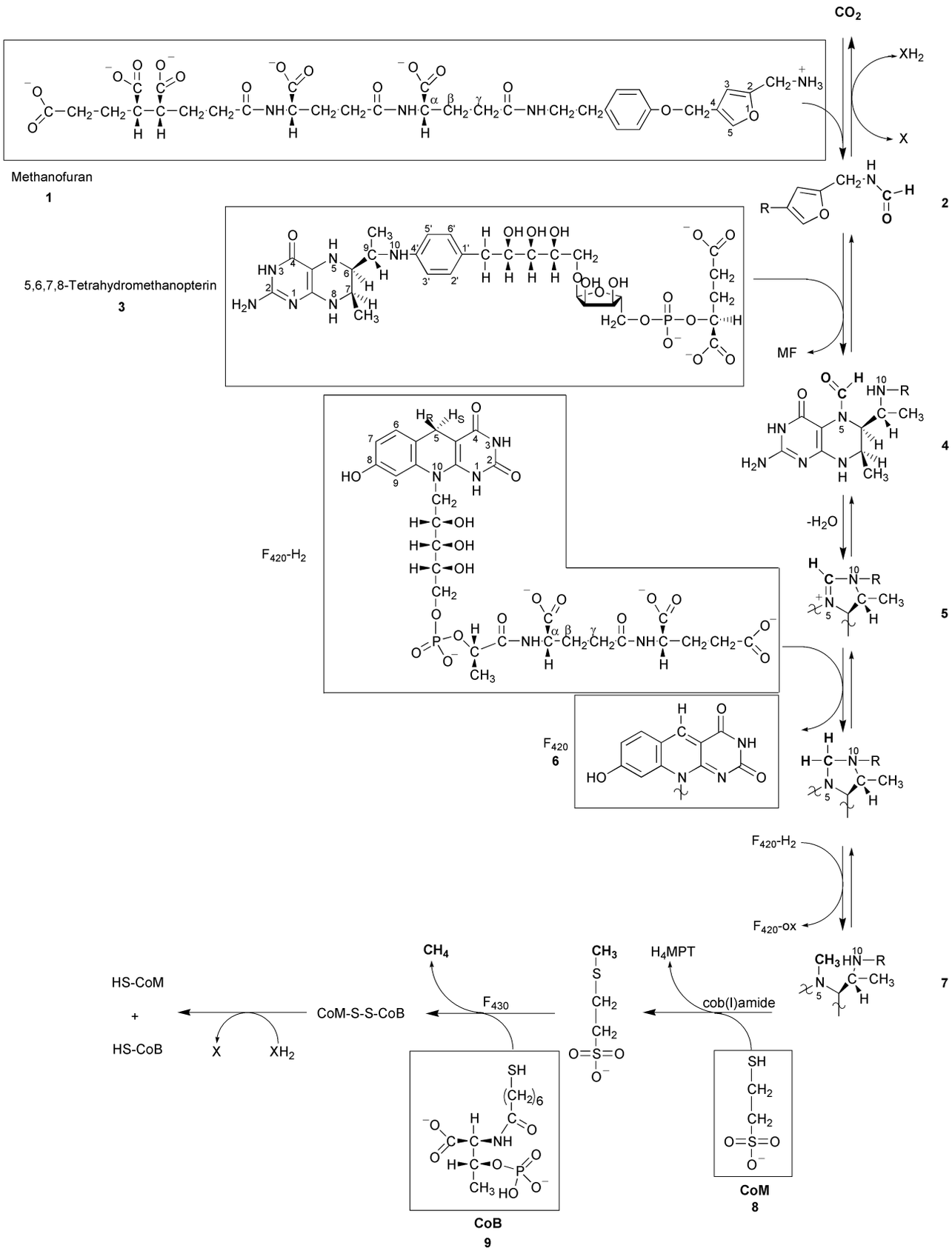

Fig. 1 Methanogenesis by the reduction of CO_2.

pathway, however, may be considered correct until the requisite enzymes have been identified and characterized, both *in vitro* and *in vivo*.

There are two traditional methods of identifying enzymes that catalyze specific reactions. Genetic analysis identifies mutant organisms missing the requisite activity and then maps the mutations on the chromosome. Biochemical analysis isolates the activity from cell homogenate. Occasionally the two methods have been combined, as in complementation assays for replication enzymes.[50] Advances in macromolecular biology and protein chemistry have helped to combine the two techniques.

The advent of complete genome sequences has invigorated physiological studies. Complete genome sequences from at least seventeen archaea are now known. In the five years since the publication of its genome sequence, *Methanococcus jannaschii* has come from obscurity to become an archaeal exemplar.[37] Theoretical metabolic reconstructions and intergenomic comparisons of biochemical pathways have identified complete, incomplete and missing pathways.[51–54] In searching for genes that function in novel pathways, we find two classes of genes to be particularly interesting.[55] Orphan genes are similar to characterized genes, but the pathway in which they are known to function is incomplete or missing in the organism's genome. Paralogs are genes that duplicated within a chromosome. In both cases homologs—evolutionarily related genes—may have acquired new functions and been recruited to new pathways.[56] Frequently an enzyme's function is not obvious from its structure.[57] In some pathways, functionally associated genes are encoded in clusters or operons on chromosomes.[58] In studying the biosynthesis of coenzyme M, we have found these gene clusters to be especially useful to identify enzymes that have novel reaction mechanisms or have no characterized homologs in the databases. As diverse genome sequences accumulate another bioinformatic technique becomes useful: concordance analysis identifies genes that are unique to genomes of organisms with a specified phenotype.[59]

Recombinant protein expression systems have vastly simplified protein purification and identification. Recombinant hyperthermophilic enzymes are particularly easy to isolate because they are usually stable to heat treatment at temperatures that denature native *E. coli* proteins. Except in the case of post-translationally modified proteins, recombinant proteins are structurally and functionally equivalent to native proteins, but much less expensive to produce. Even without automation, it is possible to produce purified recombinant protein from a gene sequence in less than two weeks. Therefore it is often simpler to clone and assay a handful of recombinant proteins to find a desired activity than it is to purify a single native protein.

In this review we survey early spectroscopic evidence for the methanogenic coenzymes' biosynthetic pathways and describe more recent results that identify the biosynthetic enzymes from genomes. For each of the five coenzyme biosyntheses we explore analogies to other pathways and emphasize their utility in discovering and characterizing the responsible enzymes.

2 Methanofuran

In the first step of the methanogenic reduction of CO_2, methanofuran **1** reacts with CO_2 to form an N-carboxymethanofuran.[19] The formylmethanofuran dehydrogenase enzyme reduces this carbamate to formylmethanofuran.[20,21] Although furans have been identified in polyketides and fatty acid derived natural products[60] and are often formed during the heating or oxidation of carbohydrates,[61] methanofuran is the only cofactor known to contain a furan moiety. Archaea (and probably bacterial methylotrophs[62]) produce at least five different methanofuran cofactors.[63] Each of these variants has a central core structure of 4-[*N*-(γ-L-glutamyl-γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan, with additional structures attached by an amide bond to the α-amino of the terminal glutamyl residue. The first methanofuran isolated, from *Methanobacterium thermoautotrophicum*, consists of the core structure attached to 1,3,4,6-hexanetetracarboxylic acid (HTCA).[64] Alternatively methanofuran *b*, from *Methanosarcina barkeri*, has two γ-linked glutamic acids[65] and methanofuran *c*, from *Methanobrevibacter smithii*, has 2-hydroxyHTCA.[63] Methanofuran and methanofuran *b* have been chemically synthesized and shown to be functionally and spectroscopically equivalent to the natural product.[66]

The biosynthesis of methanofurans involves four fragments joined by ether or amide bonds: a 2,4-disubstituted furan, tyramine, two glutamates and a variable structure such as HTCA. To study the biosynthesis of the 2-(aminomethyl)-4-(hydroxymethyl)furan, methanogen 10–16B cells were fed [1,2-$^{13}C_2$]-acetate or [2,2,2-$^{2}H_3$]acetate and derivatives of this furan product were analyzed by GC-MS.[67] The isotope incorporation patterns suggest that dihydroxyacetone-phosphate **10** (DHAP) condenses with phosphoenolpyruvate **11** (PEP) in the first step of methanofuran biosynthesis (Fig. 2(A)). After elimination of

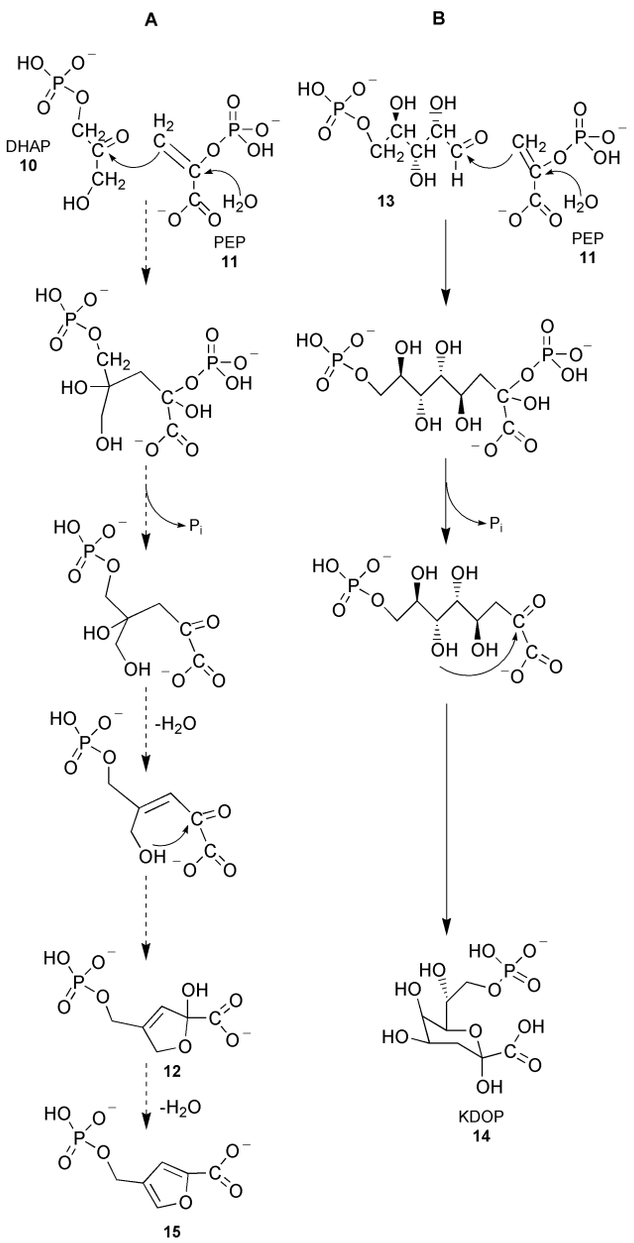

Fig. 2 Analogous condensation, cyclization reactions. Scheme A, biosynthesis of the 2,4-disubstituted furan. Scheme B, biosynthesis of 3-deoxy-D-*manno*-2-octulosonic acid 8-phosphate.

phosphate and dehydration, the intermediate cyclizes to produce the phosphate ester of the dihydrofuran **12**, which eliminates water to form **15** (Fig. 2(A)). The proposed reactions cleave the carbon–oxygen bond from PEP, analogous to the reaction described for 3-deoxy-D-*manno*-2-octulosonic acid 8-phosphate synthase (KDOPS) (Fig. 2(B)) and for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase.[68,69] In the KDOPS reaction mechanism, PEP condenses with D-arabinose-5-phosphate **13**, the 2-carbon–oxygen bond of PEP is broken and then the molecule cyclizes forming the KDOP product **14** (Fig. 2(B)). An alternative, but less energetically favorable mechanism

involves the condensation of pyruvate with DHAP, analogous to the dihydrodipicolinate synthase reaction mechanism (not shown).[70]

^{13}C NMR studies on methanofuran isolated from *M. thermoautotrophicum* Marburg cells grown on [1-^{13}C]acetate, [2-^{13}C]acetate, [1,2-$^{13}C_2$]acetate or [1-^{13}C]pyruvate support the proposed biosynthesis of the furan moiety from pyruvate/PEP and DHAP.[71] The 2,4-disubstituted furan **15** is probably reduced in an ATP and NAD(P)H-dependent reaction to form the 2-furaldehyde **16**. Dephosphorylated **16** has been previously isolated from plant rhizomes.[72] Transamination and reaction with tyramine **18** produces an ether-linked intermediate **19** (Fig. 3). Isotope incorporation data also showed that tyramine is produced from tyrosine **17**.[71]

Glutamates linked by amide bonds to the γ-carbon carboxylate have been identified in diverse cofactors including folates, F_{420}, and glutathione as well as methanofuran. The mechanism of glutamate addition in methanofuran biosynthesis may be similar to those proposed for γ-glutamylcysteine synthetase,[73] γ-glutamyltranspeptidases[74] or folylpoly-γ-glutamate synthetase.[75]

HTCA is derived from 2-oxoglutarate, acetyl-CoA and CO_2.[71] *M. thermoautotrophicum* ΔH contains significant amounts of free *meso*-HTCA with a similar pattern of isotopic label incorporation.[76,77] This pool of HTCA is metabolically inactive and the molecules may function as osmolytes. A pathway for the biosynthesis of HTCA in *Methanosarcina thermophila* TM-1 has recently been proposed based on the metabolism of several intermediates by cell-free extract (D. Howell and R. White, unpublished data).[14] In the final step of methanofuran biosynthesis, an acyl transferase is proposed to form a peptide bond from a glutamate-bound intermediate **20** and the HTCA-CoA thioester **21** (Fig. 3).

Only one protein has been shown to be specifically involved in methanofuran biosynthesis. The *mfnA* gene from *M. jannaschii* encodes an L-tyrosine decarboxylase (R. White, unpublished data). This tyramine-producing enzyme is similar to the group II pyridoxal-phosphate dependent amino acid decarboxylases.[78] Despite mechanistic similarities between the KDOPS-catalyzed aldol condensation and the proposed pathway for 2,4-disubstituted furan biosynthesis, no homolog of KDOPS has been found in the methanogen genomes. Nor have homologs of the γ-glutamyl ligases been identified.

3 Methanopterin

Methanopterin (MPT) is the intermediate C_1 carrier in methanogenesis.[79] After the formylmethanofuran:tetrahydromethanopterin formyltransferase enzyme produces *N*-5-formylmethanopterin **4**, two oxidoreductases reduce the formyl group to a methyl group (Fig. 1).[15] Methanopterin was originally identified during studies of $^{14}CO_2$ incorporation by cell-free extracts of *Methanobacterium thermoautotrophicum* ΔH and *Methanosarcina barkeri*.[80] The precursor of this isotopically labeled yellow fluorescent compound was later isolated from *M. thermoautotrophicum* and named methanopterin.[81] The structures of methanopterin from *M. thermoautotrophicum* and sarcinapterin, a similar cofactor from *M. barkeri*, were elucidated using ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and circular dichroism spectroscopy.[82–84] Similar pterins have been identified in other methanogens and bacterial methylotrophs as well as in non-methanogenic euryarchaea and crenarchaea.[85–88] Because the structures of methanopterin and sarcinapterin are substantially identical except in their dicarboxylic acid side chains, most steps in their biosyntheses are presumed to be identical and we describe both as methanopterin in the discussion below.

The structures of methanopterin and related pterins are similar to those of folates. Folates consist of pteroic acid conjugated to L-glutamate(s) whereas methanopterin contains the same pteroic acid core structure but a ribitol-containing side

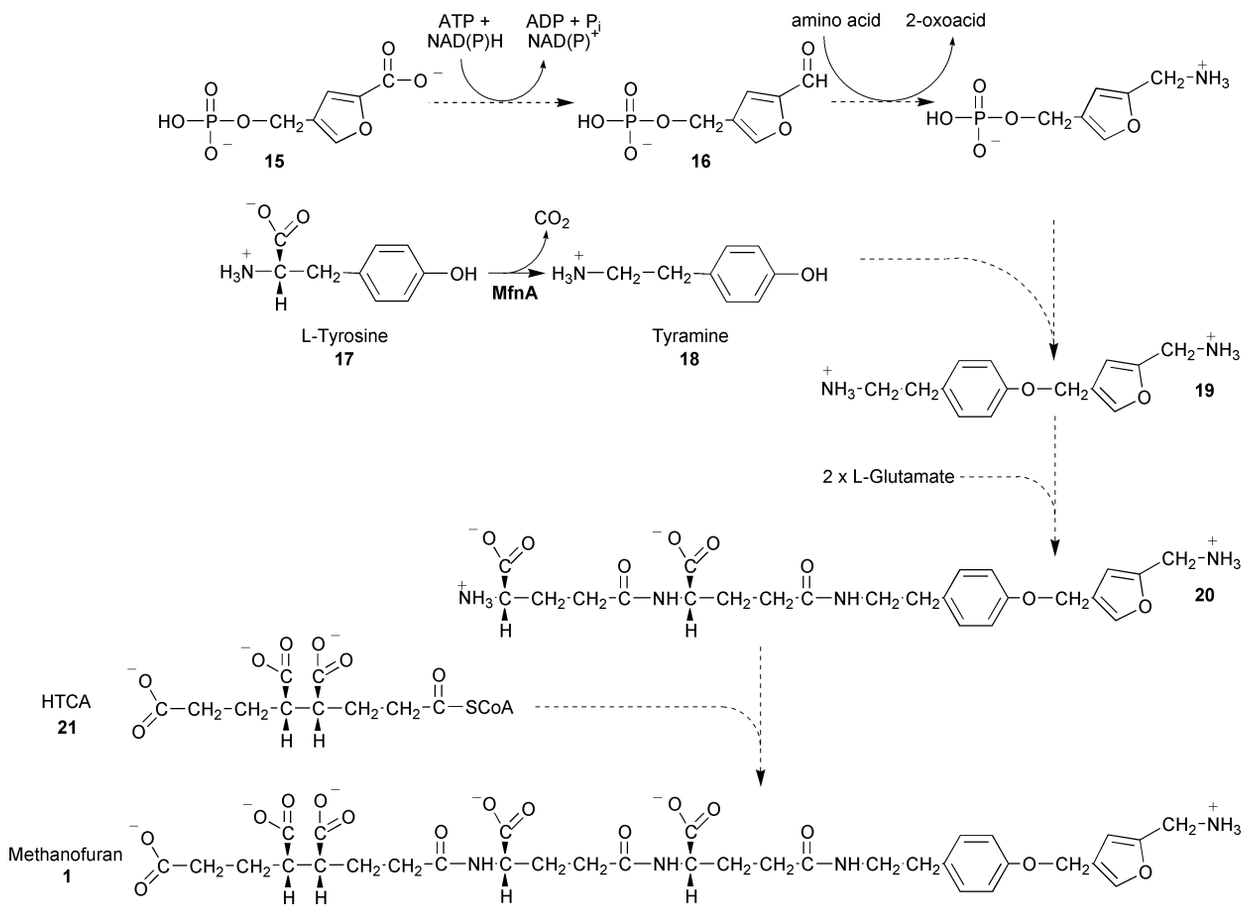

Fig. 3 Biosynthesis of methanofuran.

chain replaces its carboxylic acid (Fig. 4).[85] Both cofactors are biologically active in their reduced forms: tetrahydromethanopterin (H_4MPT) **3** and tetrahydrofolate (H_4F) **22** and both pterins have the same stereochemistry.[84,89] Both cofactors bind carbon at either their N-5 or N-5 and N-10 atoms. Although H_4MPT and H_4F are C_1 carriers in the reduction of a formyl group, the two are functionally differentiated and most enzymes specifically recognize one or the other.[90]

The pterin ring of both H_4F and H_4MPT originates from GTP.[91,92] When methanogen 10-16B was fed [^{15}N]glycine the label was incorporated with equal efficiency into the N-7 atom of guanosine and the N-5 nitrogen of H_4MPT.[91] Studies of [1-^{13}C]acetate incorporation into methanopterin from *M. thermoautotrophicum* confirm that the guanine ring of GTP is cleaved, releasing formate, and the intermediate recyclizes using the ribose side chain to form a phosphorylated 7,8-dihydroneopterin.[93] In folate biosynthesis, this product is 7,8-dihydroneopterin 3′-triphosphate; however, in *M. thermoautotrophicum* and *M. thermophila* methanopterin biosynthesis produces 7,8-dihydroneopterin 2′:3′-cyclic phosphate.[94] In both biosyntheses, the products are dephosphorylated and glycoaldehyde is released from the resulting 7,8-dihydroneopterin by a reversed aldol reaction. ATP is then used to pyrophosphorylate the 6-hydroxymethyl-7,8-dihydropterin to form **23** (Fig. 4).

The next step in folate biosynthesis condenses **23** to 4-aminobenzoate **24** (a derivative of chorismate).[92] The analogous reaction in methanopterin biosynthesis is the reaction of **23** with 4-(β-D-ribofuranosyl)aminobenzene 5′-phosphate (β-RFA-P) **25**.[95] β-RFA-P is formed in *M. thermophila* from the condensation of 4-aminobenzoate with the purine precursor 5-phospho-α-D-ribosyl-diphosphate (PRPP) **26** (Fig. 4).[96] This reaction is unique among known PRPP transferases in that one substrate (4-aminobenzoate) is decarboxylated and a C-riboside rather than an N-riboside is produced.

The resulting dihydropteroate **27** intermediate in folate biosynthesis condenses in an ATP-dependent reaction with L-glutamate and is reduced to tetrahydrofolate **22**. The analogous intermediate in methanopterin biosynthesis **28** reacts with an

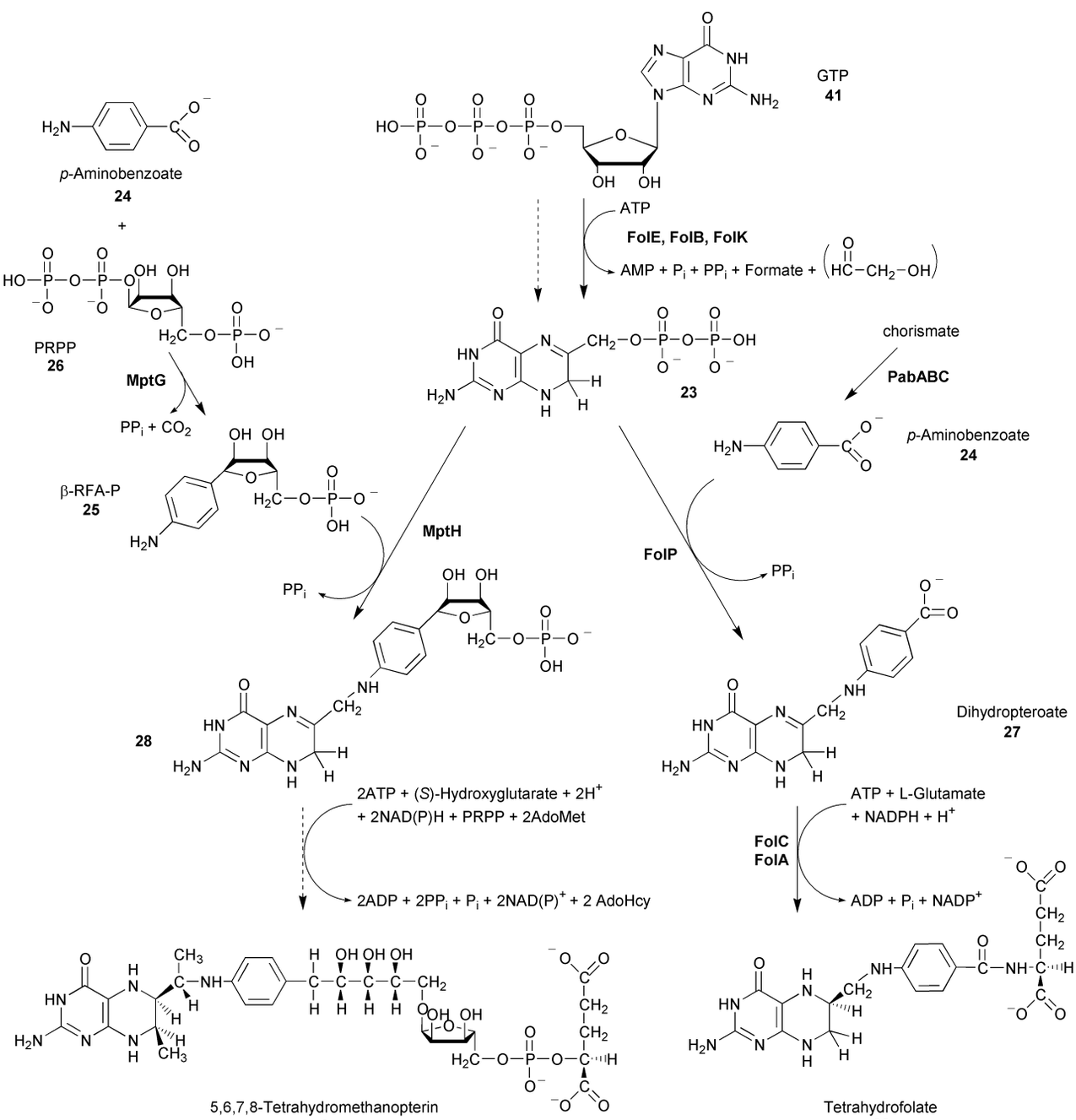

Fig. 4 Analogous biosyntheses of tetrahydromethanopterin and tetrahydrofolate.

additional molecule of PRPP before being linked to (*S*)-2-hydroxyglutarate, methylated at the C-7 and C-9 atoms by *S*-adenosyl-L-methionine (AdoMet), and reduced to 5,6,7,8-tetrahydromethanopterin **3** (Fig. 4).[95,97,98]

Despite substantial similarities between the folate and methanopterin biosynthetic pathways, few homologs of the known folate biosynthetic enzymes have been identified in methanogens. *E. coli* genes *folEBK*, which encode enzymes required for the biosynthesis of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate **23** have no recognizable homologs in euryarchaeal genomes. Genes for 4-aminobenzoate biosynthesis from chorismate (*pabAB* in *E. coli*) are paralogous to tryptophan biosynthesis genes, but have no obvious orthologs in archaea. Nevertheless, *M. jannaschii* employs a highly diverged homolog of dihydropteroate synthase to catalyze the addition of β-RFA-P to **23** (MptH in Fig. 4).[99] A 2-hydroxyacid oxidoreductase (ComC) has been identified in *M. jannaschii* and shown to reduce 2-oxoglutarate to 2-hydroxyglutarate.[100] However that enzyme has broad substrate specificity and its *in vivo* functions are unclear. Finally, the AdoMet-dependent methyltransferases that methylate pterin atoms C-7 and C-9 may be members of a large family of archaeal proteins that contain the AdoMet-binding domain (D. Graham, unpublished data). No homologs of dihydrofolate reductase have been identified in the methanogens despite the structural and stereochemical similarities between the dihydrofolate and dihydromethanopterin substrates.

4 Coenzyme F_{420}

The redox-active coenzyme F_{420} (*N*-(*N*-L-lactyl-γ-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate) **6** was first isolated from *Methanobacterium* str. M.o.H. based on the oxidized cofactor's intense absorbance peak at 420 nm and its blue-green fluorescence.[101] Cell extract from that methanogen uses electrons from hydrogen to reduce the F_{420} deazaflavin to a non-fluorescent form.[102] The fluorescence of coenzyme F_{420} was considered characteristic of hydrogen-metabolizing euryarchaea until it was identified in halobacteria[103] and in high G+C Gram-positive bacteria.[104] Some cyanobacteria produce a DNA photolyase that contains an 8-hydroxy-5-deazariboflavin cofactor.[105] The list of oxidoreductases that transfer a hydride to or from the *si* face of F_{420} grows continually.[106,107] Although F_{420} cofactors from these diverse organisms have comparable spectroscopic properties and are often functionally interchangeable, they differ in their glutamate side chains. Mature F_{420} molecules are derived from F_{420}-0 by the addition of 1–7 glutamates linked by amide bonds to the α- or γ-carbons.

The reduction potential of F_{420}/F_{420}-H_2 (and its FO precursor) is −340 to −350 mV, between the redox potentials of NAD(P)/NAD(P)H and $2H^+/H_2$.[108] At first glance, the 8-hydroxy-5-deazariboflavin moiety of F_{420} is structurally similar to riboflavin, but the substitutions lower the coenzyme's redox potential, prevent F_{420} from accepting a single electron to form a radical flavin semiquinone and render it less susceptible to aerobic oxidation. Because of these properties, it has been suggested that the chemistry of F_{420} is more similar to nicotinamide chemistry than to flavin chemistry.[18,108] However the biosynthesis of F_{420} and riboflavin from a shared precursor and the similar charge distributions of reduced 1,5-dihydrodeazariboflavin and 1,5-dihydroriboflavin rings link F_{420} to riboflavin. Similarities between F_{420} and nicotinamide hydride donors probably result from convergent evolution.

The structure of F_{420}-2 from *Methanobacterium* M.o.H. was solved by studying the acid hydrolysate fragments: SAC (γ-glutamyl-L-glutamic acid, which hydrolyzes further to L-glutamic acid), (*S*)-lactate, and chromophore F+ (which hydrolyzes further to phosphate and an acid-stable chromophore (FO)).[102] These fragments were identified using UV–Vis, IR, ^{1}H and ^{13}C NMR spectroscopy; subsequently, FO and F_{420} were synthesized for comparison with the natural product.[109,110]

The biosynthesis of F_{420} proceeds modularly, with intermediates that parallel the acid hydrolysate fragments. 7,8-Didemethyl-8-hydroxy-5-deazariboflavin **29** (FO) was identified in the supernatant of *Methanobacterium thermoautotrophicum* ΔH cultures, suggesting that it is a precursor in F_{420} biosynthesis.[111] Subsequent studies with *M. thermoautotrophicum* str. Marburg showed that the pyrimidine ring of FO is synthesized by a mechanism similar to riboflavin biosynthesis: label from [2-^{14}C]guanine, but not [8-^{14}C]guanine is incorporated into F_{420}.[112] The same organism incorporated [1-^{13}C]acetate, [2-^{13}C]acetate, [1,2-^{13}C]acetate or [1-^{13}C]pyruvate into FO to give a labeling pattern consistent with the pyrimidine ring and ribityl chain originating from guanosine and the phenolic ring and deazaflavin C-5 from tyrosine.[113]

The biosynthesis of FO by CofGH enzymes (Fig. 5(B)) resembles a pathway recently proposed for the biosynthesis of thiazole phosphate **34**, a precursor of thiamin.[14,114] In the ThiGH-catalyzed biosynthesis of thiazole (Fig. 5(A)), L-tyrosine **17** condenses with 1-deoxy-D-xylulose-5-phosphate **30** (DXP) to form a Schiff's base. The nucleophilic enamine attacks a ThiF-ThiS protein acyldisulfide producing a ThiS protein-bound thiolate and releasing quinone methide **31**, which is hydrated to form **32**. This thiolate intermediate cyclizes, displacing ThiS. Finally, **33** is decarboxylated and dehydrated to form thiazole-phosphate **34**. Several of the early steps in thiazole-phosphate biosynthesis have analogs in FO biosynthesis: 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione **35**, an intermediate in riboflavin biosynthesis, condenses with 4-hydroxypyruvate **36**, a precursor of L-tyrosine, to form a Schiff's base. Rather than releasing a quinone methide from **37**, the Schiff's base cyclizes, converting the β-carbon from 4-hydroxypyruvate into C-5 of the nascent deazaflavin. After deamination and the release of C-1 and C-2 from 4-hydroxypyruvate as glyoxylate **38**, the compound cyclizes to form FO **29**. The analogy of these two pathways is shown in Fig. 5.

Biosynthesis of the phosphodiester bond and lactate moiety of F_{420} is unusually complicated.[115] Rather than phosphorylating FO to make the F+ fragment, *M. jannaschii* and *Methanosarcina thermophila* TM-1 incorporate GTP-activated (*S*)-2-phospholactate. This mechanism is similar to one demonstrated for adenosylcobalamin (AdoCbl) **44** biosynthesis in *Salmonella enterica* (Fig. 6(A)).[116] In the adenosylcobalamin pathway, the CobU enzyme phosphorylates adenosylcobinamide (AdoCbi) **39** then guanylates it, displacing pyrophosphate from GTP **41**. The CobS enzyme catalyzes the nucleophilic attack of α-ribazole **42** on the activated AdoCbi-GDP **43**, releasing GMP and AdoCbl **44**. In F_{420}-0 biosynthesis, the CofC enzyme catalyzes the analogous guanylation of (*S*)-2-phospholactate **45** producing lactyl(2)diphospho-(5′)-guanosine **46** (LPPG). The CofD enzyme uses FO **29** to displace GMP resulting in the lactate phosphodiester of FO (F_{420}-0) **47** (Fig. 6(B)).[115]

Subsequent steps in the maturation of F_{420} include the condensation of multiple L-glutamates, linked by amide bonds to the α- or γ-carbons. Analogous γ-linked glutamates are polymerized by folylpoly-γ-glutamate synthetase in folate biosynthesis[75] and by unidentified enzymes in methanofuran biosynthesis. F_{420} isolated from *M. jannaschii* appears to contain an α-linked glutamate, in addition to the γ-linked glutamates (M. Graupner and R. White, unpublished data). α-Linked glutamates have also been identified in folates isolated from *Escherichia coli*.[117,118]

The enzymology of F_{420} biosynthesis has been studied recently in *M. jannaschii* by biochemical methods (M. Graupner and R. White, unpublished data) and by genetic methods in *Mycobacterium bovis*.[119] Assuming that homologous enzymes catalyze the FO or F_{420} biosynthetic reactions in different organisms, gene candidates can also be identified by their

phylogenetic distribution in genome sequences of euryarchaea, high G+C Gram-positive bacteria and cyanobacteria that produce deazariboflavin cofactors.

Five *M. jannaschii* genes involved in F_{420} biosynthesis have been cloned and expressed in *E. coli* (M. Graupner, H. Xu and R. White, unpublished data). The *cofC* gene is an ortholog of the *cobY* cobalamin biosynthetic gene described in *M. thermoautotrophicum*.[116] Therefore this enzyme appears to catalyze the guanylation of both (*S*)-2-phospholactate and adenosylcobinamide phosphate. The *cofD* gene was originally identified in a screen of *Mycobacterium bovis* PA-824 antibiotic-resistant mutants.[119] The CofD protein has no significant sequence similarity to the CobS protein that catalyzes the analogous reaction in cobalamin biosynthesis (Fig. 6(B)). The CofE protein, which adds one L-glutamate to F_{420}-0 to make F_{420}-1, has no similarity to previously characterized proteins.

The FO biosynthetic gene identified in *M. bovis* produces a protein with two domains (L. Daniels, personal communication); in *M. jannaschii* these domains are encoded by separate genes, *cofG* and *cofH*. Although they catalyze analogous

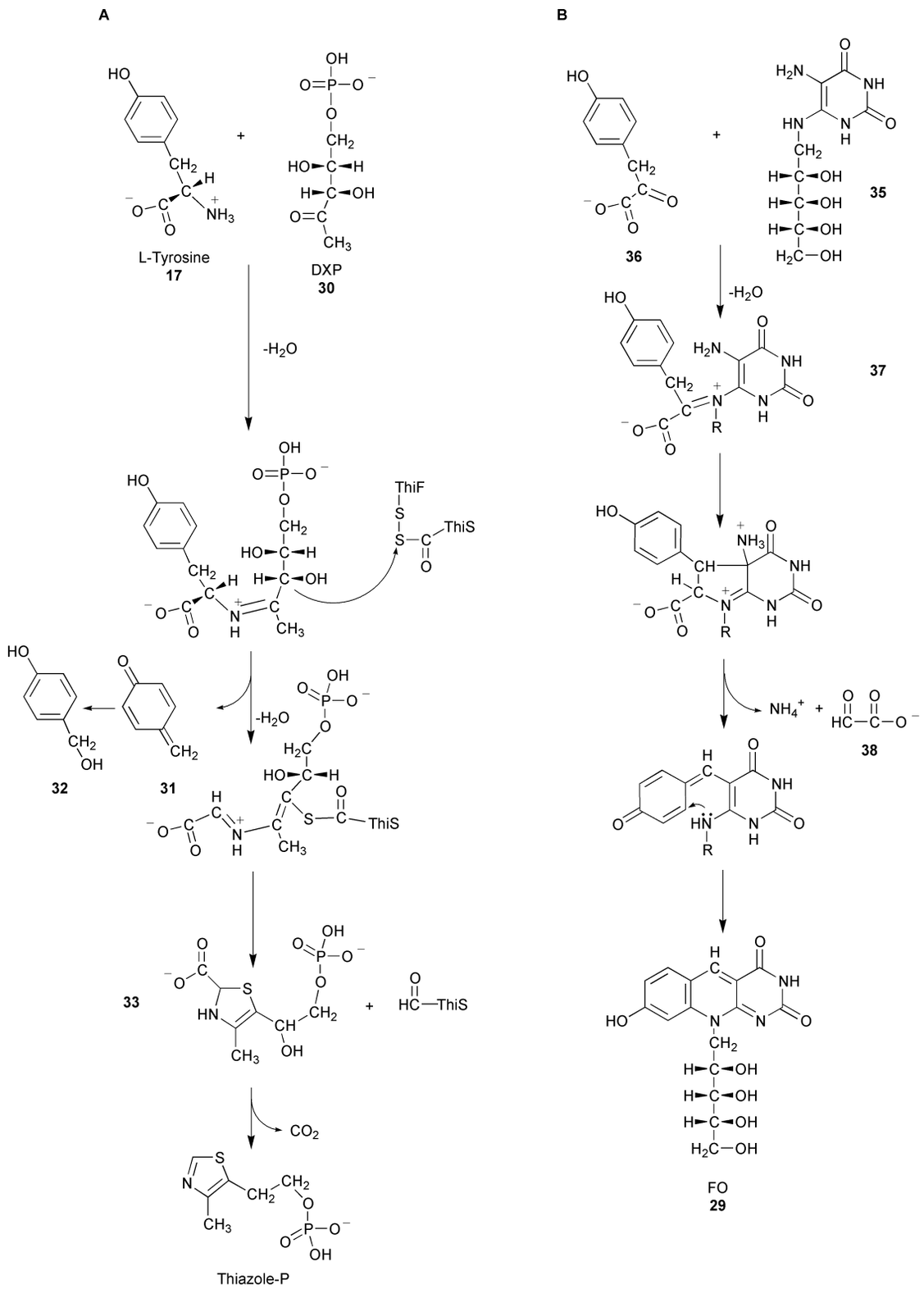

Fig. 5 Analogous condensation and cyclization reactions. Scheme A, biosynthesis of thiazole phosphate. Scheme B, biosynthesis of FO.

reactions, the only similarity between CofGH and ThiGH proteins lies in an iron–sulfur cluster motif found in *S*-adenosylmethionine-dependent enzymes that catalyze free radical reactions.[120] This finding suggests that the mechanisms of FO and thiazole phosphate syntheses may be more complicated than those shown in Fig. 5.

5 Coenzyme M

Coenzyme M **8** (CoM; 2-mercaptoethanesulfonic acid) is the smallest known organic cofactor (M_r 142), yet it is the essential terminal methyl carrier in methanogenesis. The structure of the oxidized $(S\text{-CoM})_2$ disulfide was determined by Taylor and Wolfe using ^{1}H NMR and IR spectroscopy.[121] CoM has also been used therapeutically as a mucolytic and uroprotective agent during chemotherapy.[122] Originally characterized in 1971 as one of several coenzymes involved in the formation of methane by the archaeon *Methanobacterium* str. M.o.H.,[123] CoM was considered unique to methanogens until 1999 when it

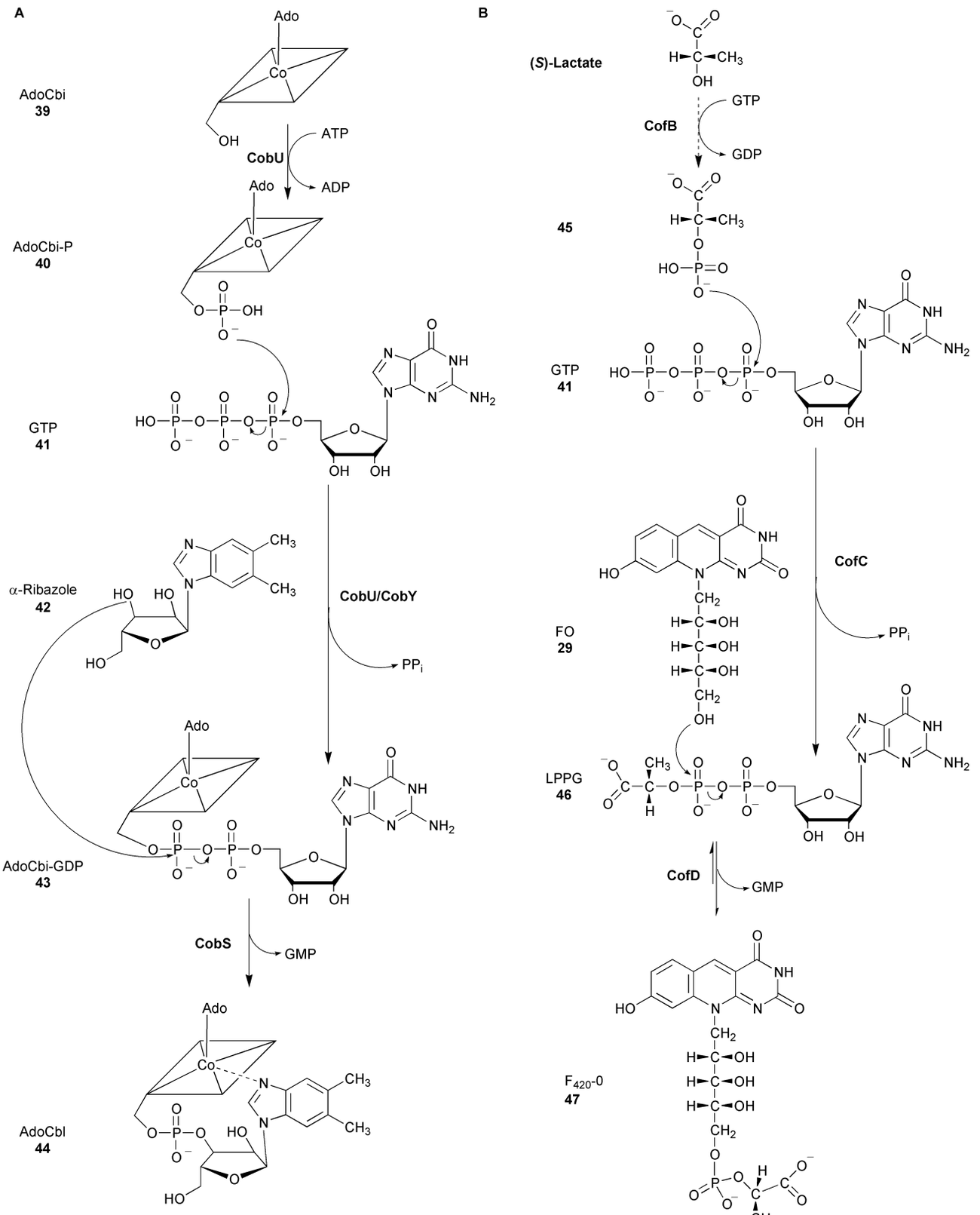

Fig. 6 Analogous guanylation reactions. Scheme A, biosynthesis of adenosylcobalamin. Scheme B, biosynthesis of coenzyme F_{420}-0.

was discovered as a cofactor in the alkene oxidation pathway of *Xanthobacter* str. Py2.[124]

In both methanogenesis and alkene oxidation, the thiol group of CoM forms a thioether bond with the alkyl substrate that is irreversibly broken by substitution to form a heterodisulfide bond with either coenzyme B (methanogenesis) or a cysteine residue in protein component II (alkene oxidation). In both pathways this disulfide bond is reductively cleaved to regenerate coenzyme M. Despite the small size of CoM, at least six proteins are required for its biosynthesis (Fig. 7).

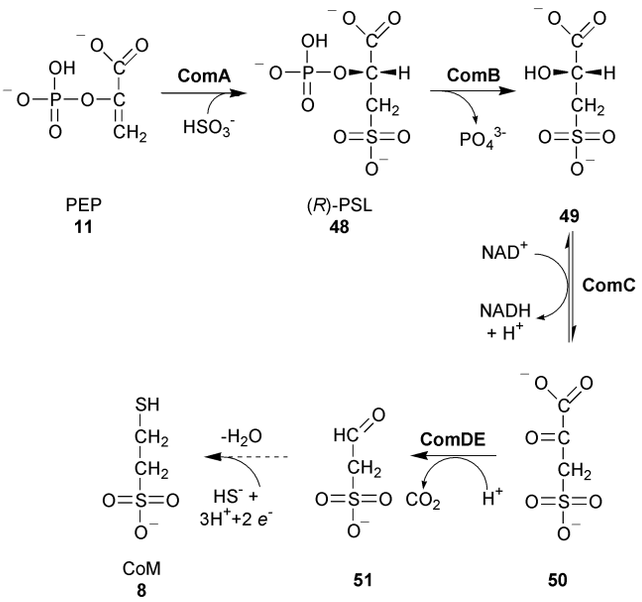

Fig. 7 Biosynthesis of coenzyme M.

The CoM biosynthetic pathway was elucidated by stable isotope incorporation studies, reviewed previously.[125] To determine the origin of the CoM carbon skeleton [1,2-$^{13}C_2$]acetate was fed to methanogenic rumen isolate 10–16B. CoM was isolated from this organism and derivatized with methyl iodide and diazomethane for analysis by GC-MS. The derivatized CoM contained both labeled carbons. CoM isolated from cells fed [2,2,2-$^{2}H_3$]acetate was shown to contain 0–2 deuterium atoms attached to the C-1 atom, consistent with a phosphoenolpyruvate **11** precursor.[126]

Cell-free extracts of *Methanobacterium formicicum* were used to demonstrate that $H^{34}SO_3^-$ formed the sulfonate group and sulfur from [^{34}S]-L-cysteine was incorporated into the thiol group of CoM.[126,127] Intermediates in the CoM biosynthetic pathway were isolated by strong anion exchange chromatography from a reaction mixture containing *M. formicicum* cell-free extract, PEP, bisulfite and cysteine. Sulfolactate **49**, sulfopyruvate **50** and sulfoacetaldehyde **51** derivatives were all identified by GC-MS, confirming the proposed biosynthetic scheme (Fig. 7).[128] In further support of this scheme, (*RS*)-sulfo[3,3-^{2}H]lactic acid was synthesized, fed to strain 10–16B, and shown to be incorporated into CoM *in vivo*.

Subsequently, four CoM biosynthetic enzymes have been identified in *Methanococcus jannaschii*: ComA condenses sulfite with PEP (D. Graham and R. White, unpublished data), ComB hydrolyzes phosphosulfolactate **48**,[129] ComC oxidizes (*R*)-sulfolactate (L-sulfolactate)[130] and ComDE decarboxylates sulfopyruvate to form sulfoacetaldehyde.[131] The respective genes were identified by their proximity to other CoM biosynthetic genes on the chromosome and by their phylogenetic distribution (present in all methanogen genomes and, for *comABC*, present in *Bacillus subtilis*, which produces sulfolactate). Candidate genes were cloned into *E. coli* and the recombinant enzymes were purified and assayed *in vitro*.

The first CoM biosynthetic enzyme, phosphoenolpyruvate sulfonotransferase (ComA), is one of the first identified enzymes that form sulfonates. Despite extensive studies of sulfonate degradation,[132] sulfonate biosynthesis is less well understood. ComA has no sequence similarity to the previously characterized UDP-glucose sulfonotransferase (SQD1) involved in sulfolipid biosynthesis.[133] Although both ComA and SQD1 sulfonotransferases use sulfite, the origin and persistence of sulfite *in vivo* is not known. The sulfonation of PEP occurs non-enzymatically *in vitro*; however, the ComA enzyme catalyzes the reaction and presumably determines the stereochemistry of the phosphosulfolactate product. The *M. jannaschii comA* gene has recently been cloned and expressed for *in vitro* studies (unpublished data).

The phosphosulfolactate phosphatase (ComB) is a Mg^{2+}-dependent acid phosphatase that hydrolyzes a variety of L-2-phosphocarboxylic acids.[129] Curiously, it specifically hydrolyzes only (*S*)-2-phospholactate, but hydrolyzes both stereoisomers of 2-phosphosulfolactate and 2-phosphomalate, presumably by binding the enantiomers in opposite orientations. ComB has no sequence similarity to previously characterized phosphatases, although homologous, uncharacterized genes have been identified in numerous microbial genomes.

(*R*)-Sulfolactate dehydrogenase (ComC) was the first CoM biosynthetic enzyme identified, based on the supposition that the enzyme would be similar to previously characterized NAD(P)-dependent 2-hydroxyacid dehydrogenases.[130] Indeed ComC is homologous to the *E. coli* ureidoglycolate dehydrogenase enzyme.[134] A broad-specificity oxidoreductase, ComC oxidizes several L-2-hydroxycarboxylic acids including (*R*)-sulfolactate, (*S*)-malate, (*S*)-lactate and (*S*)-2-hydroxyglutarate. Unlike previously characterized 2-hydroxyacid dehydrogenases, this enzyme transfers the pro-4*S* hydrogen of NADH to C-2 of 2-oxoacid substrates.[100]

The reaction catalyzed by sulfopyruvate decarboxylase (ComDE) is analogous to the decarboxylation of phosphonopyruvate described in *Streptomyces* spp.[135] Several species of *Streptomyces* produce phosphonate natural products that are biosynthesized from PEP using phosphomutase and phosphonopyruvate decarboxylase enzymes: fosfomycin,[136] bialaphos[135] and phosphinothricin tripeptide.[137] The genome sequence of *M. jannaschii* encodes a homolog of these thiamin pyrophosphate-dependent decarboxylases, expressed as two subunits.[37] These *M. jannaschii* genes were cloned and expressed in *E. coli* and the recombinant ComDE protein was shown to decarboxylate sulfopyruvate but not phosphonopyruvate.[131] The ComDE subunits form an $\alpha_6\beta_6$ heterododecamer that is stabilized by phosphate and inactivated by exposure to oxygen. However the inactivated enzyme could be readily reactivated by treatment with dithionite and methylviologen. Together, the two subunits of ComDE are homologous to phosphonopyruvate decarboxylase enzymes that have been previously characterized in *Streptomyces* spp.[135]

No enzyme has yet been identified to catalyze the final step(s) of CoM biosynthesis—the transformation of an aldehyde to a thiol. In the presence of sulfide, dithionite and methylviologen, sulfoacetaldehyde is converted into CoM non-enzymatically. This conversion is consistent with earlier organic syntheses that converted aldehydes into thiols when incubated with sulfides[138] or with sulfides and a reductant.[139] Nevertheless, L-cysteine rather than sulfide may be the relevant sulfur source *in vivo*. Incubation of *M. formicium* cell-free extract with sulfoacetaldehyde and [^{34}S]-L-cysteine led to nearly complete incorporation into [^{34}S]-CoM.[127] Reactions containing both L-cysteine and Na_2S produced less CoM than reactions containing L-cysteine alone. Although sulfur from L-cysteine is efficiently incorporated into the CoM thiol, it is not known whether the cysteine first reacts directly with sulfoacetaldehyde (forming 2-(sulfomethyl)thiazolidine-4-carboxylic acid)[127] or whether cysteine contributes sulfur to a biosynthetic enzyme, as proposed

for biotin, lipoic acid, molybdopterin, iron–sulfur cluster and thiamin biosyntheses.[140–142] An analogous reaction is required in coenzyme B biosynthesis, described in the next section.[143]

The four identified CoM biosynthetic enzymes from *M. jannaschii* have homologs in *M. thermoautotrophicum*. *B. subtilis* has homologs of *comABC*, which presumably function in sulfolactate and sulfopyruvate biosyntheses. CoM-containing *Xanthobacter* str. Py2 also has at least one CoM biosynthetic enzyme encoded on a linear plasmid.[144] However, the partial genome sequence of *Methanosarcina barkeri* str. Fusaro (http://www.jgi.doe.gov/) contains no homolog of *comA*, *comB* or *comC*, suggesting that *Methanosarcina* spp. may biosynthesize sulfopyruvate by a different pathway, possibly by oxidizing cysteine to form a cysteic acid intermediate. Experiments feeding [2,2,2-$^2H_3$]acetate to *Methanosarcina thermophila* TM-1 showed acetate incorporation into CoM, but could not distinguish among possible pathways.[126]

6 Coenzyme B

Coenzyme B **9** (CoB; HS-HTP; 7-mercaptoheptanoylthreonine phosphate) partners with coenzyme M in the final step of methanogenesis (Fig. 1).[15] The methyl-coenzyme M reductase enzyme (MCR) specifically recognizes two functional groups in CoB: a thiol group displaces CH_4 from methyl-CoM and a L-threonine phosphate group binds to basic amino acids in MCR.[145] Component B (later called coenzyme B) was originally identified as one of three chromatographically separated fractions required to reconstitute MCR.[29] The structure of CoB was determined using ^{1}H NMR, ^{13}C NMR, ^{1}H COSY NMR and fast-atom-bombardment mass spectrometry.[146] Chemically synthesized CoB is spectroscopically and functionally equivalent to the natural product.[147] CoB functions solely in methanogenesis and is the only methanogenic cofactor still considered to be unique to methanogens.

The biosynthesis of CoB may be considered in two parts, similar to lipid biosynthesis: the formation of a hydrophobic 7-mercaptoheptanoate **55** group and the addition and phosphorylation of an L-threonine **56** headgroup (Fig. 8).[125] When methanogenic rumen isolate 10-16B or *Methanococcus voltae* PS were fed [1,2-$^{13}C_2$]acetate, 7-mercaptoheptanoate atoms C-7 and four of the five atoms C-2 through C-6 were labeled; C-1 presumably came from CO_2. However, when fed [2,2,2-$^2H_3$]acetate, these organisms produced 7-mercaptoheptanoate with substantial amounts of deuterium only at C-2 or C-3. This labeling pattern is not consistent with acetate assimilation by the canonical fatty acid biosynthesis pathway, which incorporates deuterium at alternating carbons.[148] Alternatively, the observed isotope incorporation patterns are consistent with a 2-oxoglutarate precursor. All four deuterium atoms from [2,2,3,3-$^2H_4$]succinate fed to *M. voltae* were incorporated into 7-mercaptoheptanoate, confirming the 2-oxoglutarate precursor. Because deuterium incorporation data from the acetate feeding experiments is inconsistent with the acetate incorporation and reduction reactions of fatty acid biosynthesis, 2-oxoglutarate **52** was proposed to be extended by a 2-oxoacid elongation pathway (Fig. 9).[149,150]

Analogous 2-oxoacid elongation reactions are ubiquitous.[151] In each set of reactions acetate is transferred from acetyl-CoA to a 2-oxoacid. After isomerization, the 2-hydroxy-3-carboxy-acid is oxidatively decarboxylated to produce a new 2-oxoacid that is one methylene group longer than the original substrate. In the citric acid pathway, oxaloacetate **58** is converted into 2-oxoglutarate **52** by citrate synthase, aconitase and isocitrate dehydrogenase enzymes (Fig. 9(A)).[152] Some microbes, including *M. jannaschii*, produce 2-oxobutyrate **60** (a precursor of L-isoleucine) from pyruvate **59** by the citramalate pathway (Fig. 9(B)).[153,154] The canonical precursor of L-leucine, 2-oxoisocaproate **62**, is made from 2-oxoisovalerate **61** by the isopropylmalate pathway (Fig. 9(C)).[155] Fungi, yeast and some

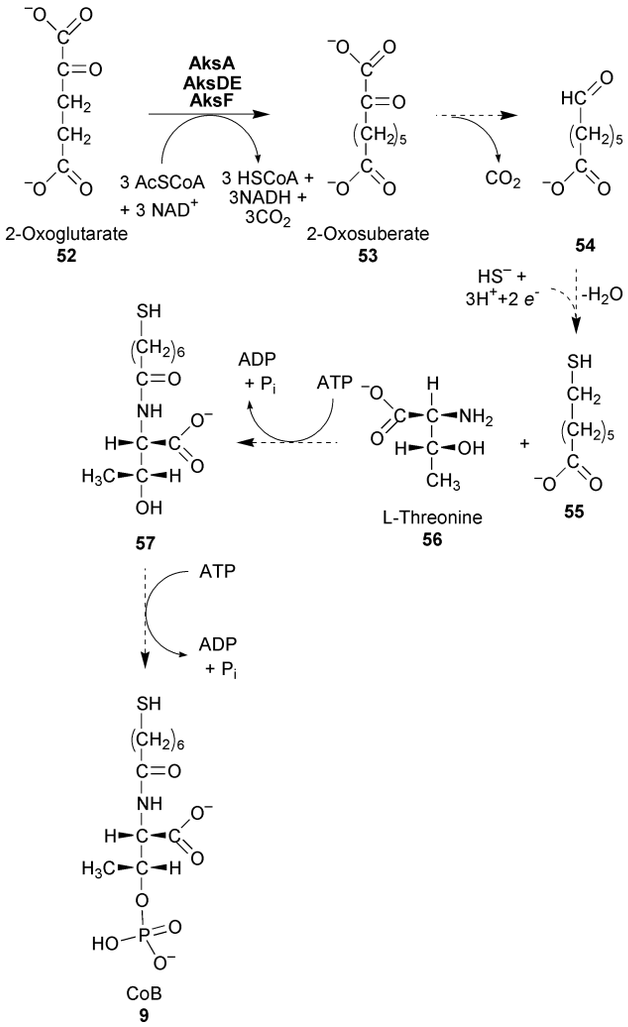

Fig. 8 Biosynthesis of coenzyme B.

bacteria produce 2-oxoadipate **65** for L-lysine biosynthesis by a homocitrate pathway (Fig. 9(D)).[156] In CoB biosynthesis, the 2-oxosuberate **53** precursor of 7-mercaptoheptanoate is produced by a remarkable three iterations of the pathway (Fig. 9(D)-(F)). Analogous successive reactions are also the basis for a proposed prebiotic cycle of carbon fixation.[157]

The next two steps in the biosynthesis of CoB have analogs in CoM biosynthesis (Fig. 7 and Fig. 8). Methanogens decarboxylate 2-oxosuberate to produce an aldehyde, 7-oxoheptanoate **54**. Conversion of the aldehyde into a thiol, using sulfur from cysteine, generates 7-mercaptoheptanoate **55**.[143] As in CoM biosynthesis, cell-free extract from *M. voltae* cleaves *S*-(6-carboxyhexyl)cysteine and uses the cysteine–aldehyde conjugate, 2(*RS*)-(5-carboxypentyl)thiazolidine-4(*R*)-carboxylic acid, as a precursor.[143] However only one-half of 7-mercaptoheptanoate produced in these experiments contained sulfur from an isotopically labeled thiazolidine, suggesting either that the sulfur is exchanged or that the thiazolidine is not a direct precursor. It is not known whether the methanogens produce this cysteine conjugate nor whether the observed cleavage activity is specific. In an alternative scenario cysteine could contribute sulfur to an enzyme that catalyzes the thiolation and reduction of 7-oxoheptanoate.

The final steps in CoB biosynthesis both depend on ATP: 7-mercaptoheptanoate condenses with L-threonine **56** and the product is phosphorylated to make CoB **9**. [7,7-$^2H_2$]-7-Mercaptoheptanoic acid fed to *M. voltae* and to methanogen 10–16B was incorporated into CoB, as were DL-[3,4,4,4-$^2H_4$]threonine and [7,7-$^2H_2$]-*N*-(7-mercaptoheptanol)-threonine.[158] Curiously, deuterium atoms at C-7 of labeled

7-mercaptoheptanoate were significantly exchanged during growth. Further studies showed that the threonylation reaction requires ATP and probably proceeds through a 7-mercaptoheptanoyl phosphate intermediate.[159] An analogous amide-bonded threonine has been identified in N^6-threonylcarbamoyladenosine, a modified nucleoside found in tRNA molecules from most organisms, including *M. jannaschii*.[160,161] Finally, cell-free extracts from both *M. voltae* and *Methanosarcina thermophila* phosphorylate **57** to produce CoB.[158]

Enzymes catalyzing the 2-oxoacid elongation steps of CoB biosynthesis were presumed to be homologous to the enzymes of leucine biosynthesis. The *M. jannaschii* gene *aksA*, which is homologous to isopropylmalate synthase (*leuA*), was cloned and expressed in *E. coli.* Recombinant protein catalyzed the nucleophilic addition of acetate to 2-oxoglutarate **52**,

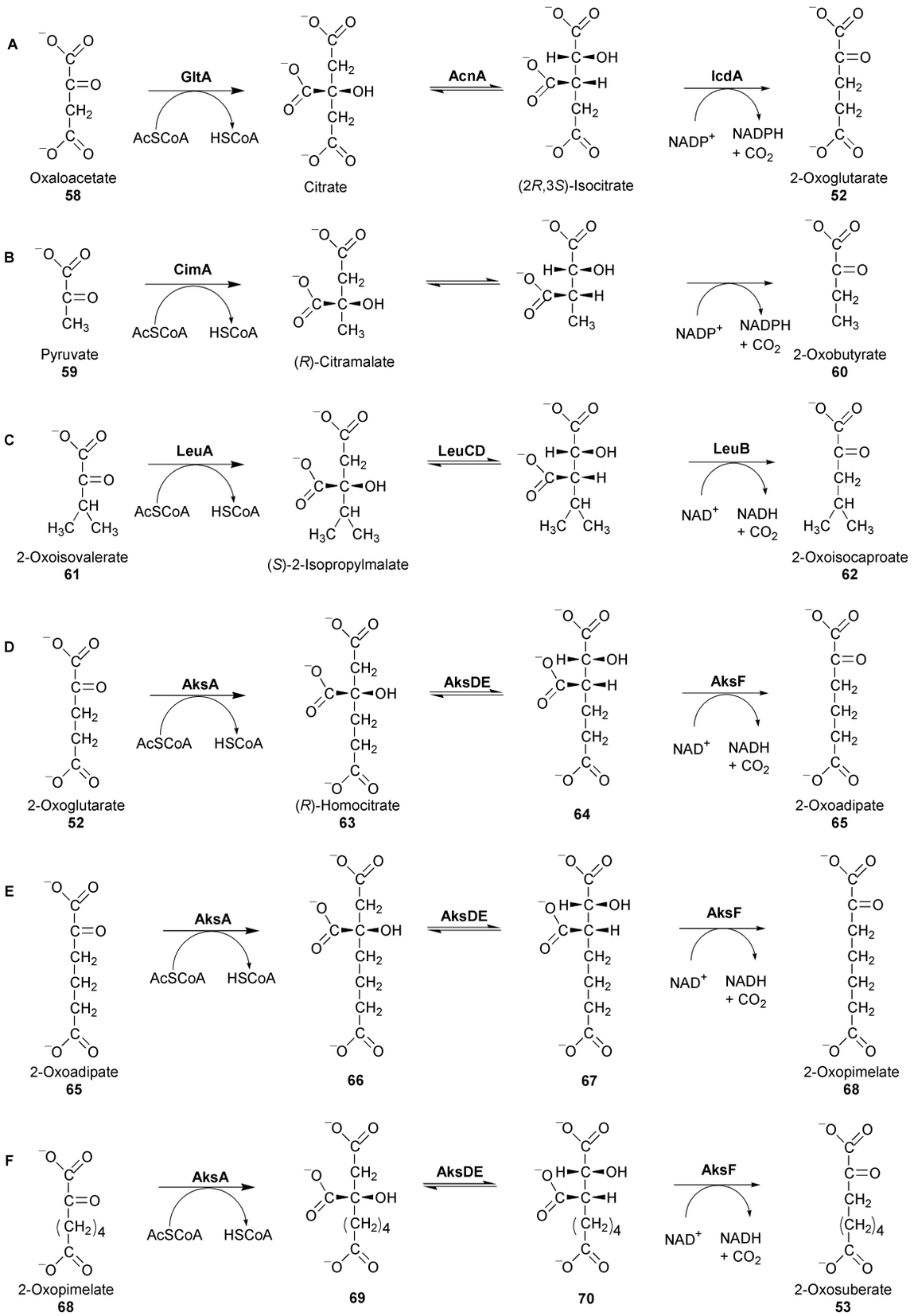

Fig. 9 Analogous 2-oxoacid elongation reactions.

2-oxoadipate **65** or 2-oxopimelate **68** from acetyl-CoA (Fig. 9(D)–(F)).[150] Therefore a single enzyme catalyzes all three analogous condensation reactions. Unexpectedly, (*S*)-homocitrate and *trans*-homoaconitate were detected as products of the AksA-catalyzed reaction of 2-oxoglutarate with acetyl-CoA; however these products may have been made by native aconitase-like enzymes in the recombinant cell extract. Another *M. jannaschii* gene similar to *leuA*, c*imA*, was shown to encode (*R*)-citramalate synthase, the analogous reaction in the citramalate pathway (Fig. 9(C)).[162]

Proteins AksD and AksE from *M. jannaschii* are homologous to the two subunits of isopropylmalate dehydratase (LeuC and LeuD) (M. Graupner and R. White, unpublished results). The AksDE enzyme catalyzes the isomerization of (*R*)-homocitrate **63** and isohomocitrate **64** by way of a *cis*-homoaconitate intermediate. AksDE also isomerizes (*R*)-(homo)$_3$-citrate **69** and presumably (*R*)-(homo)$_2$-citrate **66** as well Fig. 9(D)–(F)). Finally, the AksF enzyme from *M. jannaschii* oxidizes and decarboxylates **64**, **67** and **70** in an NAD^+-dependent reaction.[163] This reaction is analogous to the 3-isopropylmalate dehydrogenase reaction and the *aksF* gene is homologous to *leuB* (Fig. 9(C)–(F)).

The four remaining enzymes required for CoB biosynthesis have not yet been identified. Although the decarboxylation of 2-oxosuberate is analogous to the ComDE-catalyzed decarboxylation of sulfopyruvate, ComDE does not use 2-oxosuberate as a substrate (M. Graupner and R. White, unpublished results). The replacement of the 7-oxoheptanoate aldehyde with a thiol is analogous to the final step in the synthesis of CoM; however, that enzyme(s) has not yet been identified either. The enzyme that condenses 7-mercaptoheptanoate with L-threonine may be homologous to the unidentified N^6-threonylcarbamoyladenosine biosynthetic enzyme.

7 Discussion

Three of the six methanogenic coenzymes, methanopterin, F_{420} and F_{430}, are structurally similar to well-known bacterial and eukaryotic coenzymes. Tetrahydromethanopterin is a modified folate and many steps in its biosynthesis are analogous to tetrahydrofolate formation. However complete genome sequences of several methanogens lack genes similar to the folate biosynthetic genes. Coenzyme F_{420} is a deazariboflavin that shares a common precursor with riboflavin. Although several genes for riboflavin biosynthesis have not yet been identified in methanogens, archaea apparently have a eukaryal-type riboflavin biosynthetic pathway (M. Graupner and R. White, unpublished data). The steps in F_{420} biosynthesis that branch off from riboflavin biosynthesis (the formation of FO and F_{420}-0) are analogous to recently characterized reactions in thiamin and adenosylcobalamin biosynthesis. However this similarity led to the identification of only one gene involved in F_{420} biosynthesis (*cofC*). Similar to tetrahydrofolates, native forms of F_{420} have extensive polyglutamate side chains, although the core structure (FO) alone is an active coenzyme.[75,164] Yet methanogen genomes have no predicted proteins similar to γ-glutamylsynthetases.

Two of the remaining methanogenic coenzymes are structurally similar. Both CoM and CoB have redox-active thiol groups at one end and acidic groups at the other end. However their biosyntheses are dissimilar: the only analogous reactions are the conversions of aldehydes into thiols. Relatively unfamiliar enzymes catalyze familiar reactions in CoM biosynthesis. In CoB biosynthesis, enzymes of the 2-oxoacid elongation cycle are clearly related to enzymes from central metabolism. The remaining reactions have biochemical precedents, but no recognizable enzymes. Conversely, the heterodisulfide reductase enzyme, which reduces the CoM–S–S–CoB disulfide, is an iron–sulfur flavoprotein that is similar to other disulfide reductases.[165]

Methanofuran, a short polymer with peptide and ether bond linkages, is the most unusual of the methanogenic coenzymes. Nevertheless, the *N*-furfurylformamide moiety alone is a pseudo-substrate for the methanogenic formylmethanofuran dehydrogenase enzyme.[166] While the enzymology of methanofuran biosynthesis is mostly unknown, the formylmethanofuran dehydrogenase belongs to a well-known group of molybdopterin-dependent oxidoreductases.[167]

A.J. Kluyver developed the concept of "unity in diversity" to describe the remarkable consistency he observed in microbial biochemistry.[168] In his studies of fermentation processes, Kluyver found that diverse microorganisms used similar reactions to metabolize sugars. Kluyver's theory of biochemical unity fared well in subsequent studies of nucleotide, amino acid and sugar biosyntheses that looked highly conserved to enzymologists. Methanogenesis studies, which were also pioneered in Kluyver's laboratory, showed that even this novel pathway is composed of familiar reactions.[11]

But this biochemical unity looked imperiled when similar reactions were sometimes shown to be catalyzed by dissimilar enzymes. As described in this review, numerous reactions in coenzyme biosynthesis are familiar, but the responsible enzymes are unfamiliar or unrecognizable. Clearly the evolution of coenzyme biosynthetic pathways was more complicated than originally believed. Genes have been recruited to the pathways from diverse and largely unknown sources. Relatives of these genes have been recruited into unrelated biosyntheses, such as secondary metabolite production in the actinomycetes. A pathway analogous to fungal nicotinic acid biosynthesis produces the aromatic moiety of actinomycin D.[169] A gene encoding a decarboxylase in CoM biosynthesis has been recruited to make fosfomycin,[136] bialaphos[135] and phosphinothricin tripeptide.[137] This lineage has also acquired the pathway for coenzyme F_{420} biosynthesis in its entirety.[119]

The unity of biochemistry lies in mechanism, not in structure. The evolutionary process is rooted in gene structure, variation and inheritance, which are the sources of diversity. But phenotypic expression and selection are subject to mechanistic constraints. We are only beginning to understand how those constraints interact with genetic variation to produce biochemical diversity.[57] The Genomic era does not dispense with genetics and biochemistry, rather it provides a much-needed map for Biology.

8 Acknowledgements

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