Radical Enzymes

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Bacterial Methanogenesis Proceeds by a Radical Mechanism**

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Most of the biologically produced methane on earth originates from the decarboxylation of acetate and from $CO_2 + H_2$, formate, methanol, and/or methylamine. These processes are mediated by methanogenic archaea that thrive in strictly anoxic sediments, sewage plants, and animal intestines.[1] Therefore the occurrence of methane-saturated oxic seawater has been a long-standing biogeochemical problem. Recently this problem could be solved by the detection of methylphosphonate (Me-P) and elucidation of its biosynthesis in *Nitrosopumilus maritimus*, a very abundant archaeon in the open sea. The known hydrolysis of Me-P by many aerobically growing bacteria, for example, *Escherichia coli*, leads to methane and phosphate. Although methylation of phosphite to Me-P and its hydrolysis appear to be simple chemical steps, the complex biochemical pathways have been elusive until three papers were published by the groups of Metcalf and van der Donk in 2012[2] as well as by Raushel et al. in 2011 and 2013.[3]

The naturally occurring phosphonates stem from phosphoenolpyruvate (PEP) which isomerizes to 3-phosphonopyruvate. The thiamine diphosphate dependent decarboxylation leads to phosphonoacetaldehyde, which is reduced by NADH to (2-hydroxyethyl)phosphonate. Finally an Fe^{II}-containing dioxygenase catalyzes the cleavage to bicarbonate and Me-P.[2] About 20 years ago it was shown that cell suspensions of *E. coli* catalyze the conversion of chiral (*R*)- and (*S*)-[1-^{2}H$_1$,1-^{3}H]ethylphosphonates to >50% racemic (*R*)- and (*S*)-[1-^{2}H$_1$,1-^{3}H]ethane, respectively.[4] This result was explained by a mechanism with an ethyl radical as an intermediate, to which a hydrogen atom was added with retention of configuration at almost the same rate as that of the rotation of the methylene radical around the C–C bond.

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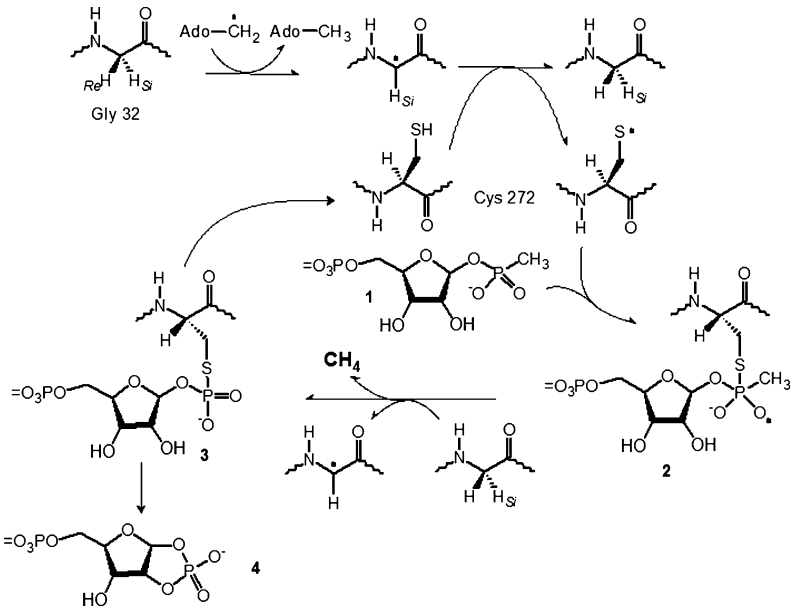
The first paper by the group of Raushel[3a] describes the expression of all 14 genes of the Me-P cluster of *E. coli*; four of them were obtained as inactive recombinant proteins fused with glutathione S-transferase to facilitate purification as well as to increase their stability and solubility. The proteins were mixed with the putative substrates and the reactions were initiated by adding the blood coagulation factor Xa. This protease cleaves off the transferase domain and the active enzyme is released. Initially Me-P reacts with ATP to give adenine and 5-triphospho-α-D-ribose-1-Me-P, which subsequently hydrolyzes to diphosphate and 5-phosphoribose-1-Me-P (**1**, Scheme 1). This under physiological conditions irreversible hydrolysis ensures the complete conversion of Me-P to the ribose derivative **1**, which can be regarded as "activated Me-P". The next step is catalyzed by the actual C-P lyase, which requires S-adenosyl-methionine (SAM) and dithionite as reducing agent leading to methane and 5-phosphoribose-1,2-cyclic-phosphate (**4**). The latter hydrolyzes to ribose-1,5-bisphosphate, which is used for the resynthesis of ATP. Energetically, this methanogenesis is very costly, because per mol methane 3 ATP, 1 PEP, and 1 NADH are consumed. In contrast, anaerobic methanogenesis from $CO_2 + 4\,H_2$ is an exergonic process that provides ATP for growth of the organism.[1] This evokes the question of the biological role of Me-P. Metcalf et al. found it esterified with saccharide cell-wall components of *N. maritimus*. The degradation of the walls affords phosphate, a main nutrient in the oligotrophic seawater.[2]

The requirement of catalytic amounts of SAM and dithionite by the C-P lyase could be due to the involvement of a glycyl radical enzyme. This family comprises six enzymes, all of which contain a stable glycyl radical in their polypeptide chain. Upon addition of substrate, the glycyl radical oxidizes a cysteine residue to a thiyl radical, the catalyst of the radical reaction, for example, the cleavage of pyruvate with coenzyme A (CoA) to acetyl-CoA and formate catalyzed by pyruvate formate lyase of *E. coli*.[5] Other glycyl radical enzymes are involved in the reduction of ribonucleotides to 2′-deoxyribonucleotides,[6] the decarboxylation of 4-hydroxyphenylacetate,[7] the dehydration of glycerol,[8] and the addition of fumarate to toluene[9] or alkanes.[10] The glycyl radical is generated by action of an activase which contains a $[4Fe\text{-}4S]^{2+}$ cluster coordinated by three cysteines within a conserved sequence motif CX_3CX_2C (C = cysteine, X = any



Scheme 1. Proposed mechanism of C-P lyase (adapted from Ref. [3a]). Ado-CH_3 = 5′-deoxyadenosine. The glycyl radical formed in reaction **2**→**3** initiates the next turnover.

amino acid) found in thousands of SAM-dependent radical enzymes. Upon binding of SAM at the fourth iron center of the reduced $[4Fe\text{-}4S]^{+}$ cluster, one electron is transferred to the sulfonium ion causing fragmentation of the molecule to methionine and a 5′-deoxyadenosyl radical (Ado-$CH_2^{\bullet}$). This radical abstracts the *Si* hydrogen of the specific glycine to give 5′-deoxyadenosine (Ado-CH_3) and the active glycyl-radical-containing enzyme, which in the absence of oxygen could catalyze unlimited turnovers.

The C-P lyase contains four conserved cysteines which do not fit into the sequence motif mentioned above.[3b] Site-directed mutagenesis of each individual cysteine to alanine revealed that three of the cysteines are required to coordinate a $[4Fe\text{-}4S]^{2+}$ cluster, whereas the fourth, Cys272, is essential for activity. Like in the activator of glycyl radical enzymes, SAM fragments to methionine and Ado-$CH_2^{\bullet}$, which abstracts the *Re* hydrogen from Gly32 of the same protein molecule (Scheme 1). This was shown by growing the C-P lyase producing *E. coli* strain on a glucose/mineral medium supplemented with 20 mM (*R*)-[2-$^2H_1$]glycine. The formed glycyl radical oxidizes Cys272 to a thiyl radical, which attacks the phosphonate-P of 5-phosphoribose-1-Me-P (**1**) yielding a covalently bound thiophosphonate radical intermediate **2**. Fragmentation of this radical to 5-phosphoribose-1-thiophosphate (**3**) creates a methyl radical that immediately catches the *Si* hydrogen of Gly32 to yield methane with retention of configuration. An intramolecular attack of the 2-OH group of 5-phosphoribose-1-thiophosphate at the thiophosphate moiety releases the product 5-phosphoribose-1,2-cyclic-phosphate (**4**) from the enzyme. The now free thiol of Cys272 together with the radical at Gly32 is ready for the next turnover without employing another SAM. Using the "mutated" substrate 5-phospho-2-deoxyribose-1-Me-P, the thiophosphate intermediate could be trapped and identified.[3b]

This new mechanism starts in a manner similar to that of pyruvate formate lyase, in which the glycyl radical just acts as radical storage and the reformed glycine residue can be regarded as spectator of the thiyl-radical-induced radical reaction. In the second step of the mechanism, however, the thiyl radical is involved in covalent catalysis and the same glycine residue acts as a donor of the fourth hydrogen of methane. Both functions are carried out separately by the *Re* and *Si* hydrogens of Gly32. Thus Ado-$CH_2^{\bullet}$ abstracts the *Re* hydrogen from the front (Scheme 1) and if Cys272 adds its H at the same side, the methyl radical generated from the substrate must approach the glycyl radical from the back. This nicely separates radical generation and methane formation by an angle of about 109°.[3b]

The mechanism of the C-P lyase provides a fine example of convergent evolution. There is no sequence similarity between the C-P lyase and pyruvate formate lyase + its activating enzyme. Hence the two lyases appear to have evolved from different origins but lead to the same glycyl radical stabilized by the captodative effect. Surprisingly the C-P lyase with a more complex mechanism is a much smaller protein (281 amino acids) than pyruvate formate lyase (810) and its activator (308).

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