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Role of rare earth elements in methanol oxidation

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For decades rare earth elements (or lanthanides) were considered not to be involved in biological processes, until their discovery in the active site of the XoxF-type methanol dehydrogenase of the methanotrophic bacterium *Methylacidiphilum fumariolicum* SolV. Follow-up studies revealed the presence of lanthanides in other pyrroloquinoline quinone-containing enzymes involved in alcohol metabolism. This review discusses the biochemistry of the lanthanide-dependent enzymes and the ability of these metals of influencing the gene expression and the type of methanol dehydrogenase used by microorganisms. Furthermore, it highlights novel insights on the uptake mechanism of rare earth elements into bacterial cells.

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metabolism (Figure 1) of the extremophilic bacterium *Methylacidiphilum fumariolicum* SolV [3^{••}]. This microorganism was completely dependent on REEs for growth and the crystal structure of its methanol dehydrogenase (MDH) revealed a lanthanide ion in the active site. The enzyme was encoded by the gene *xoxF*, a homologue of the calcium-dependent MDH *mxafi*, so far considered to be the only enzyme capable of methanol oxidation in methanotrophic and methylotrophic bacteria. This study initiated a completely new field of research that explored the role of lanthanides in biological systems and expanded it to enzymes outside methane metabolism. Furthermore, the addition of REEs to cultivation methods permitted the isolation of novel and uncharacterized bacteria from a variety of different habitats [4–8,9^{••}], allowing researchers to study microorganisms so far considered uncultivable. Currently, this area of study is growing with exciting new discoveries, which will be highlighted in this review.

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Introduction

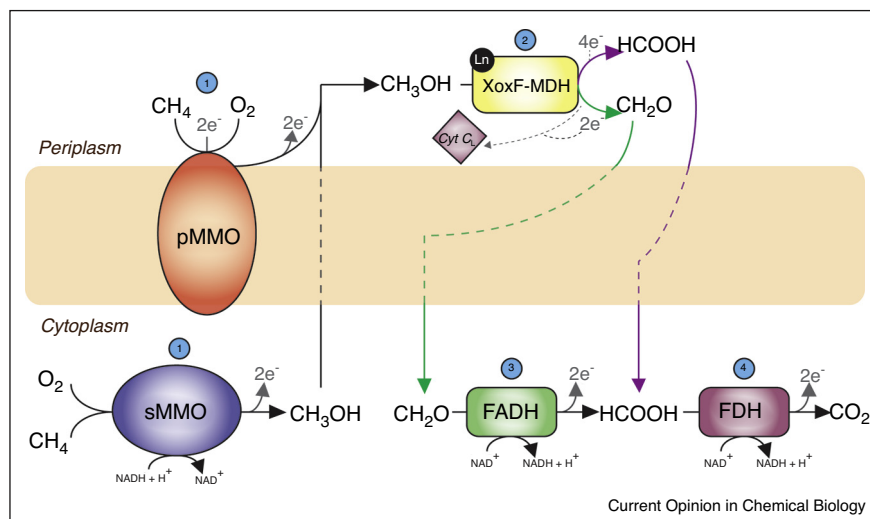
Rare earth elements (REEs) are a group of elements that includes lanthanides (Ln) from lanthanum to lutetium in the periodic table, plus yttrium and scandium. These metals are often divided in ‘light’ lanthanides (LREEs) which comprise elements with atomic number (*Z*) from 57 to 63 (La–Eu), and ‘heavy’ lanthanides (HREEs) indicating elements with *Z* from 64 to 71 (Gd–Lu). Despite their name, REEs are very abundant in the environment, with a concentration that, on average, represents 0.015% of the Earth crust [1]. Lanthanides are usually present in a trivalent form, except for cerium (Ce³⁺, Ce⁴⁺) and europium (Eu²⁺, Eu³⁺) and their ionic radius decreases with increase of atomic number, a feature known as ‘lanthanide contraction’ [1,2]. Besides their chemical properties, REEs were considered not to be involved in biological processes, but this dogma was challenged by the discovery of a lanthanide-dependent enzyme involved in methane

Biochemical characteristics of lanthanide-dependent enzymes

The lanthanide-dependent methanol dehydrogenase (XoxF-MDH) was purified from different microorganisms and it was shown to be a α_2 homodimer with periplasmic localization and a pyrroloquinoline quinone (PQQ) cofactor (Figure 2) [3^{••},10–12]. When compared to the calcium dependent MDH MxaF, XoxF showed a substitution of an alanine with an aspartate residue at the active site (Asp³⁰¹ — numbering based on *M. fumariolicum* SolV), to coordinate the REE ion. Lanthanides are stronger Lewis acids than calcium and, as shown by Density Function Theory (DFT) calculations, they represent an advantage in the redox cycling of the PQQ [13] and in the formation of the nucleophilic agent [14] compared to Ca²⁺. Furthermore, XoxF showed optimal activity at pH 7 and no ammonium activation was needed, while MxaFI performed best at pH 9 and had to be activated [15,16]. In addition, both enzymes can oxidize a range of primary alcohols and formaldehyde [17[•]] but Ln-MDH had higher affinity for methanol and faster conversion rates [3^{••},13]. Another difference between the two MDHs involves the oxidation of methanol to formaldehyde by Ca-MDH and directly to formate by Ln-MDH in a 4 electrons process [3^{••},13]. However, it was recently demonstrated that XoxF purified from *Methylobacterium extorquens* AM1 produced formaldehyde as final product [18], challenging the assumption that all Ln-MDHs would generate formate.

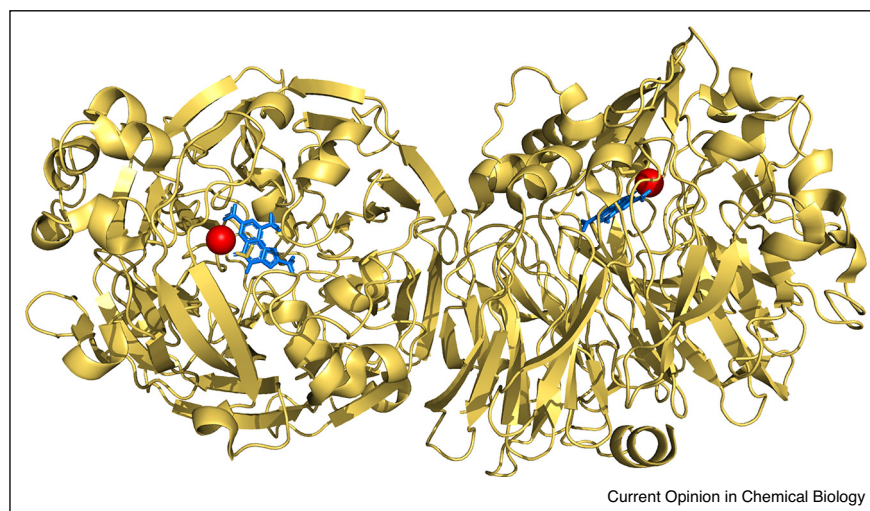
Experimental data showed that purified XoxF worked with different lanthanides, but the activity was higher

Figure 1



Aerobic methane oxidation pathway in Ln-dependent bacteria. The oxidation of methane (CH_4) to methanol (CH_3OH) (1) is catalyzed by the methane monooxygenase (MMO). Bacteria can have two types of MMOs: a soluble, iron-binding protein, called sMMO and/or a particulate methane monooxygenase (pMMO) that is a membrane-bound enzyme and uses copper as cofactor. The methanol produced by MMO is oxidized by XoxF MDH (2), that shuttles electrons to a C_1 cytochrome [41,42], encoded by the gene *xoxG* [31] (or the *xoxJG* fusion gene in thermophilic Verrucomicrobia), and releases formaldehyde (CH_2O) or formate (HCOOH) as product, which are finally converted to CO_2 by (3) formaldehyde dehydrogenase (FADH) and (4) formate dehydrogenase (FDH). In *M. fumariolicum* SolV only pMMO is present and methanol is converted directly to formate.

Figure 2



X-ray crystal structure of *M. fumariolicum* SolV XoxF-type methanol dehydrogenase illustrating the dimeric structure with the PPQ co-factor shown in blue and the lanthanide ion in red.

with the light REEs compared to the heavy ones [3^{••},12,19,20[•],21[•]] — (Table 1). This data supported *in vivo* observations, where growth rate and/or gene expression was influenced by the type of lanthanide used, with a clear preference for light REEs [12,22–24]. To test the effect of a heavy REE in the active site of the enzyme, the crystal structure of the XoxF enzyme from a culture of

M. fumariolicum SolV grown with europium (Eu^{3+}) was analyzed [20[•]]. No significant structural change was observed when comparing it with the mixed-Ln XoxF from the same organism, but the Eu^{3+} culture had a slower growth rate and lower enzymatic activity. These results supported what was suggested before by Pol *et al.* [3^{••}]: a decrease in ionic radius along the lanthanide series

Table 1

Affinity constants (K_M) for methanol of Ln-dependent enzymes^a

Enzyme	Type	Lanthanide	K_M (μM)	Strain	Reference
Methanol DHG ^b	XoxF	Ce ³⁺	29	<i>Bradyrhizobium</i> sp.	[12]
Methanol DHG	XoxF	La ³⁺ , Ce ³⁺ , Pr ³⁺ , Nd ³⁺	0.8 ± 0.3	<i>M. fumariolicum</i> SolV	[3**]
Alcohol DHG ^c	ExaF	La ³⁺	5980	<i>M. extorquens</i> AM1	[25]
Methanol DHG	XoxF	Eu ³⁺	3.6 ± 0.4	<i>M. fumariolicum</i> SolV	[20*]
Methanol DHG	XoxF	Eu ³⁺	0.9 ± 0.2	<i>M. fumariolicum</i> SolV	[21*]
Methanol DHG	XoxF	Eu ³⁺ , Lu ³⁺	0.8 ± 0.4	<i>M. fumariolicum</i> SolV	[21*]
Methanol DHG	XoxF	Eu ³⁺ , La ³⁺	1.3 ± 0.2	<i>M. fumariolicum</i> SolV	[21*]
Methanol DHG	XoxF1	La ³⁺	44	<i>M. extorquens</i> AM1	[18]
Methanol DHG	XoxF1	Nd ³⁺	29	<i>M. extorquens</i> AM1	[18]
Methanol DHG	XoxF4-1	Ce ³⁺	55 ± 32	<i>M. mobilis</i> JLW8	[43]
Methanol DHG	XoxF4-2	Ce ³⁺	42 ± 18	<i>M. mobilis</i> JLW8	[43]
Methanol DHG	XoxF5	Ce ³⁺	39 ± 11	<i>Methylomonas</i> sp. LW13	[43]

^a Enzymes are tested in different assay conditions by different authors. DHG = dehydrogenase.

^b MDH in *Bradyrhizobium* was described as the product of a *mxoF* gene in the original publication. Analysis of the protein, however, suggested it is a XoxF type.

^c K_M value for methanol of alcohol dehydrogenase PedH in *Pseudomonas putida* KT2440 was not reported. K_M for ethanol was 177 ± 31 μM with Pr³⁺.

could affect the ability of incorporating the metal ion in the active site, resulting in a decreased affinity for the substrate. The lower efficiency of the enzyme was linked to the slower growth rate determined *in vivo*. A difference in enzymatic activity was also observed in XoxF purified from *Mb. extorquens* AM1, when assayed with lanthanum (La³⁺) and neodymium (Nd³⁺) [18]; in particular, the activity was higher in the presence of Nd³⁺ compared to La³⁺, despite the fact that both elements are considered light lanthanides. Novel insights about the preference of the enzyme for specific REEs came from the work of Lumpe *et al.* [21*]. DFT calculations were used to explain why Nd³⁺ and praseodymium (Pr³⁺), among the whole lanthanide group, resulted in the highest activity for reconstituted XoxF purified from *M. fumariolicum* SolV. They proposed that different factors influenced the specific activity of the enzyme: the decreased ionic radius of the metal ion, the preference in coordination number, ligand exchange rates, substrate orientation and activation and hydrogen bonding.

Besides their role in methanol oxidation, REEs were recently shown to take part in multi-carbon metabolism, when the first lanthanide-dependent ethanol dehydrogenase (ExaF) was discovered in *Mb. extorquens* AM1 [25]. Furthermore, an ethanol dehydrogenase (PedH) whose activity was related to the presence of REEs, was purified from the non-methylotrophic bacterium *Pseudomonas putida* KT2440 [19]. Thanks to these recent findings, the biological role of lanthanides was expanded to a broader range of enzymes and microorganisms and elicited research on the role of lanthanides in genetic regulation.

Lanthanides regulate the expression of different methanol dehydrogenases

The expression of both types of methanol dehydrogenases appears to be correlated with the presence of

REEs. This phenomenon is known as the ‘lanthanide switch’ and it refers to the upregulation of the lanthanide-dependent MDH gene *xoxF* and downregulation of the calcium-dependent MDH *mxoFI* genes in the presence of lanthanides, in both methano- and methylotrophic bacteria [23–25,26*,27,28**]. Since the discovery of the switch, it became obvious that this genetic regulation was not as straightforward as first described. In fact, experimental data in *Methylosinus trichosporium* OB3b showed that the lanthanide switch was completely overruled by copper [22,26*,27,28**,29], that is used as a cofactor for the particulate methane monooxygenase (pMMO). When copper was present, cerium had minimum effect on the expression of *mxoFI*. However, the same response was not observed in other bacterial species. Experiments performed in *Methylomicrobium buryatense* 5GB1C showed that XoxF was the preferred enzyme for methanol oxidation, even when calcium was present in 100-fold higher concentrations than lanthanide, and that copper had little effect on the expression of *xoxF*- encoded methanol dehydrogenase [30]. The overexpression of *xoxF* at higher calcium concentrations compared to lanthanides was also observed in *Mb. extorquens* AM1, and it was suggested that a condition may exist in which *mxoFI* and *xoxF* were expressed at the same time [23]. The co-expression of *mxoFI* and *xoxF* was also detected in *Ms. trichosporium* OB3b [29] and in *Methylomonas* sp. strain LW13 [31], when grown with both calcium and cerium in the medium. Interestingly, evidences showed that *xoxF* was required for the expression of *mxoF* in *Mb. extorquens* AM1 [23,32] and in *Methylobacterium aquaticum* 22A [24], since *xoxF* deletion mutants were not able to grow in the presence of calcium.

Although the active role that REEs have in the expression of the different MDHs has been well documented, the mechanism by which lanthanides operate the switch is

not completely clarified. It is known that *mxbDM* and *mxoQE* genes have a role in the regulation of the lanthanide switch in *Mb. extorquens* AM1 [23,32]. The genes *mxbD* and *mxoQ* encode sensor kinases, whereas *mxoE* and *mxoM* encode response regulators [23,24]. Indications of a regulatory function for MxbD was also observed in the methylotrophic bacterium *Mb. aquaticum* 22A [24], where *mxoD* was upregulated in presence of calcium. In *Mm. buryatense* 5GB1C, instead, an important function was identified for *mxoB* and *mxoY*; in fact, it was proposed that a putative lanthanum-binding protein activated the orphan response regulator MxB in absence of REEs and MxB induced the *mxo* operon and repressed *xoxF* [30]. Furthermore, a mutation in the histidine kinase *mxoY* gene (glutamate 147 → glycine), allowed constant expression of *mxoB* and *mxoF*, indicating the involvement of MxB in the lanthanide switch regulation [33].

Besides the mechanism of regulation, it soon became clear that the expression of the two MDHs was not solely linked to the presence of lanthanides. Recently, it was shown that a mixed community composed of a methanotrophic and a non-methanotrophic methylotroph could be established, and that the methanol produced by the methane oxidizer was used by the methylotroph as energy source [28^{••},31,34]. Under these conditions, a switch in the type of methanol dehydrogenase used was observed: when grown with the methylotroph the expression of the MDH in the methanotroph shifted from *xoxF* to *mxoFI* in the presence of lanthanum. It was speculated that the methylotroph induced the methanotrophic bacterium to perform a reaction carried out by MxBFI instead of XoxF; based on reported K_M values for methanol [3^{••}], in fact, the reaction catalyzed by MxBFI would be less efficient compared to XoxF, leading to an increased loss of methanol from the methanotroph, that enabled the methylotroph to survive. Furthermore, it was reported that the supernatant retrieved from the coculture had the same inhibitory effect towards *xoxF* expression. This allowed to hypothesize the presence of a secreted compound able to regulate the expression of the two MDHs in the methane oxidizing bacterium *Methylobacter tundripaludum* 31/32 [28^{••}]. To date, this putative compound has yet to be identified. Other experimental data showed that, in mixed communities, the lanthanide switch was also influenced by the nitrogen source (with *xoxF* more expressed when ammonium was supplied instead of nitrate), methane and oxygen concentrations [34]. With nitrate present, in fact, high methane concentrations selected for *mxoF* overexpression, low methane and low oxygen partial pressures for *xoxF*, high methane and low oxygen concentrations, instead, allowed the transcription of both. To better understand the regulation, insight into the uptake of rare earth elements is crucial.

Rare earth elements uptake by bacteria

In 2010, one of the biggest natural disasters in history was reported on the *Deepwater Horizon* drilling rig in the Gulf

of Mexico, where a large oil spill led to the release of natural gas in the ocean. At the oil spill site, REEs were rapidly removed from the water column at a depth that also revealed the presence of bacteria containing the *xoxF* methanol dehydrogenase, as shown by metagenomic data [35]. Other studies reported the microbial removal of REEs in solution, and the measurements were usually carried out by applying Inductively Coupled Plasma Mass Spectrometry (ICP-MS) [3^{••},22,27,36]. Alternatively, a fast and easy colorimetric assay was recently developed for specific detection of lanthanides in a range of 0.1–10 μM , delivering results in accordance with ICP-MS data [37]. Several authors speculated on how these metals, despite the low solubility, were transferred into the cell. The transport could be either passive, mediated by secreted compounds or more selectively controlled by transporters, as suggested in studies that documented the genetic regulation of MDH by REEs [22,38]. It was proposed that carboxylate and phosphate groups on the bacterial cell surface mediated the absorption in *Bacillus subtilis* and *Escherichia coli* [39]. Furthermore, indications for the presence of three different pH-dependent lanthanide binding sites in *Roseobacter* sp. were reported. Sites with higher pK_a values bound the light, more basic lanthanides and those with lower pK_a the heavier ones [36]. Also, TonB dependent transporters could have an important role in the lanthanide uptake system of *Ms. trichosporium* OB3b; the hypothesis was based on the presence of a certain sequence (CGA(T/C)(G/A)TGACC) in the promoter region of genes whose expression was influenced by the presence of REEs [22]. More indications came from transcriptome studies in the same strain, where the upregulation of a gene encoding a TonB dependent transporter was possibly linked to the presence of cerium in the medium [29]. In addition, TonB transporters and TonB dependent receptors seemed to be conserved in the genome of some *xoxF*-containing microorganisms [7,40]. Whether they are really involved in REEs uptake needs to be established.

Conclusions

REEs are essential for methanol metabolism since they are needed for an active XoxF-type MDH. The enzymatic activity and the growth rate of methanotrophic and methylotrophic bacteria are influenced by the type of lanthanide available, with a clear preference for LREEs. Moreover, their presence in solution regulates the gene expression between the calcium and the Ln-dependent MDH genes, a phenomenon known as 'lanthanide switch'. Despite the novel discoveries, many questions still need to be answered in this emerging field of research. For example, how are these metals transported into the cell? New insights in this uptake mechanism could help clarify the regulation mechanism for the gene expression and the physiological behavior of bacteria observed *in vivo*. Furthermore, the role of REEs is so far confined to alcohol metabolism. It is tempting to

speculate that we are just starting to understand the importance of lanthanides in biological systems, and their function may be expanded to other pathways in the near future.

Conflict of interest statement

Nothing declared.

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