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MINI-REVIEW

PQQ-dependent methanol dehydrogenases: rare-earth elements make a difference

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Abstract Methanol dehydrogenase (MDH) catalyzes the first step in methanol use by methylotrophic bacteria and the second step in methane conversion by methanotrophs. Gram-negative bacteria possess an MDH with pyrroloquinoline quinone (PQQ) as its catalytic center. This MDH belongs to the broad class of eight-bladed β propeller quinoproteins, which comprise a range of other alcohol and aldehyde dehydrogenases. A well-investigated MDH is the heterotetrameric MxaFI-MDH, which is composed of two large catalytic subunits (MxaF) and two small subunits (MxaI). MxaFI-MDHs bind calcium as a cofactor that assists PQQ in catalysis. Genomic analyses indicated the existence of another MDH distantly related to the MxaFI-MDHs. Recently, several of these so-called XoxF-MDHs have been isolated. XoxF-MDHs described thus far are homodimeric proteins lacking the small subunit and possess a rare-earth element (REE) instead of calcium. The presence of such REE may confer XoxF-MDHs a superior catalytic efficiency. Moreover, XoxF-MDHs are able to oxidize methanol to formate, rather than to formaldehyde as MxaFI-MDHs do. While structures of MxaFI- and XoxF-MDH are conserved, also regarding the binding of PQQ, the accommodation of a REE requires the presence of a specific aspartate residue near the catalytic site. XoxF-MDHs containing such REE-binding motif are abundantly present in genomes of methylotrophic and methanotrophic microorganisms and also in organisms that hitherto are not known for such lifestyle. Moreover, sequence analyses suggest that XoxF-MDHs represent only a small part of putative REE-containing quinoproteins, together covering an unexploited potential of metabolic functions.

Keywords Methanol dehydrogenase · Alcohol dehydrogenase · Pyrroloquinoline quinone · Rare-earth elements · Methylotrophy · Methanotrophy

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Introduction

Methanol serves as the carbon and energy source for a large variety of obligate and facultative methylotrophic microorganisms. Phototrophic prokaryotes may use methanol as the electron donor for anoxygenic photosynthesis and respiration (Quayle and Pfennig 1975; Sahm et al. 1976). Plants excrete methanol from their root systems and leaves (Fall and Benson 1996; Sy et al. 2005; Abanda-Nkpwatt et al. 2006). Here, methylotrophs are particularly abundant as plant pathogens and nitrogen-fixing symbionts (Vorholt 2012). They even assist in plant development by providing plants with pyrroloquinoline quinone (PQQ) (Choi et al. 2008). Next, methanol is, after methane, the second most abundant organic compound in the atmosphere, and methanol deposited in the ocean surface provides marine microbes with an important carbon and energy source (Yang et al. 2013). In addition to being a primary substrate, methanol is the first intermediate in methane oxidation in methanotrophs that thrive on the oxygen-dependent oxidation of methane, a common end product of the anaerobic degradation of organic materials. In both methanotrophs and methylotrophs, methanol is oxidized by methanol dehydrogenase (MDH) to toxic formaldehyde, which is either further oxidized to CO_2 or serves as the starting substrate for two convenient cell carbon fixation pathways: the serine pathway and the ribulose monophosphate (RuMP) cycle.

The diversity of microbial species that are capable of methanol conversion is reflected in the diversity of MDHs. Gram-positive bacteria harbor the NAD(P)-dependent nicotinoprotein MDH in their cytoplasm (Hektor et al. 2000, 2002). In Gram-negative methylo- and methanotrophs, an unrelated MDH is localized in the periplasm. The particular MDH, which is also present in microorganisms belonging to the deep-branching phyla *Verrucomicrobia*, the NC10 bacteria and *Acidobacteria*, and which is the topic of the present minireview, contains PQQ as its catalytic cofactor. In addition, PQQ-dependent MDHs occur as several types. A well-investigated type is the so-called MxaFI-MDH (see for reviews, Anthony and Ghosh 1998; Anthony and Williams 2003; Anthony 2004). Presently, the crystal structures of MxaFI-MDHs from six different species have been resolved (Xia et al. 1992, 1996, 1999, 2003; Ghosh et al. 1994, 1995; Williams et al. 2005; Nojiri et al. 2006; Choi et al. 2011). MxaFI-MDHs are heterotetrameric enzymes ($\alpha_2\beta_2$) composed of two large (α, MxaF) and two small (β, MxaI) subunits. The large subunits represent the catalytic part of the enzyme carrying one PQQ and one calcium atom per subunit. The function of the small subunits, which tightly wrap against the large subunits, is elusive. A second type of MDH, referred to as MDH2, has been implicated in methylotrophy in the *Burkholderiales* and *Rhodocyclales* (Kalyuzhnaya et al. 2008a). MDH2 is related to PQQ-containing alcohol dehydrogenases that generally display only low affinity for methanol.

Genome sequencing studies suggested the existence of yet another MDH, the XoxF-MDH (Chistoserdova and Lidstrom 1997; Giovannoni et al. 2008; Kalyuzhnaya et al. 2008b), which apparently lacks a small subunit and displays less than 50 % sequence identity to MxaFs. Nevertheless, all amino acids identified in MxaF as crucial ligands for PQQ- and Ca^{2+} binding as well as for catalysis are conserved in XoxF-MDHs. Again, XoxF-MDHs are phylogenetically diverse and at least five clades can be distinguished (Chistoserdova 2011). Genes coding for these proteins can also be detected in genomes of microorganisms that hitherto lack a known capability of methanol conversion. The function of the XoxF proteins has been enigmatic for quite some time. In model organisms, XoxF-MDHs were hardly expressed during growth under laboratory conditions, and the disruption of their encoding genes was not associated with a clear phenotype initially (Chistoserdova and Lidstrom 1997). However, XoxF genes were abundantly expressed in natural habitats, notably in the plant root systems (Delmotte et al 2009; Sowell et al. 2011; Vorholt 2012). Certain established methylo- and methanotrophs, like the thermoacidophilic *Verrucomicrobia* (Pol et al. 2007; Hou et al. 2008; Khadem et al. 2012b), the marine microbe *Methylotenara* (Bosch et al. 2009; Lapidus et al. 2011) and the phototroph *Rhodobacter sphaeroides* (Mackenzie et al. 2001) do not carry *mxaF* genes in their genomes. In these organisms, a XoxF-type protein would be the only candidate to comply with a methano- or methylotrophic lifestyle. Indeed, the deletion of the *xoxF* gene in *R. sphaeroides* resulted in the inability to use methanol for aerobic respiration and photosynthesis, indicating that the XoxF-type enzyme is a true MDH (Wilson et al. 2008). A clue to the understanding of the function of the XoxF-type enzymes came from the finding that deletion of the *mxaF* gene in *Methylobacterium extorquens* resulted in the failure of growth on methanol, which could be restored by the addition of lanthanide-group rare-earth elements (REE), like cerium(III) and lanthanum(III), to the growth media (Nakagawa et al. 2012). Under these conditions, a XoxF-MDH was specifically induced. Similarly, the addition of REE to growth media of *Methylobacterium radiotolerans* (Hibi et al. 2011) and *Bradyrhizobium* species (Fitriyanto et al. 2011) invoked both growth on methanol and the functional expression of XoxF-MDHs. Furthermore, growth of the Verrucomicrobium *Methylacidiphilum fumariolicum* SolV strictly depended on the addition of mudpot water from its natural environment (Pol et al. 2014). Mudpot water that turned out to be relatively rich in REEs could be fully replaced by mineral media containing submicromolar concentrations of REEs, Ce^{3+} in particular. Importantly, purification and subsequent resolution of the crystal structure of the XoxF-MDH from *M. fumariolicum* established the presence of a lanthanide at the catalytic site (Pol et al. 2014).

The presence of a lanthanide at the active site might confer XoxF-MDHs a catalytic efficiency (k_{cat}/K_m) in methanol oxidation that is superior to their calcium-containing MxaFI counterparts. In this minireview, we will outline the phylogenetic relationship between MxaFI- and XoxF-MDHs, point to common properties of and remarkable differences in the way both MDH systems are organized at the genetic level, address the question of what make the REE-containing MDHs more efficient catalysts, and discuss the catabolic and anabolic consequences of possessing different MDHs. Supported by sequence analyses, we will argue that REE-containing PQQ proteins are widespread in nature where they may catalyze yet to be disclosed oxidations of a potentially broad range of alcohol and aldehyde substrates.

Phylogeny of methanol dehydrogenases

MDHs belong to the large class of eight-bladed β propeller (PQQ-containing) quinoproteins, more specifically to type I of this class. The soluble proteins are about 600 amino acids in size. They are localized in the periplasm and use a cognate cytochrome *c* (cyt c_L) as the electron acceptor for substrate oxidation. MDHs share the type I classification with ethanol- and alcohol dehydrogenases (ADHs) (Fig. 1) (Matsushita et al. 2002; Toyama et al. 2004). Types II and III ADHs are

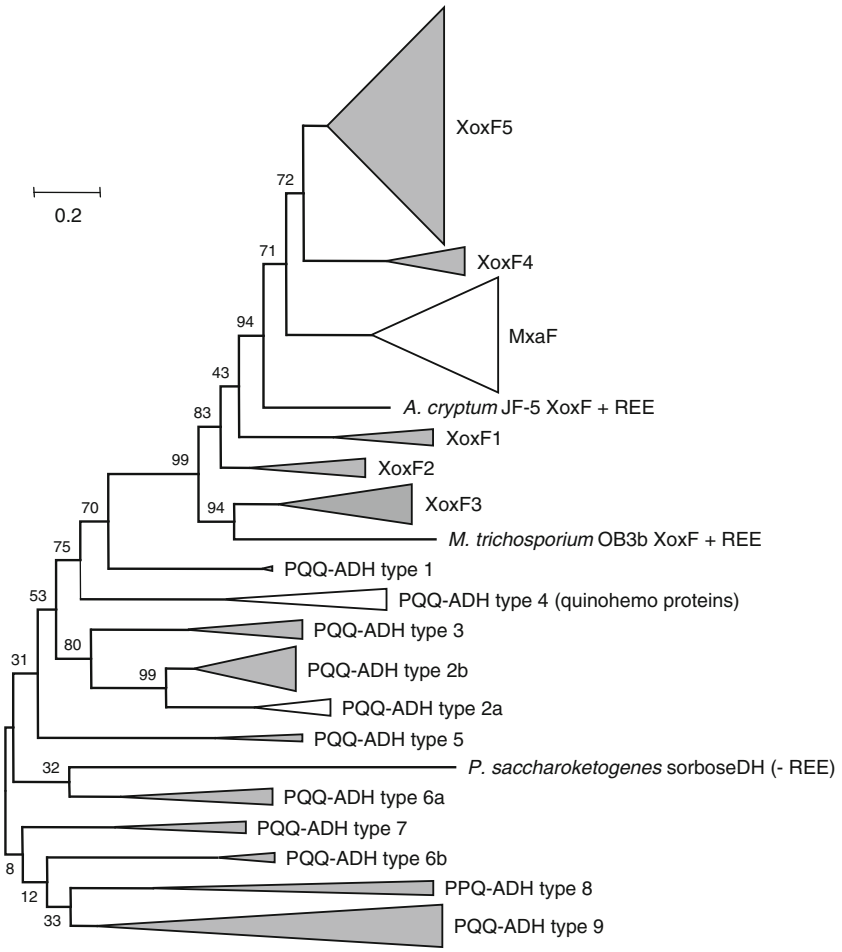

Fig. 1 Phylogenetic tree of types I and II alcohol dehydrogenase quinoproteins. The tree shows the phylogenetic relationship of the five clades of XoxF-MDHs proposed by Chistoserdova (2011), along with MxaF-MDHs and additional divergent XoxF-type MDHs. The *lower part of the figure* comprises other types I and II alcohol dehydrogenase (*ADH*) quinoproteins, covering nine clades of calcium- and putative REE-containing proteins. The different protein families that emerge from the phylogenetic analysis are *bracketed at the right side of the panel*; clades covering proteins with a REE-binding site are *highlighted by dark grey triangles*; calcium-containing protein families are in *white*. The tree was constructed by the MEGA5 program (Tamura et al. 2011). A detailed version of the figure is shown in Fig. S1, which provides further details in tree construction in the legend. *A. cryptum JF-5 XoxF* divergent XoxF-type MDH from *A. cryptum* JF-5 (YP_001235900; Acry_2790), *M. trichosporium OB3b XoxF* divergent XoxF-type MDH from *M. trichosporium* OB3b (EFH04071), *P. saccharoketogenes* sorbose dehydrogenase, sorbose dehydrogenase from *P. saccharoketogenes* (BAB62258). *Scale bar*, number of substitutions per site

other members of this class. In type II ADHs, the quinohemoproteins, a heme *c* is bound at the C-terminal part to the PQQ-containing catalytic part present in the N-terminal region. Type III ADHs are only found in acetic acid bacteria and represent quinohemoproteins as well (Yakushi and Matsushita 2010). Like the types I and II quinoproteins, type III ADHs function in the periplasm. However, they contain two additional subunits including a triheme protein, and they are anchored in the membrane where quinone is used as the electron acceptor. The membrane-bound nature and the use of quinones apply to two other groups of eight-bladed β propeller quinoproteins: membrane-bound glucose dehydrogenases (mGDH) (Yamada et al. 2003; Anthony 2004) and type I ADH-affiliated glycerol dehydrogenases from *Gluconobacter* species (Adachi et al. 2001; Sugisawa and Hoshino 2002; Matsushita et al. 2003; Gómez-Manzo et al. 2008). For the binding to the membrane, the β propeller-structured PQQ-catalytic module is fused to transmembrane helices present in the N-terminal part of the glycerol and glucose dehydrogenases.

Phylogenetic analysis of all MxaF and XoxF catalytic subunits collected from available genomes separates them, next to the MxaF-type, in five different XoxF families (XoxF1–5) (Fig. 1; Fig. S1). While the genome of a certain microorganism may encode several XoxF paralogs and orthologs, only one MxaF copy is found, if present. The division and branching shown in the figures are the same as described by Chistoserdova (2011), and we will apply the numbering proposed by the author. In fact, two more XoxF subtypes can be distinguished with only one representative each, a protein (Acry_2790) from the acidophile *Acidiphilium cryptum*, an *Alphaproteobacterium* belonging to the class of

Rhodospirillales and a divergent XoxF3 from *Methylosinus trichosporium* OB3b (EFH04071). Family-5 XoxF proteins (XoxF5) are the largest group. This group comprises proteins from known and potential methylotrophs affiliated with the *Alpha-* and *Gammaproteobacteria*, as well as with the betaproteobacterial *Burkholderiales* and *Rhodocyclales* (Fig. S1). The XoxF5 proteins branch into subgroups in agreement with the taxonomic position of those bacteria. Within the phylogenetic tree, XoxF5 proteins from methanotrophs constitute specific sub-branches, again in correspondence with their affiliation to the *Alpha-* and *Gammaprotobacteria*. XoxF4 proteins are exclusively found in members of the betaproteobacterial *Methylophilales*. XoxF2 proteins are limited to methanotrophs belonging to the phylum *Verrucomicrobia* and to the NC10 phylum bacterium *Candidatus* 'Methylomirabilis oxyfera'. These organisms are characterized by their unique physiology. The verrucomicrobial species are thermoacidophiles (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008). *M. oxyfera* is the only organism known that is capable of anaerobic methane oxidation using an aerobic pathway; oxygen needed for the activation of methane into methanol is made by the organism itself (Ettwig et al. 2010). In addition to the XoxF2-MDH, *M. oxyfera* contains an MxaF- and a second XoxF-type protein, which is a member of the XoxF1 family (Fig. S1) (Ettwig et al. 2010). The latter enzyme from *M. oxyfera* that has been identified by us as a genuine MDH (Wu and Keltjens, unpublished result) shares its position within the small XoxF1 group with quinoproteins found in the genomes of *Methylocella silvestris* and *Methyloferula stellata*, both acidophilic methanotrophs belonging to alphaproteobacterial *Rhizobiales.* In addition to the ones mentioned, genes coding for XoxF1 proteins are detected in *Xanthomonas axonopodis* and in *Hydrocarboniphaga effuse*, members of the *Xanthomodales* class of *Gammaproteobacteria* (Fig. S1). XoxF3 proteins form the deepest branch within the MDHs (Fig. 1). This group comprises a mix and match of quinoproteins mainly from alphaproteobacterial *Rhizobiales*, some gammaproteobacterial *Methylococcales*- both including methanotrophs-, few betaproteobacterial *Methylophilales* and *Burkholderiales*, as well as a single representative of the *Acidobacteria* (*Candidatus* 'Solibacter usitatus'). Phylogenetically, XoxF3 proteins subdivide according to the taxonomic position of their representatives.

Since 1964 (Anthony and Zatman 1964a, b), MxaFI-MDHs have been studied into great detail (see for reviews: Anthony and Ghosh 1998; Anthony and Williams 2003; Anthony 2004). These enzymes were isolated from methylotrophic and methanotrophic model organisms. However, the inspection of the phylogenetic tree of MDHs (Fig. 1; Fig. S1) shows that the MxaF proteins represent only a minor fraction; most (putative) MDHs are XoxF proteins. Indeed, *mxaF* sequences from environmental samples are clearly underrepresented with respect to their *xoxF* counterparts (Kalyuzhnaya et al. 2008b; Bosch et al. 2009; Sowell et al 2011). This uneven distribution also holds for mRNA expression levels in environmental samples. Importantly, all native MxaFI-MDHs and other ethanol/ alcohol quinoproteins studied thus far contain Ca^{2+} as a cofactor, a common constituent of laboratory growth media. A second conclusion that can be drawn from inspection of the phylogenetic tree is that the development of the MxaFI-MDHs is a secondary and even later evolutionary event: the enzymes descend from a XoxF prototype. The evolution of the MxaFI-MDHs seems to have had its implications in the way they are encoded and assembled into functional enzymes.

Genomic organization of MDH systems

The genomic system underlying the functional expression of MxaFI-MDHs and the role of the different gene products are quite well understood for *M. extorquens* (Chistoserdova et al. 2003, 2007; Ward et al. 2004). Their expression requires the combined action of more than 25 genes divided over five gene clusters (*mxa, mxb, mxc, pqqABCDE*, and *pqqFG*) that are scattered across the genome. The comparison with MxaFI systems from other organisms shows that the gene organization is highly conserved, especially regarding the *mxa* gene cluster (Fig. 2; Fig. S2). In this cluster, *mxaF* codes for the MDH large (α) subunit. The gene is linked to the *mxaJ*, *mxaG*, and *mxaI* genes that encode a polypeptide belonging to the Family 3 periplasmic solute-binding proteins (COG0834, pfam13531) (Kim et al. 2012), a cognate physiological electron acceptor of methanol oxidation (cytochrome c_L), and the MDH small (β) subunit, respectively. The function of MxaJ is not clear. It might act as a chaperone in MxaFI assembly (Van Spanning et al. 1991). Alternatively, MxaJ might function in the binding of methanol or trap the reaction product formaldehyde. MxaJ is usually not present in purified MxaFI preparations. However, MxaJ formed part of the MxaFI complex isolated from *Acetobacter methanolicus* (Matsushita et al. 1993) and from *Methylophaga aminisulfidivorans* MPT (Kim et al. 2012). In the latter bacterium, the $\alpha_2\beta_2\gamma$ MxaFIJ complex was enriched in preparations obtained after gentle cell lysis and the complex displayed somewhat higher V_{max} values for methanol oxidation and lower K_m values towards its electron acceptors (artificial dyes, cyt c_L) as compared with the heterotetrameric ($\alpha_2\beta_2$) MxaFI. The *mxaACKLD* gene products are essential for Ca^{2+} insertion into the apoprotein (Richardson and Anthony 1992; Chistoserdova et al. 2003). The MxaC, MxaL, and MxaD proteins belong to the superclass of von Willebrand type A (vWFA) proteins harboring an additional metal ion-dependent adhesion (MIDAS) domain, which is consistent with such insertion function. MxaK is a tetratricopeptiderepeat (TPR)

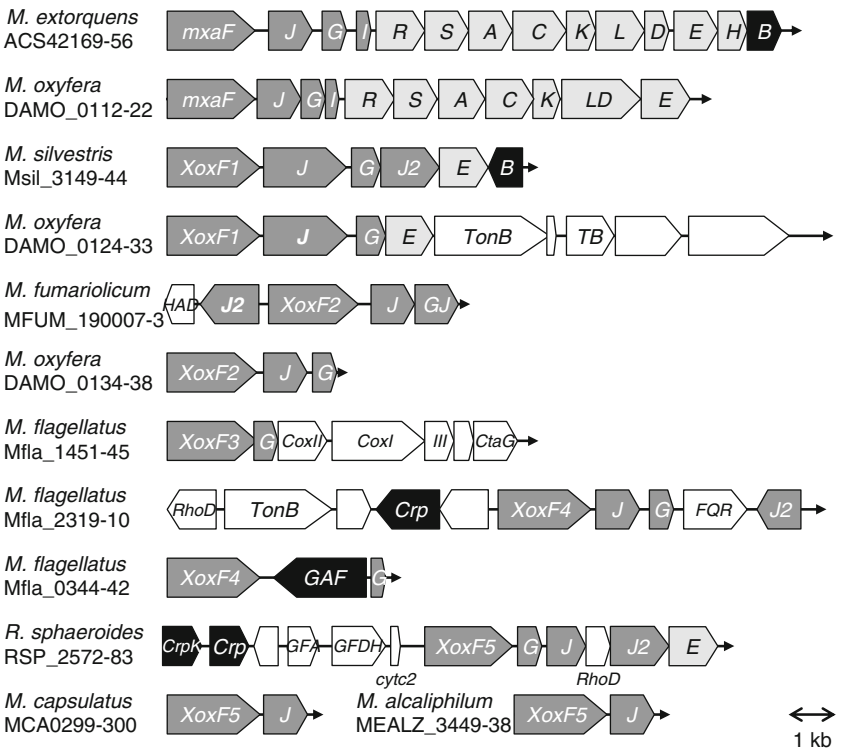

Fig. 2 Genomic organization of representatives of MxaFI and XoxF systems. Genes coding for structural proteins are displayed as *dark grey arrows with white lettering*, accessory genes in MDH assembly as *light grey arrows* (*black lettering*), and regulatory genes as *black arrows* (*white lettering*). Genes coding for proteins with an associated function in methanol metabolism are shown as *white arrows* together with their gene codes; proteins with an unknown or an unrelated function lack such coding. Gene sizes and their intergenic regions are drawn to scale (*scale bar*, 1 kb). Protein or gene identifiers are denoted underneath the names of bacterial species, reading in the order of the genes. *M. extorquens Methylobacterium extorquens* AM1, *M. oxyfera Candidatus* 'Methylomirabilis oxyfera', *M. silvestris Methylocella silvestris* BL2, *M. fumariolicum Methylacidiphilum fumariolicum* SolV, *M. flagellatus Methylobacillus flagellatus* KT, *R. sphaeroides Rhodobacter sphaeroides* 2.4.1, *M. capsulatus Methylococcus capsulatus* Bath, *M. alcaliphilum Methylomicrobium alcaliphilum* 20Z, *TB TonB*, TonB-like uptake system, *HAD* HAD superfamily hydrolase, *Cox* cytochrome *c* oxidase, *CtaG* cytochrome *c* oxidase assembly factor, *RhoD* rhodanese- containing protein, *Crp* cyclic AMP-receiving transcriptional regulator, *FQR* putative formaldehyde/quinone reductase, *GAF* GAF-modulated sigma 54-specific transcriptional regulator, *GFA* glutathione-dependent formaldehyde-activating enzyme, *GFDH* glutathione-dependent formaldehyde dehydrogenase

domain protein that might facilitate the interaction between the MDH apoprotein and accessory proteins. Proteins encoded by the *mxaRSEH* genes are thought to play a role in further MDH maturation (Anderson et al. 1990; Nunn and Lidstrom 1986a, b). Herein, MxaR is a type 3 AAA protein, a broad class of proteins related with numerous cell functions. MxaS is another vWFA protein with a MIDAS domain. Genes coding for the 40-residue YVTN family beta-propeller repeat proteins MxaE and mxaH are not always found in the *mxa* gene clusters, whereas some of these clusters encode an additional protein, termed MxaP. It may be noted that all proteins mentioned are preceded by an N-terminal signal sequence for protein export, indicating that the complex machinery for MDH maturation resides in the periplasm.

The *pqqABCDE* and *pqqFG* gene clusters encode the enzymes involved in PQQ biosynthesis (Goosen et al. 1992; Puehringer et al. 2008; Gliese et al. 2010). In *M. extorquens*, *mxbDM* and *mxcQE* code for two different two-component regulatory systems in which MxbD and MxcQ are the sensor kinases and MxbM and MxcE are the response regulators (Lidstrom et al. 1994; Springer et al. 1997). MxcE, which is a member of the LuxR family of C-terminal DNA-binding proteins with an N-terminal CheY-like receiver domain, has a close homolog in MxaB (Springer et al. 1998), its encoding gene forming part of the *mxa* cluster. These regulatory elements are not conserved and differ in species-dependent manners. Still, in many genomes *mxa* gene clusters are flanked by two-component regulators with MxcE/MxaB-related response regulators.

In comparison to MxaFI-MDHs, the genomic organization of the XoxF-MDHs is simpler (Fig. 2; Fig. S2). First, XoxF systems are devoid of a gene that codes for the small subunit (*mxaI*). In agreement herewith, all but one known XoxF-MDHs are homodimeric (α_2) proteins lacking the small subunit (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012; Pol et al. 2014). The notable exception is the XoxF1-MDH ($\alpha_2\beta_2$) MDH from *M. oxyfera* which binds a small subunit derived from the *mxaI* gene in the same gene cluster

(Fig. 2) (Wu and Keltjens, unpublished result). Next, genes coding for Ca^{2+} insertion (*mxaACKLD*) and (most of the ones) for further protein maturation (*mxaRSEH*) are absent in XoxF systems. However, in the genome of *Methyloversatilis universalis* FAM5, which lacks the MxaFI system, two closely connected *xoxF5* genes (METUNv1_03887 and METUNv1_03889; sequence identity, 92.5 %) are associated with *mxaACKLDRS* genes (Fig. S2). As described below, XoxF-MDHs are equipped to use REEs as their cofactor. Apparently, the restructuring of the XoxF-MDH into a species (MxaFI) in which the catalytic properties of the much more accessible calcium are exploited required at least some assistance by accessory proteins.

There are no general patterns in the way the XoxF-MDH systems are organized at the genomic level (Fig. S2). Generally, *xoxF* genes are linked to genes (*xoxG*) coding for class 1 *c*-type (cytochrome c_L) proteins and/or—more frequently—to genes that encode the periplasmic solute-binding protein (*xoxF*). Herein, the *xoxG* and *xoxJ* genes are homologs of *mxaG* and *mxaJ* denoted above. The associated heme *c*'s likely serve as the immediate electron acceptors. In XoxF2 systems, the *xoxJ* and *xoxG* genes may be fused. *XoxF* genes also occur as stand-alones, i.e., they are not linked to other genes required for MDH activity. This is especially the case for XoxF5 proteins of which several gene copies can be found in a genome, sharing a 75–95 % sequence identity. XoxF systems, however simple, may be clustered with regulatory genes—their nature being species-specific—, genes coding for related metabolic functions like formaldehyde oxidation or genes encoding putative accessory proteins. An example of the latter is the TonB uptake system found in the XoxF1 gene cluster of *M. oxyfera* (Fig. 2). Even though it is present in other parts of their genomes, this particular TonB is highly conserved in all organisms carrying *xoxF* genes, where it might act in the acquirement/uptake of REEs from the environment. XoxF5 systems in *Bradyrhizobium*, *Mesorhizobium*, *Paracoccus denitrificans*, and in *R. sphaeroides* represent a noteworthy example of functionally connected genes. In these organisms, the *XoxF5* gene inventory is clustered with the one of a glutathione (GSH)-dependent formaldehyde dehydrogenase system (Fig. 2; Fig. S2). Deletion of the latter in *P. denitrificans* and in *R. sphaeroides* resulted in the loss of methanol conversion by these organisms (Ras et al.1995; Wilson et al. 2008). Moreover, the aberrant XoxF from *A. cryptum* is clustered with such formaldehyde dehydrogenase (Fig. S2). Similarly, one set of XoxF4 genes in each *Methylophilales* species that harbors this system is linked to a gene that codes for a putative membrane-associated dye-linked formaldehyde dehydrogenase (see "Catabolism and anabolism in methylotrophs and methanotrophs"). XoxF3-MDHs provide another example of a functionally connected gene system. *XoxF3* genes appear to be consistently clustered with the set of genes that code for an entire cytochrome *c* oxidase (Fig. 2; Fig. S2). If expressed as an assembly, methanol oxidation could be directly coupled to oxygen respiration to sustain the proton-motive force for ATP synthesis.

Catalysis and catalytic mechanism of methanol dehydrogenases

MDHs catalyze the two-electron oxidation of methanol into formaldehyde (Eq. 1). To a varying degree, MxaFI-MDHs use other primary alcohols as substrates (Table 1). These enzymes are also capable of oxidizing formaldehyde into formate (Eq. 2), but with considerable lower V_{max} and lower substrate affinity. These reactions are commonly assayed by following the phenazine methosulfate (PMS)-mediated reduction of dichlorophenol indophenol (DCPIP) (Anthony and Zatman 1967a, b). In the dye-coupled assays, the Ca^{2+}-containing enzymes require a nonphysiological high pH (8–11) for maximal activity (Anthony 2004). In addition, they strictly depend on ammonia or methylamine for activation. Remarkably, a high pH and ammonia activation is not required in assays with the physiological electron acceptor cyt c_L. Nevertheless, the requirement of ammonia for activation is also seen for other types I and II ADH quinoproteins that prefer ethanol and alcohol substrates (Table 1). In those ADHs, catalytic potentials relate to the specific function that a certain enzyme is tuned for, but methanol is only a poor substrate.

$$CH_3OH \rightarrow HCHO + 2H^+ + 2e^- \left(E_0' = -182\ \text{mV}\right) \quad (1)$$

$$HCHO + H_2O \rightarrow HCOO^- + 3H^+ + 2e^- \left(E_0' = -534\ \text{mV}\right) \quad (2)$$

XoxF-MDHs are not well characterized with respect to their enzyme kinetics. In fact, members of the XoxF3- and XoxF4-MDHs, as well as the divergent XoxF proteins from *A. cryptum* and *M. trichosporium* OB3b remain to be isolated. However still limited, data suggest that XoxF-type MDHs oxidize methanol with higher rates and higher affinity than MxaFI-MDHs (Table 1). This does not hold for the XoxF5 protein described from *M. extorquens*, but this enzyme was obtained by heterologous overexpression (Schmidt et al. 2010); therefore, it is conceivable that it was purified in an only partially active state with calcium as the cofactor. Indeed, the same protein obtained after growth in the presence of La^{3+} and containing this REE displayed a 100-fold higher specific activity (Nakagawa et al. 2012). Currently, the best understood XoxF-MDH is the enzyme from *M. fumariolicum* SolV (Pol et al. 2014). Like the MxaFI-MDHs, this enzyme is capable of oxidizing a range of primary alcohols, besides its preferred substrate, methanol (Table 1). In addition,

Table 1 Catalytic and structural properties of methanol dehydrogenases (MDH), types I and II alcohol dehydrogenases (ADH)

Source	MxaFI-MDH															XoxF-MDH		
	M. methylotrophicus			*Hyphomicrobium* WC			*Pseudomonas* W1			*Methylosinus* sp. W1			*Methylocystis* sp. GB25			*M. fumariolicum* solV XoxF2		
Catalytic properties	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})
MeOH	100 (6.45)	0.020	800	100 (1.7)	0.014	290	100 (1.5)	0.015	240	100 (2.8)	0.45	13	100	0.34	13	100 (4.4)	0.0008	11,600
EtOH	76	0.028	430	100	0.016	250	93	0.016	200	114	3.58	2	96	0.89	5	100	0.003	3,100
1-PropOH	94	0.280	50	106	0.036	120	100	0.040	90	91	3.69	1	43	1.10	1	100	0.007	1,330
S-(+)-1,2-Propanediol																		
S-(-)-1,2-Propanediol																		
2-PropOH	n.r.			n.r.			n.r.			n.r.			1	n.r.		–		
1-ButOH	89	0.285	50	100	0.040	100	93	0.041	80	172	6.38	2	42	0.19	10	100	0.006	1,550
Formaldehyde	81	1.010	15	82	3.200	1	80	3.300	1	146	n.r		78	0.10	34	100	0.007	1,330
Subunit	$\alpha_2\beta_2$			$\alpha_2\beta_2$			$\alpha_2\beta_2$			α_2			α_2			α_2		
Mol. Mass (kDa)	148.5			141			141			125			125			127		
Reference	Ghosh and Quayle (1981)			Sperl et al. (1974)			Sperl et al. (1974)			Grosse et al. (1998)			Grosse et al. (1997)			Pol et al. (2014)		

Source	XoxF-MDH						Type I ADH								
	Bradyrhizobium sp. XoxF5			*M. extorquens* AM1 XoxF5[c]			*Pseudomonas aeruginosa* EtOH-DH			*Pseudomonas* BB1 EtOH-DH			*Pseudomonas putida* EtOH-DH		
Catalytic properties	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})
MeOH	100 (15)	0.029	1,090	100 (0.015)	0.011	3	62	94	0.1	79	31.7	0.8	n.r.	n.r.	
EtOH	90	n.r.		160	0.014	4	100 (5.63)	0.014	860	100 (15.3)	0.011	2,970	100 (26.3)	0.163	370
1-PropOH	90	n.r.		n.r.			138	0.021	790	103	0.013	2,580	103	n.r.	
S-(+)-1,2-Propanediol															
S-(-)-1,2-Propanediol															
2-PropOH	70	n.r.		n.r.			115	0.68	20	142	2.6	18	31	n.r.	
1-ButOH	70	n.r.		n.r.			115	0.37	37	n.r.	n.r.		87	1.62	32
Formaldehyde	n.r	n.r.		93	0.065	0.5	29	n.r.		256	0.04	2,090	n.r.	.	
Subunit	α_2			α_2			α_2			α_2			α_2		
Mol. Mass (kDa)	126			126			128			128			137.5		
Reference	Fitriyanto et al. (2011)			Schmidt et al. (2010)			Görisch and Rupp (1989)			Dijkstra et al. (1985)			Toyama et al. (1995)		

Source	Type I ADH						Type II ADH (quinohemoproteins)								
	Thauera butanivorans ButOH-DH[d]			*M petroleiphilum* MDH2[e]			*Pseudomonas putida* ADH-IIB			*Pseudomonas putida* ADH-IIG			*Comamonas testosteroni* ADH-DH		
Catalytic properties	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})
MeOH	0.7	n.r.		100	0.29	1.7	n.r.			n.r.			n.r.		
EtOH	30	n.r.		109	0.10	5.4	100 (15.4)	0.163	115	100 (11.5)	33.2	0.4	70 (10.5)	5.0	3
1-PropOH	22	n.r.		111	0.09	6.1	n.r.			n.r.			80	0.06	245

Table 1 (continued)

Source	Type I ADH						Type II ADH (quinohemoproteins)								
	Thauera butanivorans ButOH-DH[d]			*M petroleiphilum* MDH2[e]			*Pseudomonas putida* ADH-IIB			*Pseudomonas putida* ADH-IIG			*Comamonas testosteroni* ADH-DH		
Catalytic properties	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})	V_{max}[a] (%)	K_m (mM)	k_{eff}[b] (s^{-1} mM^{-1})
S-(+)-1,2-Propanediol							17	3.71	1	183	0.055	480	n.r.		
S-(-)-1,2-Propanediol							46	4.58	2	106	3.32	5	n.r.		
2-PropOH	30	n.r.		n.r.	n.r.										
1-ButOH	100 (0.24)	n.r.		100 (0.23)	0.10	5.0	201	0.015	2,500	74	0.043	250	85	0.005	3,100
Formaldehyde	n.r.			n.r.			n.r.			n.r.			20	3.0	1
Subunit	α_2						α_1			α_1			α_1		
Mol. Mass (kDa)							72.6			75.3			73.3		
Reference	Vangnai et al. (2002)			Kalyuzhnaya et al. (2008)			Toyama et al. (1995, 2005)			Toyama et al. (1995, 2005)			Groen et al. (1986)		

M. methylotrophicus Methylophilus methylytrophicus, *M. fumariolicum Methylacidiphilium fumariolicum*, *M. extorquens Methylobacterium extorquens* AM1, *MeOH* methanol, *EtOH* ethanol, *PropOH* propanol, *ButOH* butanol, *n.r.* not reported

[a] Percentage of V_{max}, which is given in parentheses (μmol min^{-1} mg of $protein^{-1}$)

[b] $k_{eff}=k_{cat}/K_m$

[c] Heterologously expressed (see text)

[d] Partially purified

[e] Partially purified (46 %)

formaldehyde is used with high rate and high affinity. Actually, methanol is converted by a four-electron oxidation directly into formate. Alcohols other than methanol are oxidized to their corresponding aldehydes. The direct oxidation of methanol into formate might explain the absence of dedicated formaldehyde dehydrogenases in *M. fumariolicum* (Pol et al. 2014). The two-step oxidation also might explain why *M. fumariolicum* has to rely on the Calvin–Benson–Bassham (CBB) cycle for autotrophic CO_2 fixation (see below) (Khadem et al. 2012a). Other remarkable properties of the *M. fumariolicum* MDH are that the enzyme shows optimal activity at neutral pH and that ammonium is not needed for activation.

While different mechanisms have been envisaged for methanol (and formaldehyde) oxidation, the common understanding—not only for MDHs, but for all quinoproteins—, is that the reaction involves a base-catalyzed proton abstraction in concert with the hydride transfer from the substrate to the C-5 of the PQQ cofactor (Fig. 3) (Anthony 1996, 2004; Anthony et al. 1994; Zheng et al. 2001). After still elusive tautomerization of the intermediate, reduced PQQ ($PQQH_2$) is formed. The subsequent transfer of two electrons in two steps to the one-electron carrier cyt c_L would complete the oxidation process (Davidson 2004). The presence of PQQ in its semiquinone state in crystal structures (Anthony 1996; Pol et al. 2014) and the strong peak in EPR spectra in as-isolated proteins (Sato et al. 2001) is consistent with a two-step oxidation. In addition to PQQ, two moieties in close vicinity to this cofactor play a key role in catalysis. The first one is an aspartate (Asp^{303}); unless mentioned otherwise, the numbering of amino acids is based on the *M. extorquens* MxaFI protein (Anthony and Ghosh 1998). This aspartate is conserved in nearly all eight-bladed β propeller quinoproteins and it has been suggested to act as the base in proton abstraction from alcohols (Fig. 3a) (White et al. 1993; Anthony et al. 1994, 1996; Afolabi et al. 2001; Zheng et al. 2001). However, it should be noted that computer simulations indicate such function for another amino acid, viz. Glu^{177} (or Asp^{177} in other quinoproteins) (Reddy and Bruice 2003, 2004; Zhang et al. 2007). In the MDH structures, Asp^{303} is the only amino acid near the active site that binds a water molecule (Zheng et al. 2001). By the abstraction of a proton from the hydrogen-bonded water, the resultant hydroxyl is prepared for a nucleophilic attack on formaldehyde (Fig. 3b). In this way, Asp^{303}

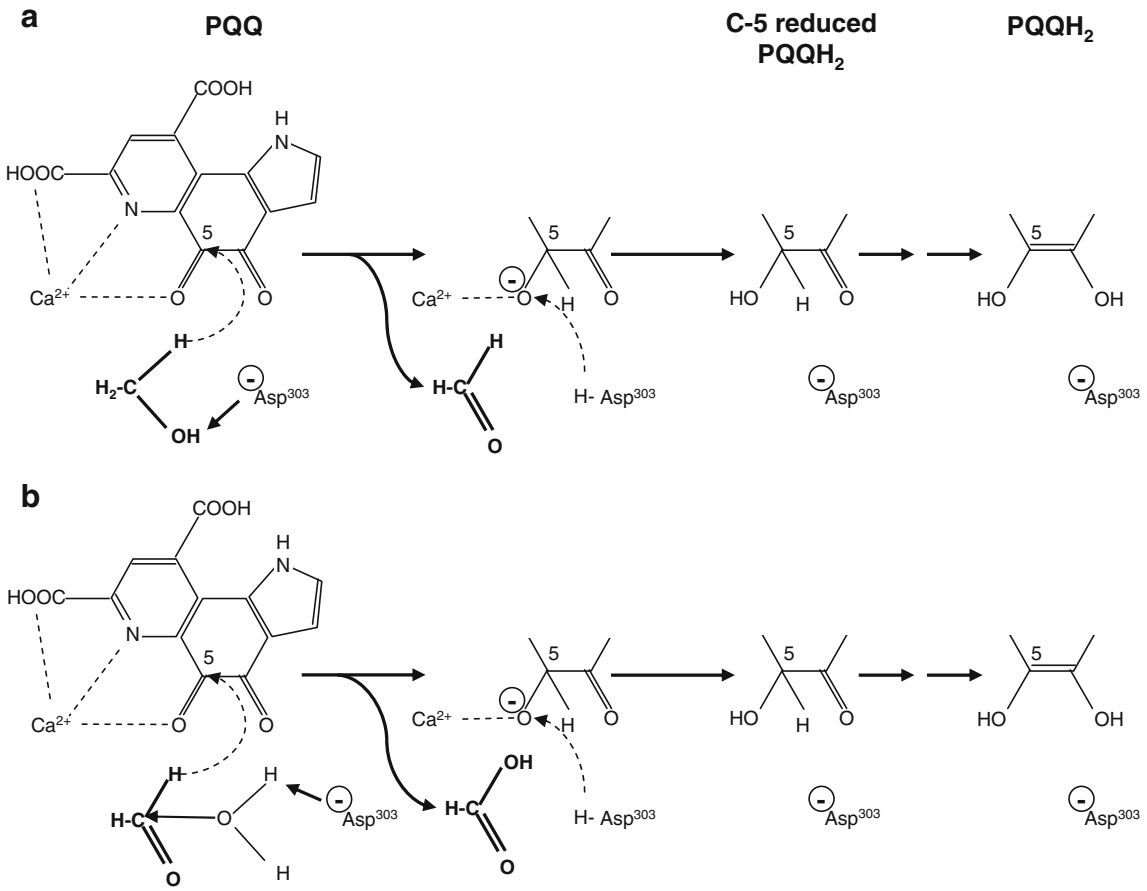

Fig. 3 Catalytic mechanism of **a** methanol oxidation and **b** formaldehyde oxidation by MDH quinoprotein. The reactive C5 atom of PQQ is indicated; the catalytic Asp^{303} is numbered as observed in the MxaFI crystal structure of *Methylobacterium extorquens* AM1 (PDB: 1W6S) (Williams et al. 2005). The figure is adapted from Oubrie et al. (2002)

might assist in formaldehyde oxidation as well (Eq. 2) (Oubrie et al. 2002). The second key component is the active site metal (Ca^{2+} or a REE). By acting as a Lewis acid, Ca^{2+} polarizes the C5-O5 bond in PQQ, thus facilitating the nucleophilic addition to C5 of the hydride from the substrate (methanol, formaldehyde) (Anthony et al. 1994; Dewanti and Duine 2000; Oubrie and Dijkstra 2000; Zheng et al. 2001). In support of this view, the substitution of Ca^{2+} in MDH by Ba^{2+}, a stronger Lewis acid, decreased the activation energy for methanol oxidation by 50 % resulting in a 2-fold higher V_{max} value in methanol conversion (Goodwin and Anthony 1996). However, the K_m for methanol increased more than 1,000-fold by this substitution. Ba^{2+} (2.41 Å) is bigger than Ca^{2+} (1.95 Å) and the larger size might interfere with the proper binding and orientation of methanol. REEs are much stronger Lewis acids than Ca^{2+} and Ba^{2+}. This property seems to be exploited in XoxF-MDHs, but the proper accommodation of a REE apparently requires a specific modification near the PQQ catalytic site.

Structure of methanol dehydrogenases

MDHs are structured by eight sets (W1–8) of four-stranded (A–D), W-shaped antiparallel β sheets (Fig. S3). Herein, W8D is located at the N terminus, while W8A–C are present at the C-terminal part of the protein. Glycine residues on each D-strand interact with (usually) threonines in tryptophan-rich stretches on the next D-strand ("tryptophan docking") (Ghosh et al. 1995). The result is the tight packing of the protein backbone into eight propeller-like peptide sequences according to a pseudo 8-fold symmetry axis (Fig. 4a–c). This overall architecture is fully conserved, not only in all MDHs with known crystal structures, including the XoxF2-MDH from *M. fumariolicum* (Pol et al. 2014), but in all structurally resolved eight-bladed β propeller quinoproteins (see for reviews, Anthony and Williams 2003; Anthony 2004; Toyama et al. 2004). PQQ is located central to the propellers, buried deep inside a hydrophobic pocket (Fig. 4d–f). In MxaF, the cofactor is noncovalently bound by a series of in-plane hydrogen bonds to side chains and carbonyl atoms of a conserved set of amino acids (Figs. S3 and S4). PQQ is kept in an equatorial position by Trp^{265} at its base. One side of the PQQ tricyclic ring structure stacks on the side chain of Trp^{243}. The other side interacts with the highly unusual disulfide bond between the adjacent Cys^{103} and Cys^{104} (Blake et al. 1994) found in various other quinoproteins (Fig. 4d–f; Figs. S3 and S4). These cysteine residues form part of an eight-membered ring and are crucial in electron transfer from $PQQH_2$ to the external electron acceptor MxaG (cyt c_L) (Mennenga et al. 2009). Furthermore, in all MxaFI-MDHs with known crystal structures Ca^{2+} is coordinated to O5, N6 and O7 of PQQ, to the OE1 and OE2 oxygen atoms of Glu^{177}, and to the OD1 oxygen of Asn^{261} (Fig. 4d). The catalytic Asp^{303} is also within the coordination sphere of Ca^{2+}. In XoxF2-MDH from *M. fumariolicum*, all amino acids implicated with the binding of PQQ and of Ca^{2+} are also conserved, both regarding their position in the amino acid sequence and their spatial orientation in the structure (Fig. 4a, d; Fig. S3). However, in the verrucomicrobial MDH, the diffracting atom at the catalytic site could only be modeled as a lanthanide, Ce^{3+} in particular (Pol et al. 2014). As mentioned previously, growth of the organism depended on submicromolar concentrations of Ce^{3+}, but other REEs (La, Nd, and Pr) could replace Ce^{3+} to the same extent. REEs of higher atomic numbers than Nd (Sm, Eu, and Gd) were less supportive of growth (Pol et al. 2014). These observations could indicate that XoxF-MDHs are promiscuous in the use of REEs. Importantly, the larger atom seen in the crystal structure was coordinated to one more amino acid, located two positions downstream from the catalytic aspartate, namely Asp^{301} (numbering according to *M. fumariolicum* XoxF structure). In all MxaFs, an alanine is found at this position (Fig. S3). Two more amino acid substitutions seem to be required to accommodate the larger lanthanide: the replacement of Pro^{264}, conserved in MxaFs, by the smaller threonine, and the replacement of Ala^{176} by a glycine. By the presence of Pro^{264} in MxaFs, the protein backbone is slightly changed, forcing the upstream Ca^{2+}-coordinating Asn^{261} further away from the metal. Despite the significant difference in size between Ca^{2+} and lanthanides, bond lengths of these metals to PQQ and to the coordinating Glu^{177} differ less than 0.3 Å (Pol et al. 2014). It is tempting to speculate that the small subunit (MxaI) in MxaFI-MDHs facilitates a proper Ca^{2+} coordination. In agreement, homodimeric MxaF-MDHs purified from *Methylosinus* and *Methylocystis* species that somehow lacked the small subunits displayed 10- to 50-fold higher K_m values for methanol than other MxaFI-MDHs, while V_{max} values were comparable (Table 1) (Grosse et al. 1997, 1998). In addition to various primary alcohols, formaldehyde and other aldehydes were oxidized at high rates by the *Methylosinus* and *Methylocystis* MDHs. As a result, the homodimeric enzymes converted methanol directly into formate, just like the *M. fumariolicum* XoxF2-MDH.

As mentioned, MDHs and other types I and II ADHs share a highly similar overall structure. Moreover, the amino acids responsible for binding PQQ and the metal cofactors are conserved (Figs. S3 and S4). However, the quinoproteins markedly differ in substrate specificities (Table 1). A factor that contributes to the substrate specificity is the volume of the PQQ-containing hydrophobic pocket where catalysis takes place (Toyama et al. 2004, 2005). As calculated from known crystal structures, this volume is smallest for MDH (18 $Å^3$), larger in ethanol dehydrogenase (62 $Å^3$), and even larger in the alcohol dehydrogenases IIB (120 $Å^3$) and IIG (150 $Å^3$) from *Pseudomonas putida*, the last two exhibiting the

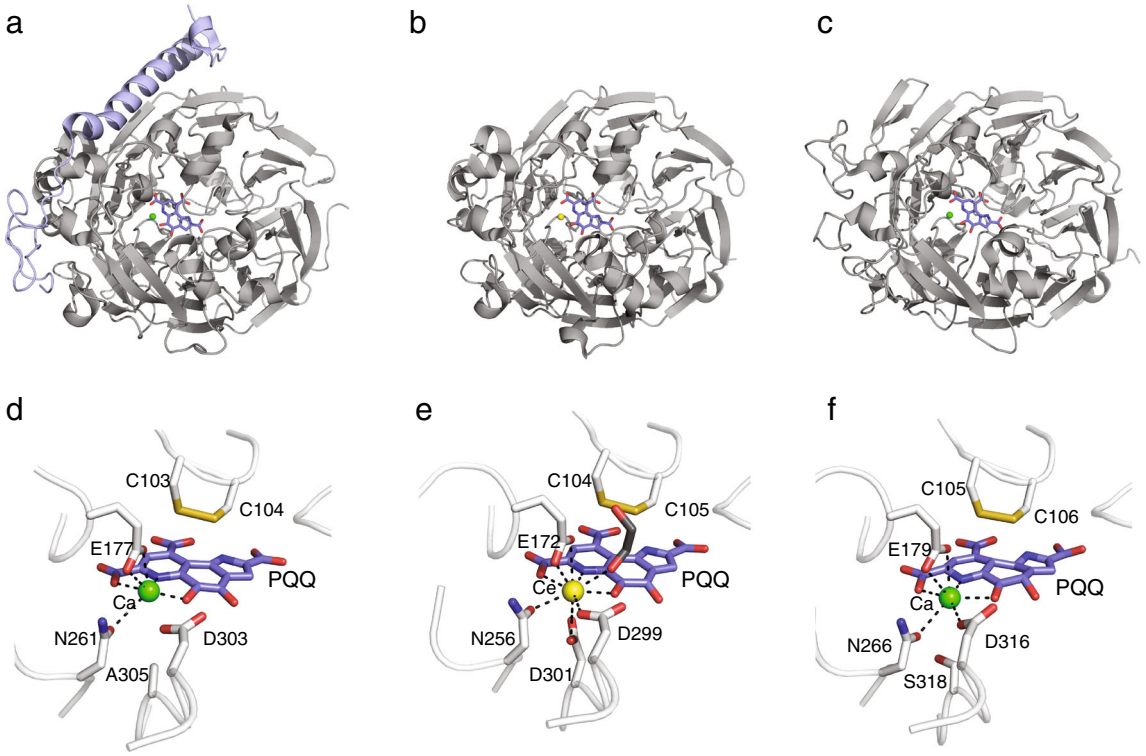

Fig. 4 Structures of methanol and ethanol dehydrogenase quinoproteins. Bottom views of **a** MxaFI-MDH of *M. extorquens* AM1 (PDB: 1W6S) (Williams et al. 2005), **b** XoxF-MDH from *M. fumariolicum* SolV (PDB: 4MAE) (Pol et al. 2014), and **c** ethanol dehydrogenase from *P. aeruginosa* (PDB: 1FLG) (Keitel et al. 2000). **d–f** Detailed view of the PQQ catalytic site of MxaFI-MDH, XoxF-MDH, and ethanol dehydrogenase, respectively. The MxaI small subunit in (**a**) is shown in *blue*. PQQ is displayed in *purple*. Calcium atoms are colored in *green* and cerium in *yellow*. Adjacent cysteines that make a characteristic disulfide bond are shown in *yellow* as well. In (**e**), a polyethylene glycol fragment resolved in the XoxF crystal structure is colored *grey*. Amino acids are numbered according to their positions in the respective structures. Figures were prepared in PyMOL (DeLano 2002)

broadest substrate spectrum (Table 1). During catalysis, the hydrophobic pocket in MDH is closed by Leu^{556}, acting as a lid for substrate access and product exit (Toyama et al. 2004). In ethanol dehydrogenase, Phe^{408} and Leu^{409} function similarly (Keitel et al. 2000), whereas in ADH IIB and the quinohemoprotein alcohol dehydrogenase from *Comamonas testosteroni*, both broad-spectrum alcohol dehydrogenases, the side chains of two aromatic amino acids (Phe^{419} and Phe^{425} and Trp^{440} and Phe^{606}, respectively) form a wider lid (Fig. 4d–f) (Chen et al. 2002; Oubrie et al. 2002; Toyama et al. 2005). In the polypeptide sequences, these amino acids and other ones involved in structuring the active site cavity are notably found in a loop structure (L4) including a segment with high sequence variability (S2) in between the propellers W5 and W6, and another variable segment (S3) in front of W8 (Figs. S3 and S4) (Toyama et al. 2004). A specific feature of MDH is the presence of two cysteines (Cys^{386} and Cys^{415}) around L4. These cysteines make a second disulfide bond in the protein structure, next to Cys^{103}–Cys^{104}.

Phylogenetic analysis described above divided the XoxF-MDHs into at least five families. The structure-based multiple sequence alignment of representatives of these subtypes, including MxaFs, supports this division (Fig. S3). Firstly, peptides that shape the eight β propellers in the crystal structures are conserved in the amino acid sequences. Next, the amino acids that bind PQQ and the central metal (Ca^{2+} and Ce^{3+}), the lid to catalytic site (Leu^{556}), and both Cys^{386} and Cys^{415} are conserved. Deviations mainly concern functionally equivalent substitutions. Importantly, the additional amino acid involved in lanthanide binding in *M. fumariolicum* MDH (Asp^{301}) is found in essentially all XoxF-MDHs. A remarkable aspect is that the amino acids in MxaFs binding to the small subunit are also conserved to quite an extent in XoxFs (Fig. S3), although the latter proteins do not contain a small subunit. These similarities strongly suggest that all XoxFs function as MDH.

Apart from similarities, a number of differences may be noted from the sequence alignment of the MxaF and XoxF large subunits (Fig. S3). These differences correspond with the phylogenetic classification described above. While sequence identities of members that belong to the same XoxF family tend to be higher than 65–70 %, identities between large subunits from different families are considerably less (<50 %). Next, XoxF families are characterized by specific insertions in or small deletions from two subsequent beta propeller strands or beta propeller blades (Fig. S3). This does

not hold for XoxF3-MDHs that lack any of such insertions or deletions. Sequence variations are largest in Loop L4, including segment S2, and S3, which are important in governing the catalytic activity and specificity. However, amino acid sequences of members of the same XoxF family are conserved in these parts. It is not possible to predict how these variations translate into differences in catalytic properties. Only purification and characterization of the proteins can resolve this issue. Furthermore, all XoxFs could bind a lanthanide, but it is not known if they do so. It is conceivable, that they are not only promiscuous in the use of REEs, but certain species might also incorporate the more available Ca^{2+} at the expense of catalytic activity. In this respect, it is conspicuous that in the genome of *M. universalis* XoxF5-encoding genes are present together with the Ca^{2+} insertion machinery (*mxaACKLDRS*). As said, only enzyme purification can give the answer regarding the nature of the metal in a specific enzyme and its relationship to enzyme kinetics.

Catabolism and anabolism in methylotrophs and methanotrophs

XoxF-MDHs provide methylotrophs with the means to metabolize methanol at very low concentrations, which is a definite competitive advantage in natural systems. However, these enzymes might have a drawback.

Formaldehyde, the product of MDH, is both a powerful electron donor (Eq. 1) and the building block for two efficient routes of autotrophic carbon fixation, the RuMP cycle and the serine pathway of which the overall reactions are described by Eqs. 3 and 4a, b, c, respectively.

$$3HCHO + ATP \rightarrow 1GAP + ADP \quad (3)$$

$$HCHO + CO_2 + 2NAD(P)H + 2ATP + HS{-}CoA \rightarrow acetyl{-}S{-}CoA + ADP + 2Pi + 2NAD(P)^+ \quad (4a)$$

$$1.5HCHO + 1.5CO_2 + 3NAD(P)H + 3ATP \rightarrow 1GAP + 3NAD(P)^+ + 3ADP + 2Pi \quad (4b)$$

$$2HCHO + 1CO_2 + 2NAD(P)H + 4ATP \rightarrow 1GAP + 2NAD(P)^+ + 4ADP + 3Pi \quad (4c)$$

$$3CO_2 + 6NAD(P)H + 9ATP \rightarrow 1GAP + 6NAD(P)^+ + 9ADP + 8Pi \quad (5)$$

In the RuMP cycle, all cell carbon is derived from formaldehyde. As formaldehyde and its fixation product, glyceraldehyde-3-phosphate (GAP), are at the same redox level, no reducing power is needed. In the serine pathway, half of the fixed carbon stems from CO_2, which has to be reduced to the formaldehyde level. Written as such in Eq. 4a, the serine pathway is incomplete and does not account for the long-standing enigma of how the starting substrate, glyoxylate, is regenerated. This enigma was solved by the elucidation of ethylmalonyl-CoA (EMCP) pathway in which glyoxalate is recovered by two carboxylation reactions (Erb et al. 2007). In microorganisms that employ the serine pathway but lack the EMCP part, the classic glyoxalate shunt might serve for regeneration (Peyraud et al. 2009). Taking into account those replenishment routes and calculating the serine pathway as leading to GAP as the carbon fixation product, the net process of the EMCP-dependent serine pathway can be reformulated as Eq. 4b. Similarly, the overall reaction of the serine pathway that is supported by the glyoxalate shunt is described by Eq. 4c. One may note that the EMCP variant (Eq. 4b) needs one ATP less but requires more reductive power from NAD(P)H as compared with the glyoxalate alternative (Eq. 4c). Methylotrophs and methanotrophs utilize both the RuMP cycle and the serine pathway for cell carbon synthesis (Table 2). While *Gammaproteobacteria* commonly take advantage of the RuMP cycle, *Alphaproteobacteria* tend to employ the serine pathway. In *Betaproteobacteria,* both pathways are found, yet in a taxonomic-class-related manner. Curiously, in the genomes of many methylo- and methanotrophs the complete inventory of the CBB cycle can be detected as well, which is the most energy-demanding carbon fixation route with respect to both ATP and NAD(P)H requirements (Eq. 5).

Reduced cytochrome *c* (cyt c_L) formed during methanol oxidation serves respiration (Fig. 5) (Anthony 1992). With oxygen as the terminal electron acceptor, one ATP can be synthesized in case of proton-pumping cytochrome *c* oxidases, or less than one ATP in case of high oxygen-affinity, nonproton pumping terminal oxidases, which are also found in the genomes of methylo- and methanotrophs. In energy metabolism, the second product of the MDH reaction, toxic formaldehyde, is further oxidized. Quite recently, it was found that formaldehyde can also be disproportionated by *Methylomicrobium alcaliphilum*, and presumably also by other gammaproteobacterial methanotrophs, supporting a fermentative-like lifestyle under low-oxygen conditions (Kalyuzhnaya et al. 2013). Methylo- and methanotrophs can oxidize formaldehyde by different, species-dependent routes (Table 2). In three of these routes, the reducing power of formaldehyde is stored as NAD(P)H. Many species employ the tetrahydromethanopterin (H_4MPT)-dependent route for formaldehyde oxidation (Chistoserdova et al. 1998; see for reviews, Chistoserdova et al. 2009; Chistoserdova 2011). After formaldehyde import into the cytoplasm, the compound is bound to H_4MPT. The product thereof, 5,10-methylene-

Table 2 Metabolic modules in methylotrophs and methanotrophs

Phylum	Order	Species	Catabolism									Anabolism								
			CH_4 oxidation		CH_3OH oxidation		HCHO oxidation				Bc_1	HCOOH reduction				CO_2 fixation				
			pMMO	sMMO	MxaFI	XoxF	H_4MPT	GSH	FQR	PGDH		PurU	Ftfl	MtdA	FolD	Ser	EMCP	ICL	RUMP	CBB
Alphaproteobacteria	*Rhizobiales*	*M. trichosporium*	+	+	+	+	+	−	+	−	+	−	+	+	−	+	+	−	−	−
		M. silvestris	−	+	+	+	+	+	−	−	+	−	+	−	+	+	+	−	−	+
		M. extorquens	−	−	+	+	+	−	+	+	+	−	+	+	−	+	+	−	−	−
		M. radiotolerans	−	−	+	+	+	+	+	+	+	−	+	+	−	+	+	−	−	−
		H. denitrificans	−	−	+	+	+	−	+	+	+	−	+	−	+	+	+	+	−	−
		F. pelagi	−	−	−	+	+	−	−	−	+	−	+	−	+	+	−	+	−	+
		B. diazoefficiens	−	−	−	+	−	+	+	+	+	+	−	−	+	+	−	+	−	+
		B. japonicum	−	−	−	+	−	+	+	+	+	+	−	−	+	+	−	+	−	+
		S. medicae	−	−	−	+	−	+	+	+	+	+	+	−	+	+	−	+	−	+
	Rhodobacteriales	*P.denitrificans*	−	−	+	+	−	+	?	+	+	+	+	−	+	−	+	−	−	+
		R. sphaeroides	−	−	−	+	−	+	+	−	+	+	+	−	+	+	+	−	−	+
		R. pomeroyi	−	−	−	+	−	+	−	+	+	−	+	−	+	+	+	+	−	−
	Rhodospiralles	*G. bethesdensis*	−	−	+	+	+	−	+	+	−	−	+	+	−	+	+	+	−	−
		A. cryptum	−	−	−	+	−	+	+	+	−	+	+	−	+	+	−	+	−	+
Betaproteobacteria	*Methylophilales*	*M. flagellatus*	−	−	+	+	+	−	+	+	−	+	+	−	+	−	−	+	+	−
		M. glucosetrophus	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	+	+	−
		M. mobilis	−	−	−	+	+	−	+	+	+	+	−	−	+	−	−	+	+	−
		Methylophil2181	−	−	−	+	−	−	+	−	−	−	+	−	+	−	−	+	+	−
	Burkholderiales	*M. petroleiphilum*	−	−	+	+	+	−	−	−	+	+	+	+	+	+	−	+	−	+
		V. paradoxus	−	−	−	+	−	+	+	+	+	+	−	−	+	+	−	+	−	+
		B. phymatum	−	−	−	+	+	+	+	+	+	+	+	−	+	+	−	+	−	+
	Rhodocyclades	*M. universalis*	−	−	−	+	+	−	−	−	+	+	+	+	−	+	−	+	−	+
Gammaproteobacteria	*Methylococcales*	*M. capsulatus*	+	+	+	+	+	−	+	+	+	−	+	+	−	+	−	−	+	+
		M. methanica	+	+	−	+	+	−	+	+	+	−	+	+	−	−	−	−	+	−
		M. alcaliphilum	+	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	+	−
	Thiotrichales	*M. thiooxidans*	−	−	+	+	+	−	−	+	−	+	+	−	+	−	−	+	+	−
Verrucomicrobia		*M. fumariolicum*	+	−	−	+	−	−	−	+	−	−	+	−	+	−	−	−	−	+
NC10 Bacteria		*M. oxyfera*	+	−	+	+	+	−	−	+	+	+	+	−	+	−	−	+	−	+
Acidobacteria		*Ca* 'S. usitatus'	−	−	−	+	−	+	−	+	−	+	−	−	+	−	−	+	−	−

pMMO particulate methane monooxygenase, *sMMO* soluble methanemonooxygenase, *MxaFI* MxaFI methanol dehydrogenase, *XoxF* XoxF methanol dehydrogenase, *H4MPT* H4MPT-dependent route of formaldehyde oxidation, *GSH* glutathione-dependent route of formaldehyde oxidation, *FQR* putative formaldehyde:quinone reductase, *PGDH* 6-phosphogluconate dehydrogenase, decarboxylating, *Bc1* Bc1 complex, *PurU* formyltetrahydrofolate deformylase, *Ftfl* formate-tetrahydrofolate ligase, *Ser* serine pathway of CO_2 fixation, *EMCP* ethylmalonyl-CoA pathway of acetyl-CoA regeneration, *ICL* glyoxalate shunt, *RUMP* ribulose monophosphate cycle, *CBB* Calvin–Benson–Bassham cycle, *M. trichosporium Methylosinus trichosporium* OB3b, *M. silvestris Methylocella silvestris* BL2, *M. extorquens Methylobacterium extorquens* AM1, *M. radiotolerans Methylobacterium radiotolerans* JCM 2831, *H. denitrificans Hyphomicrobium denitrificans* ATCC 51888, *F. pelagi Fulvimarina pelagi*, *B. diazoefficiens Bradyrhizobium diazoefficiens* USDA 110, *B. japonicum Bradyrhizobium japonicum* USDA 6, *S. medicae Sinorhizobium medicae* WSM419, *P.denitrificans Paracoccus denitrificans* PD1222, *R. sphaeroides Rhodobacter sphaeroides* 2.4.1, *R. pomeroyi Ruegeria pomeroyi*, *G. bethesdensis Granulibacter bethesdensis*, *A. cryptum Acidiphilium cryptum* JF-5, *M. flagellatus Methylobacillus flagellatus* KT, *M. glucosetrophus Methylovorus glucosetrophus* SIP3-4, *M. mobilis Methylotenera mobilis* JLW8, *Methylophil2181 Methylophilales* 2181, *M. petroleiphilum Methylibium petroleiphilum* PM1, *V. paradoxus Variovorax paradoxus* S110, *B. phymatum Burkholderia phymatum*, *M. universalis Methyloversatilis universalis* FAM5, *M. capsulatus Methylococcus capsulatus* Bath, *M. methanica* Methylomonas methanica MC09, *M. alcaliphilum Methylomicrobium alcaliphilum* 20Z, *M. thiooxidans Methylophaga thiooxidans* DMS010, *M. fumariolicum Methylacidiphilum fumariolicum* SolV, *M. oxyfera Candidatus* 'Methylomirabilis oxyfera', *Ca 'S. usitatus' Candidatus* 'Solibacter usitatus' Ellin6076

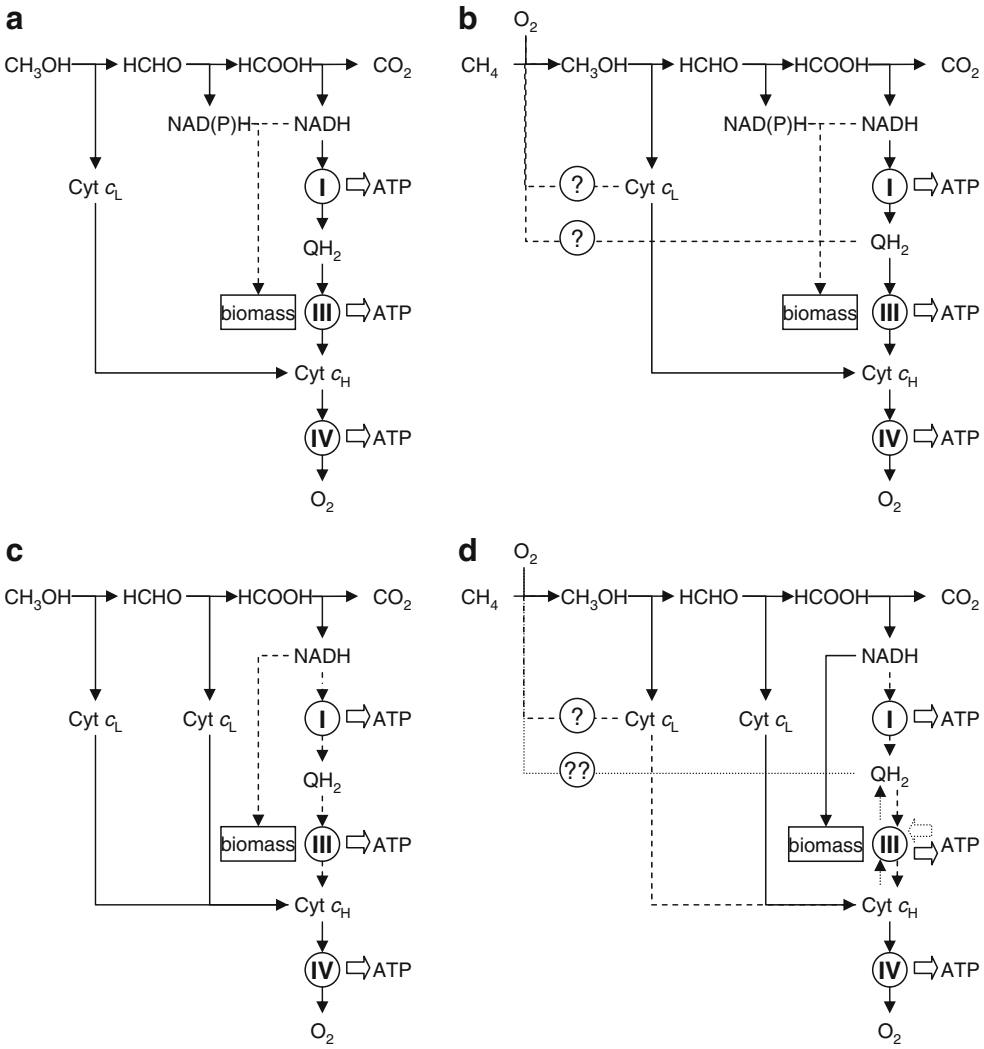

Fig. 5 Schematic overview of catabolic and anabolic processes in **a**, **c** methanol and **b**, **d** methane oxidation. In (**a**) and (**b**), methanol and methane oxidation pathways, respectively, are shown with formaldehyde as a free intermediate. In (**c**) and (**d**), it is presumed that methanol is directly oxidized to formate, i.e., without intermediary formation of free formaldehyde, as might be the case with XoxF-type MDHs. It is presently unknown if electrons needed for methane monooxygenation are derived from reduced cytochrome c_L of from reduced quinone (QH_2). The direct oxidation of methanol to formate would result either in the lack of QH_2 for methane activation or in the lack of NAD(P)H for biosynthesis. Putative generation of QH_2 in (**d**) by reversed electron transport involving complex III (bc_1) is indicated by *dotted lines*. *Roman numbers within circles* represent the respiratory complexes I, III, and IV

H_4MPT, is oxidized to the formyl (methenyl) oxidation level by NAD(P)-dependent methylene-H_4MPT dehydrogenase (MtdB) (Hagemeier et al. 2000). After rearrangement of the methenyl into the formyl group, followed by its release by the action of formyl hydrolase/lyase (Fhc) (Pomper at al. 2002), formate is oxidized to CO_2 by one of the different formate dehydrogenases (FDH) that the bacteria have at their disposal (Chistoserdova 2011). Usually, formate oxidation is coupled to the reduction of NAD^+ (Fig. 5). In the second pathway of formaldehyde oxidation, GSH acts as the C-1 carrier (Ras et al. 1995; Barber and Donohue 1998). Formaldehyde bound as hydromethyl-GSH is converted into formyl-GSH with NAD^+ as the electron acceptor, again inside the cell. The third alternative for formaldehyde oxidation coupled to $NAD(P)^+$ reduction takes place in conjunction with the RuMP cycle (Anthony 1982). Herein, 6-phosphogluconate, an intermediate of the cycle, is oxidized with the release of a CO_2 by 6-phosphogluconate dehydrogenase (PGDH). NAD(P)H formed in those processes may either serve reductive processes in cell carbon synthesis or it can be oxidized in connection with O_2 respiration; the distribution of these fluxes will depend on the cellular needs. The successive passage of the electrons from NADH oxidation through complex I, complex III (bc_1), and (one of the) terminal oxidases (complex IV) permits a maximal synthesis of three molecules of ATP (Fig. 4). Quite remarkably, not all methylotrophs possess a bc_1 complex, such as the verrucomicrobial methanotrophs (Table 2).

The depicted situation is different for methanotrophs. Methanotrophs need two electrons derived from methanol oxidation for the O_2-dependent activation of their substrate, methane (Fig. 5b, d). This activation is catalyzed by two different methane monooxygenases (MMO): a particulate, membrane-bound (pMMO) or a soluble (sMMO) enzyme (reviewed in: Kopp and Lippard 2002; Hakemian and Rosenzweig 2007; Murray and Lippard 2007; Culpepper and Rosenzweig 2012). In pMMO electrons for methane activation are most likely supplied by reduced quinone (Cook and Shiemke 2002; Choi et al. 2003; Shiemke et al. 2004), but the role of reduced cyt c_L as electron donor cannot

be ruled out (Myronova et al. 2006); sMMO uses NADH directly.

A fourth way to oxidize formaldehyde has received little attention, namely by the use of a dye-linked formaldehyde dehydrogenase (DL-FalDH). The reason for this is that DL-FalDH activities are generally low in methylo- and methanotrophs as far as investigated (Anthony 1982; Attwood 1990 and references herein). Nevertheless, three such DL-FalDHs have been purified, viz. from *Hyphomicrobium zavarzinii* (Klein et al. 1994), and from the methanotrophs *M. trichosporium* OB3b (Patel et al. 1980) and *Methylococcus capsulatus* (Zahn et al. 2001). Interestingly, the membrane-bound enzyme from *M. capsulatus* harbored covalently bound PQQ. The previously reported terminal amino acid sequence (Zahn et al. 2001) traces it now back to a protein (MCA2155) that has been annotated in the genome as sulfide/quinone reductase (SQR). SQR couples the oxidation of sulfide to the reduction of quinone. The flavin adenine dinucleotide (FAD)-containing SQR is well-defined by the resolution of its structures from *Aquifex aeolicus* (Marcia et al. 2009) and from *Acidithiobacillus ferrooxidans* (Cherney et al. 2010). SQR is distantly related to other flavoprotein disulfide reductases like GSH reductase, thioredoxin reductase, and lipoamide dehydrogenase (Marcia et al. 2010). While the comparison of PPQ-containing MCA2155 with these known SQRs reveals substantial sequence identity (~50 %), essential amino acids related with FAD binding, catalysis, access and release of the sulfur substrates and products are not well conserved in MCA2155, unlike the quinone-binding site (data not shown). Combined, the findings might suggest that MCA2155 is a new type of PQQ formaldehyde/quinone reductase. In fact, homologs of MCA2155 are found in the genomes of many methylo- and methanotrophs where these homologs have been commonly annotated as "FAD-dependent pyridine nucleotide-disulfide oxidoreductases" or "sulfide/quinone reductases" (Table 2). In the genomes of *Methylophilales*, genes coding for these homologs are clustered with the ones encoding XoxF4 proteins (Fig. 2; Fig. S2).

XoxF-MDHs can oxidize methanol directly into formate. Theoretically, such oxidation has two severe implications regarding methylotrophic catabolism and anabolism. Since cyt c_L will act as the electron acceptor for formaldehyde oxidation as well, one site for $NAD(P)^+$ reduction gets lost, leaving formate oxidation as the only option to generate NAD(P)H. Methanotrophs need one reduced quinone for each methane activation step. If reduced, quinone only is formed at the expense of NADH oxidation (by complex I), no room would be left for surplus NAD(P)H for cell carbon synthesis. However, reduced quinone could also be generated by proton-motive force driven reversed electron transport across complex III (bc_1) (Fig. 5d), but not all methanotrophs contain this complex (Table 2). In the latter case, the solution could be that pMMO takes reduced cytochrome as electron donor. The second consequence is that formaldehyde gets lost. Now, organisms would have to rely on the energy-demanding CBB cycle for CO_2 fixation (Table 2), as is the case for the *Verrucomicrobia* (Khadem et al. 2012a), for the NC10 bacterium *M. oxyfera* (Ettwig et al. 2010; Rasigraf et al. 2014) and methanol-utilizing phototrophs (Table 2). However, there is a way to circumvent the CBB scenario: reduction of formate back to formaldehyde to feed the RuMP and serine cycles.

For formate reduction, two different routes are available that both employ H_4folate as the C-1 carrier (Table 2) (Chistoserdova et al. 2009; Chistoserdova 2011). In both routes, formate is bound to H_4F in an ATP-dependent reaction catalyzed by formate tetrahydrofolate ligase (Ftfl). Microorganisms that contain the H_4MPT-dependent pathway of formaldehyde oxidation reduce formyl-H_4F by the subsequent action of two novel enzymes, methenyl-H_4F cyclohydrolase Fch (Pomper et al. 1999) and NADPH-dependent methylene-H_4folate dehydrogenase MtdA (Vorholt et al. 1998, 1999). MtdA is distantly related to MtdB mentioned before, but it uses methylene-H_4folate as the preferred substrate, unlike MtdB that preferentially converts methylene-H_4MPT. The substrate specificities of MtdB and MtdB support their role in formaldehyde catabolism and anabolism, respectively. It should be noted that the Fch/MtdA-dependent route for formate reduction is indispensable for methylotrophic growth of *M. extorquens* also under standard laboratory growth conditions, indicating that formate is an indispensible cell carbon fixation intermediate (Chistoserdova and Lidstrom 1994a, b; Crowther et al. 2008). Microorganisms that lack the Fch-MtdA enzymes dispose of the ubiquitous bifunctional methylene-H_4F dehydrogenase/methenyl-H_4 cyclohydolase (FolD) for formate reduction (Table 2). Obviously, formate reduction requires an extra ATP and an extra NAD(P)H for each molecule that is added to the RuMP or serine cycles. In this respect, the CBB cycle would not make that bad an alternative.

As pointed out, the direct oxidation of methanol to formate not only goes with the loss of formaldehyde but also with NADH generation. Thus, it is conceivable that XoxF-MDHs—perhaps in a type-specific way—release formaldehyde rather than formate, as the MxaFI proteins do. In this respect, it is interesting to recall that genes coding for $NAD(P)^+$- and GSH-dependent formaldehyde dehydrogenase and the putative formaldehyde:quinone reductase just described may be clustered with the ones that encode XoxF-MDHs (Fig. 2; Fig. S2). In addition, the clustering of the XoxF5 gene system in *Variovorax paradoxus* with the genes coding for the CBB key enzyme ribulose-1,5-bisphophate carboxylase (Rubisco) is conspicuous in these respects (Fig. S2). Anyway, how individual microbial species resolve the anabolic and catabolic issue that may go with the use of catalytically more efficient XoxF-MDHs is a wide open question than only can be resolved experimentally.

Are there any other REE-dependent quinoproteins?

In the "Structure of methanol dehydrogenases,", we noted the high sequence similarities between MxaF- and XoxF-MDHs involved in the overall structuring of these proteins and in the binding of the PQQ and metal prosthetic groups. A distinctive feature of the XoxFs was the presence of an aspartate (Asp^{301}) that was located two amino acids downstream from the catalytic aspartate in the XoxF2-MDH sequence of *M. fumariolicum* and that formed part of the REE-binding network. Bearing the structural conservation of all types I and II methanol and alcohol dehydrogenases in mind, we carried out BLAST searches, went into genomic databases of methylo- and methanotrophs, and made multiple sequence alignments to see if we could detect putative ADHs other than MDHs with the diagnostic REE-binding Asp^{301}. We readily found a multitude of these annotated as "alcohol dehydrogenases", "methanol/ethanol family PQQ-dependent dehydrogenases" or simply as "pyrrolo-quinoline quinones" and that could be split into nine clades (Fig. 1; Fig. S1). Again, phylogenetic typology was supported by multiple sequence analysis (Fig. S4). One group (no. 2) branched with known calcium-containing ethanol/alcohol-oxidizing quinoproteins. Apparently, these calcium enzymes may have their REE-containing counterparts. This did not hold for type II quinohemoproteins (group no. 4 in our tree) for which no candidate could be detected with the REE-associated aspartate. The seven other groups were not affiliated with quinoproteins with a known function, except perhaps group no. 6 that contained a distantly related sorbose dehydrogenase from *Pseudogluconobacter saccharoketogenes* (Shibata et al. 2001). Another interesting finding was that *S. usitatus* evolved a group by its own with no less than six representatives, two of which have a *c*-type heme in their N termini, which is new, and the other four lacking this heme (Fig. S4b). At this stage, it is not possible to make a prediction of the function, the new, likely REE-containing quinoproteins on the basis of the amino acid sequences alone: catalytic properties will depend on not-yet defined, subtle differences near the active site cavity. However, the new proteins might represent an undisclosed potential of dehydrogenases catalyzing the (stereospecific) conversion of a range of alcohol and aldehyde substrates. Needless to stress, that none of the proteins have been purified as yet.

Summary and perspectives

A broad class of enzymes, and MDHs in particular, use PQQ as their catalytic prosthetic group for the oxidation of alcohols or aldehydes. Except for magnesium-dependent membrane-bound glucose dehydrogenase (Anthony 2004), all quinoproteins described until a few years ago carry calcium as the cofactor in catalysis. However, recently four MDHs from four different sources were isolated that possessed REEs instead of Ca^{2+} (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012; Pol et al. 2014). These MDHs belonged to the enigmatic XoxF-type MDHs widely found in the genomes of methylo- and methanotrophs, and even in species that are not known as being methylotrophs (Fig. 1; Fig. S1). Our analyses indicate that these XoxF-MDHs, of which five clades already had been recognized (Chistoserdova 2011), could all contain REEs as their cofactor. The dependence on REEs might be an explanation for difficulties and irreproducible results in the isolation and growth of species from the environment. Furthermore, XoxF-MDHs probably represent a minor fraction of REE-containing quinoproteins, together covering an unexploited potential of catalytic functions (Fig. S1). Herewith, we might enter a new era in methylotrophy and quinoprotein research, and like any new era, this will go with many questions.

Unlike the name suggests, REEs are not particularly rare. These elements occur in each and every type of soil, sand, and sediment (Moermond et al. 2001; Weltje et al. 2002 and reference therein). The main problem is their insolubility either as carbonates or bound to organic substances. In pore and surface water, concentrations of REEs are only in the picomolar to nanomolar range, which is up to six orders of magnitude less than in the contingent solids. For the acquirement from the environment, a microorganism may need dedicated uptake systems and regulatory mechanisms that control the uptake, both of which of as-yet unknown nature. Plants and algae accumulate REEs very efficiently (Weltje et al. 2002). Thus, it is conceivable that plant-associated microorganisms benefit from this opportunity, but microorganisms most certainly will have specific REE-uptake systems themselves.

The presence of a REE in the PQQ catalytic centers could make XoxF-MDHs more efficient catalysts in methanol conversion. Questions are how this efficiency is achieved and if it holds for all XoxFs. There are still many open questions regarding the catalytic mechanism of PQQ proteins. MDHs have often been studied at nonphysiological high pH values with ammonia as an artificial activator. As pointed out by the authors (Pol et al. 2014), the XoxF-MDH from *M. fumariolicum* that already functions optimally at neutral pH and that does not need ammonia for activation might provide an excellent model to reassess the structure–function relationship of MDHs. A specific problem that might be associated with the action of XoxFs is that they efficiently oxidize not only methanol but also formaldehyde (see next). Again, the question is how formaldehyde oxidation is catalyzed and if the efficiency applies to all XoxF-MDHs. MxaFI-MDHs, which represent in fact a "modern" MDH variant, incorporate much more available Ca^{2+} as a cofactor and oxidize methanol to formaldehyde only. This retuning and

restructuring may have needed the assistance of the additional small subunit, as well as a collection of accessory proteins involved in Ca^{2+} insertion and further protein maturation.

The oxidation of methanol into formate by one enzyme, as is done by XoxF-MDHs, may have severe implications for methylo- and methanotrophic catabolism and anabolism. For a microorganism equipped with XoxFs, it will be a matter of checks and balances whether or not use those enzymes or to tune these to their own needs for survival in natural ecosystems. The catabolic and anabolic consequences are not experimentally resolved. All in all, the field of methano- and methylotrophy is a rapidly expanding puzzle of redundant anabolic and catabolic possibilities and opportunities (Chistoserdova et al. 2009; Chistoserdova 2011). Obviously, XoxF proteins add more pieces to the puzzle, but their understanding perhaps enables the puzzle to fall into place in the end.

Briefly, REE-containing MDHs—and possibly also a whole class of new quinoproteins—make a difference, both regarding their catalytic properties and metabolic consequences. Or to quote Chris Anthony, who discovered MDH quinoproteins and who spent a substantial part of his scientific career on those proteins: "If I had a lab now I'd be running around testing everything to see if I could find them" (Popkin 2013).

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