Tungsten containing aldehyde oxidoreductase (AOR)-family enzymes; past, present and future production strategies

Boes, D.M.; Schmitz, R.A.; Hagedoorn, P.L.

DOI
10.1016/bs.mie.2025.01.027

Publication date
2025

Document Version
Final published version

Published in
Methods in Enzymology

Citation (APA)
Boes, D. M., Schmitz, R. A., & Hagedoorn, P. L. (2025). Tungsten containing aldehyde oxidoreductase (AOR)-family enzymes; past, present and future production strategies. *Methods in Enzymology*, *714*, 313-336. https://doi.org/10.1016/bs.mie.2025.01.027

CHAPTER FIFTEEN

Tungsten containing aldehyde oxidoreductase (AOR)-family enzymes; past, present and future production strategies

Deborah M. Boes, Rob A. Schmitz, and Peter-Leon Hagedoorn*
Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, NL-2629HZ Delft, Netherlands
*Corresponding author. e-mail address: P.L.Hagedoorn@tudelft.nl

Contents

1. Introduction 314
2. Purification of AOR from *Pyrococcus furiosus* 318
   2.1 Preparation of *P. furiosus* growth medium 318
   2.2 Cultivation of pre-cultures of *P. furiosus* 319
   2.3 Cultivation of *P. furiosus* in a 20 L batch fermenter 320
   2.4 Cell collection, storage, and preparation of cell-free extract 321
   2.5 Purification of AOR 322
3. Recombinant expression of W-dependent enzymes 324
   3.1 Hyperthermophilic archaeal protein expression 324
   3.2 Assays 325
   3.3 Acetylene hydratase 326
   3.4 Formate dehydrogenase 328
   3.5 Glyceraldehyde-3-phosphate oxidoreductase expressed as a Mo-containing enzyme 329
   3.6 YdhV – the AOR family homolog of *E. coli* 330
4. Conclusion 332
Acknowledgements 332
References 332

Abstract

The transition metals tungsten and molybdenum are the heaviest metals found in biological systems and are embedded in the cofactor of several metalloenzymes. As a result of their redox activity, they provide great catalytic power in these enzymes and facilitate chemical reactions that would not occur using only the functionalities of natural amino acids. For their functionality these enzymes depend on a metal cofactor, which consists of at least one metal binding pterin (MPT) and a tungsten or molybdenum ion, but the complete make-up of the cofactor differs per enzyme group. One of these

Methods in Enzymology, Volume 714
ISSN 0076-6879, https://doi.org/10.1016/bs.mie.2025.01.027

enzyme groups comprises the AOR-family enzymes. These enzymes have the ability to oxidize a range of aldehyde substrates into their corresponding carboxylic acid products. Next to this, they are also the only known catalysts able to perform the thermodynamically challenging reduction reaction of carboxylic acids to aldehydes. These enzymes are currently obtained by purification from the hyperthermophilic archaeon *Pyrococcus furiosus*. This process, however, does not yield a large amount of enzyme, since it is naturally expressed at moderate levels. For that reason, other production methods need to be considered if the enzyme is to be used on a large scale. These alternatives include the use of a recombinant expression system. The recombinant expression of W-dependent enzymes in different host organisms, such as *Escherichia coli*, has already been attempted for different enzymes, but with varying success. This shows that more research on the production, and especially incorporation of the metal cofactor, is necessary to achieve a successful production and use of recombinant AOR-family enzymes.

1. Introduction

Metal-containing enzymes (metalloenzymes) contain a metal ion, a metal ion containing organic complex (e.g. heme) or an inorganic complex (e.g. FeS clusters) as cofactor, and can be used to catalyse a variety of chemical reactions under mild conditions (Cordas & Moura, 2019). Transition metals in biology are highly interesting due to their redox activity, which allows them to accept and donate electrons and thereby facilitate chemical reactions that would not occur using only the functionality of amino acid side chains. These transition metals include molybdenum (Mo) and tungsten (W) (Hille, 2002). Molybdenum is the most widely used metal amongst these two and more than 50 different Mo-enzymes have been identified to date (Hille, 2002; Mendel & Oliphant, 2024). Some organisms only have Mo-containing enzymes, some use an alternative metal with remarkably similar chemical properties, tungsten, and some organisms contain both Mo- and W-enzymes. Molybdenum and tungsten are separated in the periodic table by a group of elements called the lanthanides. These elements decrease in size of the ionic radii with an increasing atomic number. This phenomenon, which is called the lanthanide contraction, is the reason why the ionic radii of tungsten and molybdenum are very similar, despite tungsten being a much heavier element. Next to that, tungsten and molybdenum occur in nature in the same valence states and can therefore donate and receive the same number of electrons, giving them similar chemical properties (Cui & Gomes, 2021; King, 1969). Tungsten- and molybdenum-dependent enzymes can be found in various enzyme families including dehydrogenases, hydroxylases, and oxidases. Generally, these

enzymes catalyse redox reactions that are important for the metabolism of the host organism and thereby help to maintain a homeostasis of available nutrients (Arndt et al., 2019). For their functionality, these enzymes share a similar cofactor consisting of a metal centre (Mo, W) coordinated by one or two organic ligands, called metallopterins (or metal binding pterin cofactor, MPT) (Cordas & Moura, 2019).

W- and Mo-containing enzymes have been categorized into different families according to the structure of the Mo/W-cofactor they contain. W-enzymes can be divided into the DMSO reductase (DMSOR) and aldehyde oxidoreductase (AOR) families. Mo-containing enzymes can be separated into three families, namely the DMSOR, xanthine oxidase (XO), and sulphite oxidase (SO) families (Schut et al., 2021). As a result of the similar chemical properties of the two metals, some enzymes (e.g. DMSORs) are able to incorporate either metal into their cofactor, forming a W-cofactor or Mo-cofactor (Wco or Moco). Not all enzyme families, however, have this ability, and therefore some almost exclusively contain a Wco, such as AORs, and some apparently exclusively contain a Moco, such as the XO and SO families (Seelmann et al., 2020; Stewart et al., 2000). In the enzyme families that contain a Wco, two MPT molecules are present that provide four dithioline sulphur ligands to the metal. The complete make-up of the cofactor differs slightly depending on the enzyme class. In the cofactor of the DMSOR family a guanine nucleotide is added to each MPT, forming two metallopterin guanine dinucleotides (bis-MGD). Next to that, DMSORs contain an amino acid provided by the protein backbone, which serves as an additional ligand (Cys, Sec, Asp, or Ser). The other W-dependent enzyme family, AORs, only contain two MPT moieties (bis-MPT) per W without any additional ligands (Chan et al., 1995; Maia et al., 2015; Winiarska et al., 2023). To facilitate electron transfer within the protein, most W- or Mo-dependent enzymes contain additional cofactors such as an iron-sulphur (Fe-S) cluster, flavin, or heme (Seelmann et al., 2020).

In nature, the soluble forms of tungsten and molybdenum are the oxyanions tungstate (WO_4^-) and molybdate (MoO_4^-), respectively. These oxoanions are actively transported across the membrane via specific high-affinity transport systems (ABC transporters). Several ABC transporters are known: the TupABC system which exclusively transports tungstate, the WtpABC system which is able to transport tungstate as well as molybdate, but has a higher affinity for tungstate over molybdate, and the ModABC system which is generally recognized as a molybdate transporter, but has been shown to be able to bind both tungstate and molybdate in *Escherichia coli* (*E. coli*)

(Aguilar-Barajas et al., 2011; Bevers et al., 2011). The A components of all transport systems, which bind the oxyanions, share low sequence similarity. For instance, WtpA of *Pyrococcus furiosus* only has a 30% sequence similarity with ModA of *E. coli* and no homology with TupA. The other components of the transport systems, B and C, have a higher sequence similarity with homologous proteins. WtpB has a sequence similarity of 53% and 50% with ModB and TupB, respectively, and WtpC has a sequence similarity of 51% and 56% with ModC and TupC, respectively (Bevers et al., 2006).

In *E. coli*. tungsten can actively enter the cells through the transporter ModABC and passively through the sulphate transport system when a sufficiently high tungstate concentration is present in the surrounding medium (Grunden & Shanmugam, 1997).

To understand the assembly of the Wco, generally the Moco biosynthesis is used as an example, since this is the better explored biosynthesis pathway. The fact that almost all genes required for Moco synthesis are also found in genomes of organisms that exclusively produce W-containing enzymes, already shows the high similarity of the two pathways. For that reason, it is generally assumed that the biosynthetic pathway leading to the Moco and Wco includes the same intermediates and is catalysed by the same, or similar, enzymes (Bevers et al., 2008; Seelmann et al., 2020). Moco (and Wco) synthesis proceeds via a four- or five-step process, depending on the cofactor (Fig. 1). (1) First, a guanosine-5′-triphosphate (GTP) is converted to the intermediate cyclic pyranopterin monophosphate (cPMP) using the enzymes MoaA and MoaC. MoaA is a radical *S*-adenosyl-L-methionine (SAM) enzyme that contains two iron-sulphur clusters. The enzyme uses these iron-sulphur clusters and a SAM to rearrange the GTP and liberate a 5′-deoxyadenosyl radical. The second enzyme, MoaC, subsequently catalyses the cyclization of the product formed by MoaA to cPMP (Hänzelmann & Schindelin, 2004; Pang & Yokoyama, 2018). (2) Next, two thiol groups are added to the cPMP by MoaD and MoaE, forming the compound MPT. (3) The formed MPT molecule is, subsequently, adenylylated by MoaB or MogA. (4) Finally, MoeA catalyses the insertion of the metal and mediates the release of the AMP molecule forming the compound Mo-MPT, which is the cofactor found in enzymes of the SO family (Bevers et al., 2008; Leimkühler, 2020; Mendel & Leimkühler, 2015). A cytidine monophosphate (CMP) molecule can be added to the Mo-MPT by the enzyme MocA to form Mo-MCD. If a second MPT molecule is added by the enzyme MobA, the Mo-bis-MPT cofactor is formed that is found in AOR-family enzymes, amongst others. Further attachment of two guanosine

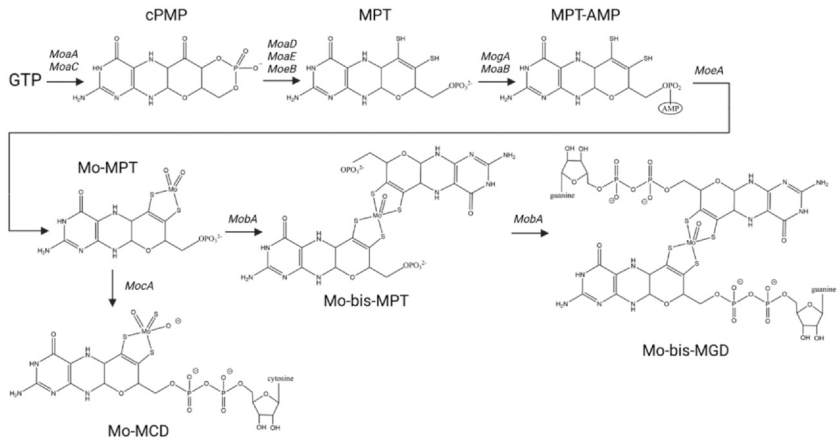

Fig. 1 Molybdenum and tungsten cofactor biosynthesis. The steps in the Moco/Wco biosynthesis and the responsible genes, from GTP to Mo-MCD, Mo-bis-MPT, or Mo-bis-MGD.

monophosphate (GMP) moieties by MobA results in the formation of the Mo-bis-MGD cofactor found in, for example, DMSOR family enzymes (Reschke et al., 2013). The enzyme MobB has also been believed to play a role in the Mo-bis-MGD formation, but the specific function and interaction of this enzyme still remains unclear (Leimkühler, 2014).

Most characterized W-containing enzymes are from hyperthermophilic archaea, such as *Pyrococcus furiosus* (*P. furiosus*). Two of these are the homodimeric aldehyde oxidoreductase (AOR) and the monomeric glyceraldehyde-3-phosphate oxidoreductase (GAPOR) (Arndt et al., 2019; Mukund & Adams, 1991, 1995). AOR can catalyse the oxidation of various aldehydes to their corresponding carboxylic acids, while GAPOR specifically converts D-glyceraldehyde-3-phosphate to 3-phosphoglycerate, with ferredoxins or viologen dyes as final electron acceptors (Fig. 2).

Since aldehydes are toxic to the cell in large quantities, it is believed that the main role of AOR enzymes is the elimination of excess aldehydes (Winiarska et al., 2023). Besides the removal of aldehydes through an oxidation reaction, AORs were shown to catalyse the reverse reduction reaction. This reversibility makes these enzymes the only known biocatalysts to catalyse the thermodynamically challenging reduction of non-activated carboxylic acids to aldehydes ($E' \approx -560$ mV), if low-potential electron donors are available (Mukund & Adams, 1991; Seelmann et al., 2020; White et al., 1989). This activity of AOR enzymes has attracted

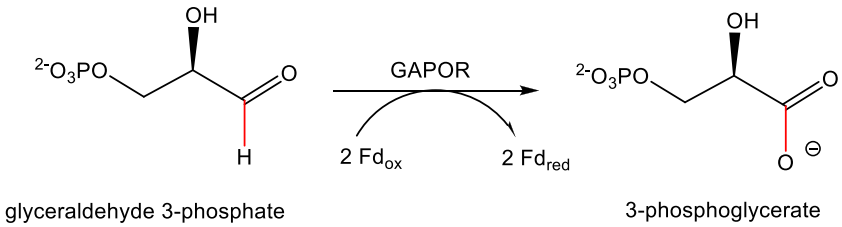

Fig. 2 Reaction catalysed by glyceraldehyde-3-phosphate oxidoreductase (GAPOR). Oxidation of D-glyceraldehyde-3-phosphate to 3-phosphoglycerate. Fd_{ox}: oxidized ferredoxin. Fd_{red}: reduced ferredoxin.

attention to be used in the production of aldehydes from carboxylic acids originating mainly from recycling processes. These aldehydes can subsequently be used in several industrial processes including the production of pharmaceutical and agricultural products. Due to lack of alternatives, these intermediates are currently obtained via chemical reactions that require the use of chemical catalysts at high temperatures and pressures, resulting in unsustainable processes (Venkitasubramanian et al., 2008).

2. Purification of AOR from *Pyrococcus furiosus*

2.1 Preparation of *P. furiosus* growth medium

Typically, AOR enzymes can be obtained from their native host, such as the hyperthermophilic archaeon *P. furiosus*, of which the AOR family enzymes have been best characterized. *P. furiosus* is cultivated in a batch culture and subsequently the enzymes are purified from the cell-free extract (CFE) using several consecutive column chromatography steps (Roy et al., 2001). *P. furiosus* is an extremophile, it grows at temperatures ranging from 70 to 105 °C, with an optimum at approximately 100 °C (Verhagen et al., 2001). The high cultivation temperature is energy demanding and requires specialized fermentation equipment that can handle liquid at a boiling temperature, e.g. stainless steel fermenters that can be sterilized in place.

To start the fermentation, *P. furiosus* is grown as an overnight batch culture in 20 L fermenters (ADI 1065, Biobench, Applikon, The Netherlands) at 90 °C. The fermenters are inoculated (2 vol%) with pre-cultures grown anaerobically overnight in incubation bottles. The following stock solutions are used to prepare medium to grow pre-cultures: (1) Solution 1 (in g L^{-1}): NaCl, 150; yeast extract, 1.25. (2) Solution 2 (in g L^{-1}): $NaHCO_3$, 10;

KH_2PO_4, 1.4; $CaCl_2{\cdot}H_2O$, 1.4; NH_4Cl, 2.5; $MgCl_2{\cdot}6H_2O$, 27; $MgSO_4{\cdot}7H_2O$, 34; KCl, 3.3; $NiCl_2$, 0.005; $Na_2WO_4{\cdot}2H_2O$, 0.033. To minimize precipitation, $NaHCO_3$ is dissolved separately before it is mixed with the solution containing the other compounds. (3) Solution 3 (in g L^{-1}): ethylenediaminetetraacetic acid (EDTA), 0.5; $ZnCl_2$, 0.05; $CoSO_4$, 0.05; $FeCl_2{\cdot}4H_2O$, 8; $MnCl_2{\cdot}4H_2O$, 0.5; H_3BO_3, 0.05. 1 mL HCl (37%) and EDTA are added before adding the metals. (4) Solution 4 (in mg per 100 mL): biotin, 2; folic acid, 2; pyridoxin, 10; riboflavin, 5; thiamine/HCl, 5; nicotinamide, 5; cyanocobalamin, 5; p-aminobenzoate (4-aminobenzoate), 5; calcium-d-pantothenate, 5; lipoic acid 5. Solution 1 and 2 are sterilized using an autoclave, whereas solution 3 and 4 are sterilized using filters (pore size 0.20 μm).

In a laminar flow cabinet, 100 mL solution 1, 50 mL solution 2, 0.5 mL solution 3, and 0.05 mL solution 4 are mixed. Subsequently, 0.25 mL resazurin (0.2 g L^{-1}), 2.5 g potato starch (Koopmans, Amersfoort), and Milli-Q water are added up to a volume of 0.5 L. This solution is referred to as "*Pfu* medium". Best results were obtained using starch that is highly soluble, such as potato starch, as commercially available corn starch was found to be less suitable.

2.2 Cultivation of pre-cultures of *P. furiosus*

For the cultivation of *P. furiosus,* cryostocks are used that were previously made using a fresh *P. furiosus* culture. To prepare the cryostocks, 0.15 mL of sterile 100% glycerol (anaerobic) and 0.85 mL *P. furiosus* culture were added to a small (2–5 mL) crimp-cap vial under anaerobic conditions. The sample was mixed, the vial capped and subsequently stored in a −80°C freezer.

To start the cultivation, sterile glass serum bottles of approximately 120 mL are filled with 30 mL *Pfu* medium. The bottles are capped and sealed with black rubber septa and aluminium crimp caps. Subsequently, the bottles are made anoxic by using an Argon/vacuum manifold (Merck, Darmstadt, Germany), using 5 cycles that last 5 min in total. Subsequently, a needle is briefly pierced through the septa to release most of the over-pressure. Hereafter, 0.5 mL of an anoxic L-cysteine solution (0.05 g mL^{-1}) is added using a syringe, resulting in medium with a slight pinkish colour (caused by reduction of resazurin) that turns colourless after static incubation at 80 °C for 1 h in a drying oven (Binder 9010–0001, model E28). Finally, the bottles are aseptically inoculated by adding 1 mL *P. furiosus* cryostocks per bottle and subsequently incubated statically in the drying oven at approximately 90 °C for 16–20 h, resulting in an optical density measured at 600 nm (OD_{600}) of 0.6–0.7 and visible turbidity (Fig. 3).

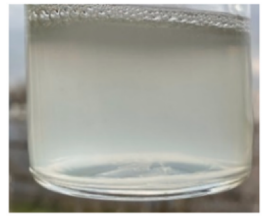

Fig. 3 *P. furiosus* pre-culture. Typical colour and turbidity of a *P. furiosus* pre-culture after 20 h static incubation in a 120 mL serum bottle at 90 °C.

Hereafter, the bottles are incubated statically at room temperature until further use. For optimal microbial growth it is imperative that the temperature inside the oven does not become too high or too low. Accordingly, a thermocouple probe is used to check the temperature throughout the incubation.

2.3 Cultivation of *P. furiosus* in a 20 L batch fermenter

Prior to the growth of *P. furiosus* in a 20 L fermenter, the fermenter is sterilized in place using pressurized steam. Hereafter, the fermenter is filled with approximately 15 L demineralized water and the temperature is increased to 90 °C. Hereafter, the pH and DO_2 probe are calibrated and the reactor is made anoxic by sparging with N_2 while stirring at 750 rpm. Subsequently, the following ingredients are sequentially added and dissolved in the fermenter (in g per 15 L water): NaCl, 434; starch, 75; $NaHCO_3$ 15; KH_2PO_4, 2.1; $CaCl_2{\cdot}H_2O$, 2.1; NH_4Cl, 3.75; $MgCl_2{\cdot}6\,H_2O$, 40.5; $MgSO_4{\cdot}7\,H_2O$, 51; KCl, 4.95. Hereafter, 7.5 mg $NiCl_2$ and 49 mg $Na_2WO_4{\cdot}2\,H_2O$ are added. Finally, 3.75 g yeast extract, 3.75 mg resazurin, 15 mL solution 3 and 1.5 mL solution 4 are added. After a few minutes, the medium turns pink, after which 7.5 g L-cysteine is added, turning the medium colourless in circa 10 min. The stirrer speed is decreased to 50 rpm and the N_2 gas flow is set at 1 L min^{-1} headspace flushing (i.e., instead of sparging the medium, which would negatively affect growth due to oxygen traces in the nitrogen gas). A bottle containing 4 M NaOH is connected to the fermenter for automated base titration and pH control is activated. Then, 300 mL pre-culture is added as inoculum and the starting pH is approximately 8.2 ± 0.1. As *P. furiosus* cells grow, the medium becomes acidified due to organic acid production and the base is automatically titrated to keep the pH at 7.0. It is not necessary to acidify the medium (to the set pH 7.0) at the start as this may disturb the inoculum. By measuring the pH and base weight on a balance, the growth

characteristics of *P. furiosus* can be observed over time (Fig. 4). In this example, the cells were harvested in late exponential phase (18.2 h after inoculation), prior to the start of the stationary phase. If another batch fermentation is started afterwards, 300 mL cells are collected for inoculation directly from the fermenter by draining hot culture into sterile (Scott) glass flask with a screwcap by filling the flask completely. If the culture becomes pink there is oxygen contamination.

2.4 Cell collection, storage, and preparation of cell-free extract

To collect *P. furiosus* cells, the fermenter is drained 20 ± 3 h after inoculation, with an average yield of 1.5 ± 0.3 g cells (wet weight) per L and OD_{600} of 0.7 ± 0.1. Cells are collected by centrifugation at 15,000 × *g* at 10 °C for 10 min, after which the supernatant is discarded. The pellet is observed as a light brown slimy paste with a sulphuric smell and with some black precipitates (possible metal sulphides) that can easily be scraped off from the centrifugation bottles. Subsequently, the pellets are stored in plastic containers under ambient conditions at −80 °C without snap-freezing. The fully grown culture and cell paste have a lot of reducing power, so the collection does not require anaerobic conditions. During and after cell lysis strict anaerobic conditions are necessary. CFE of *P. furiosus* cells is obtained by osmotic shock by mixing thawed cells with anoxic lysis buffer (30 mM Tris-HCl pH 8.4, 1 g L^{-1} $MgCl_2$, 1 mM L-cysteine, 1 mM dithiothreitol) at a 1:8 (w/v) ratio in

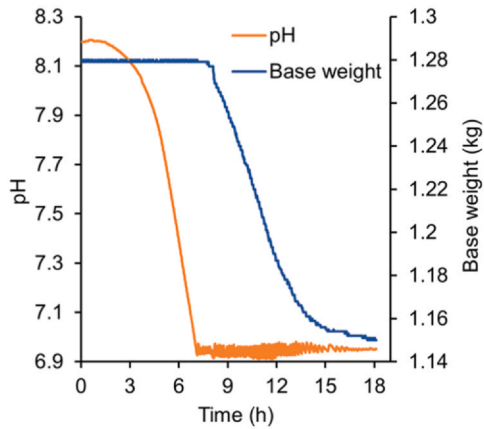

Fig. 4 Batch fermentation growth profile of *P. furiosus*. Acidification of the medium and base titration during typical growth of *P. furiosus* in a 20 L batch fermenter.

the glovebox. In addition, the lysis buffer contains a spatula tip of DNAse, RNAse and a tablet of protease inhibitor mixture. The cells are stirred for 3 h at RT in the glovebox after which the suspension is centrifuged anoxically at 20,000 × *g* for 1 h at RT. The supernatant obtained is the CFE which is stored at −80 °C under anoxic conditions until further use. Typically, 20 g of cells yield approximately 170 mL CFE with a total protein concentration of 7 ± 1 mg mL^{-1}.

2.5 Purification of AOR

AOR is purified inside a Coy anaerobic chamber ($O_2 < 2$ ppm) using an ÄKTAgo™ FPLC system (Cytiva, Marlborough, USA). All buffers are made anoxic prior to use by sparging with N_2 for 1 h. Reducing agents are freshly added to the buffers as powders inside the glovebox. All columns are run at 5 mL min^{-1} unless stated otherwise. CFE (typically 100 mL containing approximately 700 mg protein) is diluted twice in buffer A (30 mM Tris/HCl, pH 8.0, 1 mM dithiothreitol (DTT) and 1 mM L-cysteine) and loaded onto a 58 mL DEAE column equilibrated in buffer A. Hereafter, the column is washed with 2 column volume (CV) buffer A. Subsequently, a linear gradient of 5 CV to 0.5 M NaCl in buffer A is applied. Active AOR fractions eluting at 160–255 mM NaCl are pooled. The active fractions (approx. 22 mL) are diluted ten times in buffer A and loaded onto a 57 mL pre-equilibrated CHT ceramic hydroxyapatite column (Bio-Rad, Hercules, USA). Hereafter, the column is washed with 2 CV buffer A. Subsequently, a linear gradient of 5 CV to 0.3 M KPi in buffer A is applied. Active AOR fractions eluting at 15–60 mM KPi are pooled. The active fractions (approx. 10 mL) are diluted 10 times in buffer A and loaded onto an 8 mL pre-equilibrated SourceQ column. Hereafter, the column is washed with 2 CV buffer A. Subsequently, a linear gradient of 40 CV to 0.5 M NaCl in buffer A is applied. Active AOR fractions eluting at 160–180 mM NaCl are pooled. The active fractions (approx. 10 mL) are concentrated to approx. 0.5 mL using spin filters (Amicon® Ultra Centrifugal Filter, 30 kDa MWCO) and loaded onto a 24 mL Superdex 200 Increase 10/300 GL column pre-equilibrated in 30 mM Tris/HCl, 0.15 M NaCl, pH 8.0, 1 mM dithiothreitol (DTT) and 1 mM L-cysteine at 0.4 mL min^{-1}. Active AOR fractions (approx. 2 mL) are pooled and stored in elution buffer at −80 °C under anoxic conditions after snap-freezing in liquid nitrogen (Fig. 5).

The use of *P. furiosus* to produce AOR brings about some challenges including the cultivation at 90 °C, but also a relatively low enzyme yield,

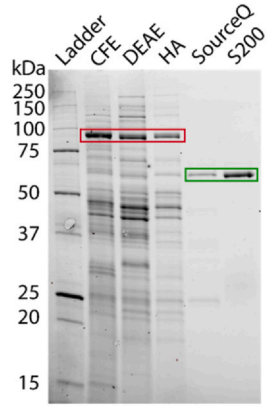

Fig. 5 SDS-PAGE gel (4–15% polyacrylamide) of protein samples obtained at various stages of the purification process. Ladder: protein standards of known size in kDa; CFE: cell-free extract; DEAE, HA, SourceQ and S200 indicate samples obtained after pooling AOR-active fractions eluting from the DEAE, hydroxyapatite, SourceQ and Superdex 200 Increase 10/300 GL columns, respectively. Red and green rectangles indicate bands containing phosphoenolpyruvate synthase and AOR, respectively. All lanes contain approx.10 µg protein.

since the enzyme is not highly expressed in the organism. This requires the cultivation of a 20 L batch reactor with 30 g wet cell weight, to obtain approximately 2.5 mg AOR, with an average enzyme activity of 1 U mg^{-1} protein at 60 °C.

This relatively low enzyme yield could be overcome by homologous expression of the desired AOR enzyme in *P. furiosus*, but unfortunately few expression methods are available to be used in this archaeon (Lipscomb et al., 2011). Some efforts have already been made to investigate the possibility of recombinant expression in organisms of which expression methods are not widely available. These organisms include *P. furiosus* and the bacterium *Aromatoleum evansii* (*A. evansii*) (Haja et al., 2020). The AOR from *Aromatoleum aromaticum* was heterologously expressed in *A. evansii*, yielding an active protein with a specific activity of 18 U mg^{-1} protein at 30 °C. The research indicates that it is possible to create an active enzyme through recombinant expression in this not so thoroughly explored organism, but it is still rather laborious and not scalable yet (Winiarska et al., 2023).

We believe that one of the most promising solutions could be the use of another host organism of which various genetic modification methods are available, such as *E. coli*, for the heterologous expression of AOR (Rosano & Ceccarelli, 2014). The choice of a suitable host organism is an important step

and each available organism has its own advantages and disadvantages. A range of prokaryotic and eukaryotic organisms that can be used are currently available, including *E. coli*, *Bacillus subtilis*, *Aromatoleum evansii*, *Pseudomonas fluorescens*, yeast, insect cells and even mammalian cells (Pouresmaeil & Azizi-Dargahlou, 2023). When deciding on the host organism of choice, all criteria for the production of a functional enzyme need to be addressed, which eliminates for example the yeast *Saccharomyces cerevisiae,* which is often used for the heterologous production of proteins. This organism does not contain a Moco biosynthesis pathway, as it does not naturally contain Mo-dependent enzymes (Perli et al., 2021). The use of, for example, an *E. coli* expression system could offer several advantages such as the option to insert gene modifications. This way protein variants can be created by the insertion of mutations, or fusion tags can be added to facilitate a simpler protein purification. Another big advantage of the use of *E. coli* is the high scalability of the process. This offers the possibility for a large scale cultivation and a resulting high amount of protein. Since *E. coli* is a mesophile, using this organism would also eliminate the need for cultivations at extremely high temperatures, which makes the whole process easier to handle and more sustainable. In addition, the protein production can be increased by placing the gene of interest under the control of a strong promotor, increasing the protein yield even more (Kim & Lee, 2008).

3. Recombinant expression of W-dependent enzymes

3.1 Hyperthermophilic archaeal protein expression

The use of microbial systems for the recombinant production of proteins has revolutionized the field of biochemistry. Because of the versatility of the process and the ability to produce virtually any protein and include genetically encoded tags and labels, this technique is used by almost all researchers that need to obtain a high amount of purified protein, while enabling protein engineering. In theory, the process to obtain a recombinant protein is pretty straightforward, but in practice several things can go wrong that affect the success of the protein production. Some examples of these risks are poor growth of the bacteria, the formation of inclusion bodies upon overexpression of the protein, and protein inactivity often caused by difficulty to incorporate (metal) cofactors (Rosano & Ceccarelli, 2014). These challenges are even more prominent if a protein from a different species is expressed (heterologous expression), for which the

bacteria do not have the right cofactor biosynthesis and protein maturation machinery. When expressing proteins from hyperthermophilic archaea in *E. coli* they are often produced as inclusion bodies (Kim & Lee, 2008). These inclusion bodies are the result of misfolded or unfolded proteins and end up as insoluble aggregates. As a result of the difference in protein structure between bacteria and archaea, over 50% of the recombinantly expressed protein can end up in inclusion bodies in a single cultivation (Kim & Lee, 2008). Besides misfolding, the inactivity of archaeal enzymes can also be due to the improper formation and insertion of other components, including cofactors and Fe-S clusters. In the case of AOR, for the protein to be active the Wco needs to be properly assembled, but *E. coli* does not naturally contain W-dependent enzymes but has a complete Moco biosynthesis pathway. As described previously, the method by which Moco and Wco are made is highly similar, but differences in the metal insertion have been reported (Schwarz et al., 2000).

3.2 Assays

If an AOR-family enzyme has been recombinantly expressed and a protein indeed has been produced, the functionality of the enzyme has to be assessed. This can be done using several assays that quantify the enzyme activity, MPT content, and the metal content of the protein. The activity of the enzyme can be assessed using a spectrophotometric assay with benzyl viologen (BV) as electron acceptor under anaerobic conditions (e.g. inside a glovebox using anaerobic solutions). Benzyl viologen changes from a transparent to blue coloured substance upon reduction. This allows for a tracking of the AOR activity by following the reduction of BV at 600 nm ($\varepsilon = 7400\ M^{-1}\ cm^{-1}$). The assay is performed under anoxic conditions in a 50 mM EPPS buffer at pH 8.0 containing 50 μM sodium dithionite and 3 mM benzyl viologen. Sodium dithionite is added to the solution to check if anaerobic conditions have been achieved, since the solution will return to a colourless substance if oxygen is still present in the surrounding atmosphere or the solutions used. If it is certain an anaerobic environment has been achieved, the addition of sodium dithionite can be omitted from the assay. First, 10–100 μL CFE is added to the cuvette and the change in absorbance is measured to visualize the background activity. Then, the reaction is initiated by the addition of crotonaldehyde, with final concentrations ranging from 0.2–5 mM (Arndt et al., 2019; Heider et al., 1995; Reschke et al., 2019).

To analyse the MPT content of the enzyme, a fluorometric assay can be performed. The molybdopterin cofactor can be oxidized into a stable fluorescent derivative called Form A (Johnson & Rajagopalan, 1982; Meckenstock et al., 1999; Rauh et al., 2004; tenBrink et al., 2011). Form A is an oxidized pterin with a 6-alkyl substituent that can be measured fluorometrically using an excitation wavelength (λ_{ex}) of 375 nm and an emission wavelength (λ_{em}) of 445 nm. A 50 μL sample of enzyme (1–2 mg mL^{-1}) is added to 200 μL 55 mM $KMnO_4$ in 0.1 M NaOH. The sample is boiled at 100 °C in a water bath for 20 min to oxidize the molybdopterin to Form A. Subsequently, 700 μL ice-cold ethanol is added to precipitate the excess $KMnO_4$. Finally, the precipitates are removed by centrifugation at 15,000 × *g* for 15 min and the supernatant is directly analysed. The pterin content in the sample is calculated by comparison with a calibration curve made using commercially available pterin-6-carboxylic acid and commercial xanthine oxidase, purchased for example from Sigma Aldrich, can serve as a positive reference for the presence of MPT (Johnson & Rajagopalan, 1982; Meckenstock et al., 1999; Rauh et al., 2004; tenBrink et al., 2011). Finally, the metal content of the protein can be analysed using an Inductively coupled plasma mass spectrometry (ICP-MS) analysis, or alternatively, using an electroanalytical procedure to simultaneously measure Mo and W in biological samples (Hagedoorn et al., 2001). For the ICP-MS analysis the metals need to be extracted from the protein sample and the sample needs to be diluted in nitric acid. For that reason, 100 μL sample (5 mg mL^{-1}) is mixed with 50 μL 65% HNO_3 and 350 μL H_2O, diluting the sample 5x. This mixture is sonicated in a ultrasonic bath for 10 min and afterwards centrifuged for 5 min at 13,500 RPM. 400 μL of the resulting supernatant is mixed with 3600 μL 2% HNO_3, resulting in a final 10-fold dilution, reaching a sample concentration of approximately 0.1 mg mL^{-1}. The samples are subsequently analysed using an PerkinElmer NexION 2000 ICP mass spectrometer (nebulizer: glass, injector: glass) and can be measured with a sensitivity starting from 2 ppm.

3.3 Acetylene hydratase

The W-dependent enzyme acetylene hydratase (AH) from the mesophile *Pelobacter acetylenicus* (*P. acetylenicus*) is a member of the DMSOR family and stands out because of its ability to catalyse a non-redox reaction despite containing typical redox cofactors, the formation of acetaldehyde from acetylene by the addition of a H_2O molecule (Fig. 6).

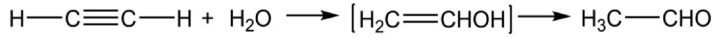

Fig. 6 Reaction catalysed by AH. AH catalyses the non-redox hydration of acetylene to acetaldehyde.

To analyse its unique chemistry, a protocol was developed to express AH in *E. coli* and produce active site variants. To produce a functional enzyme, tenBrink et al. (2011) used a chaperone system for the insertion of the Wco in the enzymes, called the TorD superfamily (tenBrink et al., 2011). This family of chaperones has been shown to facilitate the cofactor incorporation in certain molybdoenzymes and these proteins are therefore necessary to connect the last steps in the synthesis of the Moco or Wco to the incorporation of the cofactor (Ilbert et al., 2004). First, the chaperones bind to an N-terminal signal sequence which causes a delay of both the export and folding of the protein until the cofactor has been inserted. Second, the chaperones bind to another, still unknown site, to actively facilitate the incorporation of the cofactor. The chaperones that aid in the cofactor incorporation form a pair with a specific protein and cannot be exchanged for different chaperones if the original chaperone is absent (Ilbert et al., 2004). When analysing the amino acid sequence of AH, tenBrink et al. (2011) found that an N-terminal signal sequence was missing in the *P. acetylenicus* gene. To make use of this chaperone system, tenBrink and coworkers fused the N-terminal binding sites of two commonly used TorD superfamily chaperones to the AH gene, NarJ and TorD. NarJ is the chaperone used for the maturation of the nitrate reductase NarG and TorD for the trimethylamine-oxide reductase TorA (Genest et al., 2008). To make use of this chaperone system, tenBrink and coworkers therefore fused the N-terminal amino acid sequences of the NarG and TorA genes, 108 bp and 117 bp, respectively, to the AH gene. Both of these variants were cloned into a pET24a expression vector forming a NarG-AH and TorA-AH plasmid (tenBrink et al., 2011). These plasmids were used to transform three different types of *E. coli* cell lines; BL21(DE3), BL21(DE3) pLysS, and Rosetta (DE3). Different results were obtained using the three strains, indicating that the type of bacterial strain influences the protein production. The yield of the TorA-AH protein was significantly lower than the NarG-AH protein and was therefore not used for further experiments. The highest soluble expression of active AH was seen using the NarG-AH plasmid in *E. coli* Rosetta (DE3) in anaerobic minimal medium (100 mM KPi buffer, 10 mM NH_4Cl, 2 mM $MgCl_2$, and 0.5 g L^{-1} protein hydrolysate) supplemented

with 1 mL L^{-1} SL10 (antifoam), 10 mM Na_2WO_4, 1 mM Na_2S, 1 mL L^{-1} seven-vitamin solution, 0.5% glycerol, and 50 mM Na-fumarate (Widdel & Pfennig, 1981). The cells were grown anaerobically at 37 °C to an OD_{600} of 1.0, after which the expression was induced at 25 °C using 100 μM isopropyl-β-d-thiogalactopyranoside (IPTG) for 24 h (tenBrink et al., 2011).

With the addition of the NarJ chaperone binding site, the protein yield increased 1.3-fold and the specific activity of the enzyme increased 3.7-fold. When analysing the cofactor and metal content of the proteins, it was observed that the use of a chaperone system increased the MPT content from 0.17 ± 0.08 to 0.31 ± 0.09 mol MPT per mol of enzyme, the iron content increased from 1.22 ± 0.26 to 3.17 ± 0.49 mol Fe per mol protein, and the amount of tungsten increased from 0.06 ± 0.02 to 0.14 ± 0.06 mol W per mol protein. These results indicate that while the amount of correctly formed protein increased and an active enzyme was obtained, the amount of cofactor and incorporated metals remained substoichiometric. These findings suggest that the assistance of the chaperones help, but are not sufficient for a complete assembly and incorporation of the cofactor, which will require further investigation (tenBrink et al., 2011).

3.4 Formate dehydrogenase

Another example of the recombinant expression of a tungsten-containing DMSO reductase family enzyme is the production of the formate dehydrogenase (FDH) from *Clostridium ljungdahlii* in *E. coli* (Fischer et al., 2010). FDH is an enzyme that catalyses the reversible oxidation of formate to carbon dioxide with the help of an external electron acceptor for electron transfer (Fig. 7).

The activity of this enzyme is critical for the maintenance of a cellular redox balance and a proper energy metabolism. FDH is therefore widely conserved from bacteria to eukaryotes (Park et al., 2024). Because of its ability to perform a reversible oxidation of formate, FDH has recently gained interest to be used as a method to reduce atmospheric CO_2 levels by reducing the greenhouse gas CO_2 to formate, which could serve as a platform chemical for industry. To be able to do so, Moon et al. (2022) started out to characterize the FDH from *Clostridium ljungdahlii* (FDH_{CI}) by expressing the protein in *E. coli* (Moon et al., 2022).

The gene encoding FDH_{CI} was codon-optimized for expression in *E. coli* and placed on a pET22b(+) vector. To ensure a simple purification, the gene was fused to a C-terminal His_6-tag. The plasmid was used to transform *E. coli* BL21(DE3) cells and the cells were subsequently grown in

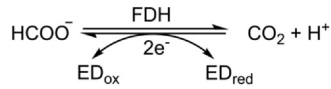

Fig. 7 Reaction catalysed by FDH. FDH catalyses the reversible oxidation of formate to carbon dioxide. ED_{ox}: oxidized electron donor. ED_{red}: reduced electron donor.

LB medium containing 100 μg mL^{-1} of ampicillin, 5 mM of sodium tungstate, and 100 μM of IPTG. The OD_{600} at which the expression is induced with IPTG is unfortunately not described. After a cultivation for 24 h at 30 °C and 130 RPM, the cells were harvested by centrifugation (Min et al., 2020). The resulting FDH_{CI} protein was shown to contain 1.76 ± 0.17 mol W per FDH_{Cl}. FDH_{CI} has been predicted to be a monomeric protein and therefore contains one W per FDH_{CI} (Li et al., 2023). The obtained value of 1.76 mol W/FDH_{CI} is therefore relatively high, but could be caused by the margin of error in the ICP-MS analysis. While testing if the protein was catalytically active, Moon and coworkers found an activity for both the formate oxidation and CO_2 reduction, which were 58 mU mg^{-1} and 9.1 mU mg^{-1}, respectively. Even though the protein originates from a strictly anaerobic organism, these specific activities were independent of the assay being performed under aerobic or anaerobic conditions. These findings show that the W-containing FDH_{CI} could be produced in *E. coli* BL21(DE3) without the need of co-expressing any chaperone or accessory protein for the metal uptake or cofactor assembly (Moon et al., 2022).

3.5 Glyceraldehyde-3-phosphate oxidoreductase expressed as a Mo-containing enzyme

Several attempts to overexpress AOR or DMSOR family enzymes in a different host organism have already been performed. Park et al. (2007) recombinantly expressed the enzyme GAPOR from the mesophile *Methanococcus maripaludis* ($GAPOR_{Mm}$) in *E. coli* (Park et al., 2007). This enzyme is a homolog to the GAPOR isolated from *P. furiosus* with 47% sequence identity, but was shown to be a Mo-dependent enzyme in contrast to its W-dependent homolog in archaea. The gene encoding $GAPOR_{Mm}$ was cloned into a pET46 Ek-LIC vector and used this vector to transform *E. coli* BL21(DE3) and Rosetta-Gami 2(DE3). Rosetta-Gami 2(DE3) cells have a double trxB/gor mutation and contain a pRARE2 plasmid, which supplies seven rare tRNAs. The trxB/gor mutation causes the thioredoxin and glutathione reductases to be inactive. Both thioredoxin

and glutaredoxin are able to reduce disulphide bonds in proteins. By inactivating their reductases, the proteins stay in their oxidized state which favours the formation of disulphide bonds during protein folding (Cassland et al., 2004; Novagen, n.d.).

Both strains were grown in M9 minimal medium including either 100 μM sodium molybdate, 100 μM sodium tungstate, or both. The cells were grown at 37 °C until an OD_{600} of 0.5–0.6 had been achieved, after which the protein production was induced using 1 mM IPTG. The bacteria were grown for another 12 h at 37 °C. The authors tested if an aerobic or anaerobic cultivation had an effect on the resulting enzyme activity, but this did not seem to be the case.

When analysing the produced recombinant enzymes, Park and coworkers found that $GAPOR_{Mm}$ is catalytically active when cultured in medium containing Mo and no W, indicating a preference for Mo as metal cofactor. Also, when equal concentrations of Mo and W were added to the medium, an inactive $GAPOR_{Mm}$ enzyme was formed, suggesting that the presence of W may even inhibit the formation of the Mo-containing Moco (M.-O. Park et al., 2007). Next to that, the production of recombinant $GAPOR_{Mm}$ in Rosetta-Gami 2(DE3) cells resulted in a 67-fold higher enzyme activity compared to GAPOR produced in BL21(DE3). Similar to the results of ten Brink et al. on AH, the specific cell-type or host used affects the enzyme production (tenBrink et al., 2011). Even in the better performing cell type Rosetta-Gami 2(DE3), a metal incorporation of maximally 0.74 W/protein was achieved. Finally, only one-fifth of the GAPOR proteins produced in BL21(DE3) has acquired an MPT cofactor and iron-sulphur cluster (M.-O. Park et al., 2007). The MPT and iron-sulphur cluster content of GAPOR produced in Rosetta-Gami 2(DE3) cells has unfortunately not been reported.

3.6 YdhV – the AOR family homolog of *E. coli*

This last example of an overexpression of an AOR-family enzyme in *E. coli* is different from the previous examples in the way that a protein is homologously expressed in *E. coli*, as it is a native protein from *E. coli* itself. It is of relevance to see how the researchers were able to obtain this enzyme and to have a better understanding of the mechanism by which the cofactor is being assimilated.

In *E. coli,* numerous MPT-containing enzymes are identified which are all shown to be Mo-containing enzymes. These enzymes can, as stated previously, be categorized into three groups; the DMSOR, SO and XO families.

Between the three families, the Moco differs from a bis-MGD in DMSOR to a single MPT in the SO family, and to an MPT with an additional CMP nucleotide (MCD) in the XO family. The proteins encoded by the *ydhYVWXUT* operon in *E. coli* were also grouped into the DMSOR family (Iobbi-Nivol & Leimkühler, 2013). The YdhV protein encoded, amongst others, by this operon has been predicted to be an oxidoreductase belonging to the family of Moco/Wco containing enzymes. Based on sequence homology of ~45% with *P. furiosus* AOR and *Geobacter metallireducens* BamBC YdhV it is, however, believed to bind a bis-MPT-cofactor, instead of the bis-MGD cofactor usually found in DMSORs. To characterize YdhV in detail, Reschke et al. (2019) performed an overexpression of YdhV in *E. coli* strain BW25113 by transforming the cells with a pTrcHis vector containing the *ydhV* gene fused to an N-terminal His_6-tag (Reschke et al., 2019). *E. coli* BW25113 is a strain in which gene disruptions can be made by using the bacteriophage λ red recombination system, which is based on homologous recombination. This strain served as the parent strain for the Keio collection, which consists of approximately 4000 single-gene deletion mutants (Grenier et al., 2014). To analyse which cofactor is being formed in the protein, mutation strains with one or two deletions were created and the formed protein was compared to the wild-type YdhV protein. These strains contained deletions of the proteins MobA and MocA involved in the formation of the bis-MGD and MCD cofactors and are called Δ*mobA*, Δ*mocA*, and Δ*mobA*/ Δ*mocA*. Precultures were grown in LB medium containing 1 mM Na_2MoO_4, 20 μM IPTG, and 150 μg mL^{-1} ampicillin. Expression cultures were started with 2 mL L^{-1} of overnight preculture in LB medium containing similar concentrations of IPTG and ampicillin, and incubated at 30 °C and 130 RPM for 24 h. The OD_{600} at which the protein expression was induced is unfortunately not described. To test the incorporation of the different metals, the medium contained varying amounts (0–100 μM) of $Na_2WO_4^{2-}$ or $Na_2MoO_4^{2-}$ in the presence of either 10 μM $Na_2MoO_4^{2-}$ or 10 μM $Na_2WO_4^{2-}$, respectively. To test an anaerobic production of YdhV similar conditions were used, but instead the cultures were incubated statically at 30 °C for 24 h.

The produced proteins were analysed using ICP-MS and a fluorometric assay to determine the MPT content. These analyses showed that the levels of molybdenum and iron present in the Δ*mobA*/Δ*mocA* deletion strains were comparable to the wildtype strain, the amount of MPT formed was similar or even higher, and no MPT with an attached nucleotide (MPT-NMP) was detected. This indicates that the formation of the cofactor in YdhV does not depend on the activity of the proteins MobA or MocA. Also, when looking

at the amount of MPT to molybdenum present in the proteins, the data showed a ratio of 2:1. Together, these findings suggest that it is possible that a bis-Mo-MPT cofactor is being formed in YdhV, a form of Moco that has not previously been found in any Mo-containing enzyme.

Finally, by varying the amount of either sodium tungstate or sodium molybdate in the medium a preference was shown for the binding of molybdenum over tungsten. Only in the absence of molybdate tungsten was incorporated in the enzyme to a level of 0.2 W/protein. Increasing the amount of molybdate in the medium resulted in a decreased amount of incorporated tungsten until no tungsten was incorporated at all. This suggests that the enzyme is indeed able to bind and incorporate tungsten into the cofactor, but has a preference for Mo insertion (Reschke et al., 2019).

4. Conclusion

As has been shown by the different investigations described above, the strain and medium used seem to have a significant effect on the formation of a functional tungsten enzyme. Homologous expression from natural W-enzyme producing organisms has been most successful and heterologous expression typically leads to substoichiometric metal incorporation. Especially the *E. coli* Rosetta and Rosetta Gami strains likely have a beneficial effect on the enzyme activity, however so far only for Mo-containing variants of the normally W-containing AOR family enzymes. For that reason our future approach includes, amongst others, the variation of cell lines and media to see the effect on protein expression. Perhaps the re-engineering of the MPT biosynthesis pathway in *E. coli* to make it more suitable for tungsten may solve the current problem. We think that by mutating and expressing several enzymes that are involved in the synthesis of the Wco, a solution might be found for this difficult heterologous expression.

Acknowledgements

This research was supported by a grant from the European Union's HORIZON-EIC-2023-PATHFINDEROPEN-01 under grant agreement No 101129798, European Innovation Council Pathfinder project W-BioCat.

References

Aguilar-Barajas, E., Díaz-Pérez, C., Ramírez-Díaz, M. I., Riveros-Rosas, H., & Cervantes, C. (2011). Bacterial transport of sulfate, molybdate, and related oxyanions. *Biometals, 24*(4), 687–707. https://doi.org/10.1007/s10534-011-9421-x.

Arndt, F., Schmitt, G., Winiarska, A., Saft, M., Seubert, A., Kahnt, J., & Heider, J. (2019). Characterization of an aldehyde oxidoreductase from the mesophilic bacterium *Aromatoleum aromaticum* EbN1, a member of a new subfamily of tungsten-containing enzymes. *Frontiers in Microbiology, 10*, 439765. https://doi.org/10.3389/fmicb.2019.00071.

Bevers, L. E., Hagedoorn, P. L., Krijger, G. C., & Hagen, W. R. (2006). Tungsten transport protein A (WtpA) in *Pyrococcus furiosus*: The first member of a new class of tungstate and molybdate transporters. *Journal of Bacteriology, 188*(18), 6498. https://doi.org/10.1128/JB.00548-06.

Bevers, L. E., Hagedoorn, P.-L., Santamaria-Araujo, J. A., Magalon, A., Hagen, W. R., & Schwarz, G. (2008). Function of MoaB proteins in the biosynthesis of the molybdenum and tungsten cofactors. *Biochemistry, 47*(3), 949–956. https://doi.org/10.1021/bi7020487.

Bevers, L. E., Schwarz, G., & Hagen, W. R. (2011). A molecular basis for tungstate selectivity in prokaryotic ABC transport systems. *Journal of Bacteriology, 193*(18), 4999. https://doi.org/10.1128/JB.05056-11.

Cassland, P., Larsson, S., Nilvebrant, N. O., & Jönsson, L. J. (2004). Heterologous expression of barley and wheat oxalate oxidase in an E. coli trxB gor double mutant. *Journal of Biotechnology, 109*(1–2), 53–62. https://doi.org/10.1016/J.JBIOTEC.2003.10.026.

Chan, M. K., Mukund, S., Kletzin, A., Adams, M. W. W., & Rees, D. C. (1995). Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. *Science, 267*(5203), 1463–1469. https://doi.org/10.1126/SCIENCE.7878465.

Cordas, C. M., & Moura, J. J. G. (2019). Molybdenum and tungsten enzymes redox properties – A brief overview. *Coordination Chemistry Reviews, 394*, 53–64. https://doi.org/10.1016/J.CCR.2019.05.005.

Cui, M., & Gomes, M. (2021). Impacts of manganese oxides on molybdenum and tungsten speciation and implications for their geochemistry in aquatic environments. *Geochimica et Cosmochimica Acta, 312*, 217–234. https://doi.org/10.1016/J.GCA.2021.07.009.

Fischer, M., Thöny, B., & Leimkühler, S. (2010). The biosynthesis of folate and pterins and their enzymology. *Comprehensive Natural Products II: Chemistry and Biology, 7*, 599–648. https://doi.org/10.1016/B978-008045382-8.00150-7.

Genest, O., Neumann, M., Seduk, F., Stöcklein, W., Méjean, V., Leimkühler, S., & Iobbi-Nivol, C. (2008). Dedicated metallochaperone connects apoenzyme and molybdenum cofactor biosynthesis components. *Journal of Biological Chemistry, 283*(31), 21433–21440. https://doi.org/10.1074/jbc.M802954200.

Grenier, F., Matteau, D., Baby, V., & Rodrigue, S. (2014). Complete genome sequence of *Escherichia coli* BW25113. *Genome Announcements, 2*(5), 1038–1052. https://doi.org/10.1128/GENOMEA.01038-14.

Grunden, A. M., & Shanmugam, K. T. (1997). Molybdate transport and regulation in bacteria. *Archives of Microbiology, 168*(5), 345–354. https://doi.org/10.1007/s002030050508.

Hagedoorn, P. L., Van't Slot, P., Van Leeuwen, H. P., & Hagen, W. R. (2001). Electroanalytical determination of tungsten and molybdenum in proteins. *Analytical Biochemistry, 297*(1), 71–78. https://doi.org/10.1006/ABIO.2001.5300.

Haja, D. K., Wu, C.-H., Ponomarenko, O., Poole, F. L., George, G. N., & Adams, M. W. W. (2020). Improving arsenic tolerance of *Pyrococcus furiosus* by heterologous expression of a respiratory arsenate reductase. *Applied and Environmental Microbiology, 86*(21), https://doi.org/10.1128/AEM.01728-20.

Hänzelmann, P., & Schindelin, H. (2004). Crystal structure of the S-adenosylmethionine-dependent enzyme MoaA and its implications for molybdenum cofactor deficiency in humans. *Proceedings of the National Academy of Sciences of the United States of America, 101*(35), 12870–12875. https://doi.org/10.1073/PNAS.0404624101.

Heider, J., Ma, K., & Adams, M. W. (1995). Purification, characterization, and metabolic function of tungsten-containing aldehyde ferredoxin oxidoreductase from the hyperthermophilic and proteolytic archaeon *Thermococcus* strain ES-1. *Journal of Bacteriology, 177*(16), 4757–4764. https://doi.org/10.1128/jb.177.16.4757-4764.1995.

Hille, R. (2002). Molybdenum and tungsten in biology. *Trends in Biochemical Sciences, 27*(7), 360–367. https://doi.org/10.1016/S0968-0004(02)02107-2.

Ilbert, M., Méjean, V., & Iobbi-Nivol, C. (2004). Functional and structural analysis of members of the TorD family, a large chaperone family dedicated to molybdoproteins. *Microbiology, 150*(4), 935–943. https://doi.org/10.1099/MIC.0.26909-0.

Iobbi-Nivol, C., & Leimkühler, S. (2013). Molybdenum enzymes, their maturation and molybdenum cofactor biosynthesis in Escherichia coli. *Biochimica et Biophysica Acta – Bioenergetics, 1827*(8–9), 1086–1101. https://doi.org/10.1016/J.BBABIO.2012.11.007.

Johnson, J. L., & Rajagopalan, K. V. (1982). Structural and metabolic relationship between the molybdenum cofactor and urothione. *Proceedings of the National Academy of Sciences of the United States of America, 79*(22), 6856–6860. https://doi.org/10.1073/pnas.79.22.6856.

Kim, S., & Lee, S. B. (2008). Soluble expression of archaeal proteins in *Escherichia coli* by using fusion-partners. *Protein Expression and Purification, 62*(1), 116–119. https://doi.org/10.1016/J.PEP.2008.06.015.

King, R. B. (1969). A possible effect of the lanthanide contraction on metal carbonyl chemistry. *Inorganic and Nuclear Chemistry Letters, 5*(11), 905–907. https://doi.org/10.1016/0020-1650(69)80011-5.

Leimkühler, S. (2014). The biosynthesis of the molybdenum cofactor in *Escherichia coli* and its connection to FeS cluster assembly and the thiolation of tRNA. *Advances in Biology, 2014*(1), 808569. https://doi.org/10.1155/2014/808569.

Leimkühler, S. (2020). The biosynthesis of the molybdenum cofactors in *Escherichia coli*. *Environmental Microbiology, 22*(6), 2007–2026. https://doi.org/10.1111/1462-2920.15003.

Li, W., Gao, Y., Sun, X., Wan, L., Ji, H., Luo, H., ... Zhang, L. (2023). Direct detection of a single [4Fe-4S] cluster in a tungsten-containing enzyme: Electrochemical conversion of CO_2 into formate by formate dehydrogenase. *Carbon Energy, 5*(5), e304. https://doi.org/10.1002/CEY2.304.

Lipscomb, G. L., Stirrett, K., Schut, G. J., Yang, F., Jenney, F. E., Scott, R. A., ... Westpheling, J. (2011). Natural competence in the hyperthermophilic archaeon *Pyrococcus furiosus* facilitates genetic manipulation: Construction of markerless deletions of genes encoding the two cytoplasmic hydrogenases. *Applied and Environmental Microbiology, 77*(7), 2232–2238. https://doi.org/10.1128/AEM.02624-10.

Maia, L. B., Moura, J. J. G., & Moura, I. (2015). Molybdenum and tungsten-dependent formate dehydrogenases. *Journal of Biological Inorganic Chemistry, 20*(2), 287–309. https://doi.org/10.1007/s00775-014-1218-2.

Meckenstock, R. U., Krieger, R., Ensign, S., Kroneck, P. M. H., & Schink, B. (1999). Acetylene hydratase of *Pelobacter acetylenicus*. *European Journal of Biochemistry, 264*(1), 176–182. https://doi.org/10.1046/J.1432-1327.1999.00600.X.

Mendel, R. R., & Leimkühler, S. (2015). The biosynthesis of the molybdenum cofactors. *Journal of Biological Inorganic Chemistry, 20*(2), 337–347. https://doi.org/10.1007/s00775-014-1173-y.

Mendel, R. R., & Oliphant, K. D. (2024). The final step in molybdenum cofactor biosynthesis—A historical view. *Molecules, 29*(18), 4458. https://doi.org/10.3390/MOLECULES29184458.

Min, K., Park, Y. S., Park, G. W., Lee, J. P., Moon, M., Ko, C. H., & Lee, J. S. (2020). Elevated conversion of CO_2 to versatile formate by a newly discovered formate dehydrogenase from *Rhodobacter aestuarii*. *Bioresource Technology, 305*, 123155. https://doi.org/10.1016/J.BIORTECH.2020.123155.

Moon, M., Park, G. W., Lee, J. P., Lee, J. S., & Min, K. (2022). Recombinant expression and characterization of formate dehydrogenase from *Clostridium ljungdahlii* (ClFDH) as CO_2 reductase for converting CO_2 to formate. *Journal of CO2 Utilization, 57*, 101876. https://doi.org/10.1016/J.JCOU.2021.101876.

Mukund, S., & Adams, M. W. W. (1991). The novel tungsten-iron-sulfur protein of the hyperthermophilic archaebacterium, *Pyrococcus furiosus*, is an aldehyde ferredoxin oxidoreductase. Evidence for its participation in a unique glycolytic pathway. *Journal of Biological Chemistry, 266*(22), 14208–14216. https://doi.org/10.1016/S0021-9258(18)98669-2.

Mukund, S., & Adams, M. W. W. (1995). Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon *Pyrococcus furiosus*. *Journal of Biological Chemistry, 270*(15), 8389–8392. https://doi.org/10.1074/jbc.270.15.8389.

Novagen. (n.d.). *Rosetta-gami™ 2(DE3) Competent Cells* – Novagen | 71351*. Retrieved December 23, 2024, from https://www.merckmillipore.com/IE/en/product/Rosetta-gami-2DE3-Competent-Cells-*-Novagen,EMD_BIO-71351?ReferrerURL=https%3A%2F%2Fwww.google.com%2F.

Pang, H., & Yokoyama, K. (2018). Lessons from the studies of a CC bond forming radical SAM enzyme in molybdenum cofactor biosynthesis. *Methods in Enzymology, 606*, 485–522. https://doi.org/10.1016/BS.MIE.2018.04.014.

Park, J., Heo, Y., Jeon, B. W., Jung, M., Kim, Y. H., Lee, H. H., & Roh, S. H. (2024). Structure of recombinant formate dehydrogenase from *Methylobacterium extorquens* (MeFDH1). *Scientific Reports, 14*(1), 1–10. https://doi.org/10.1038/s41598-024-54205-7.

Park, M.-O., Mizutani, T., & Jones, P. R. (2007). Glyceraldehyde-3-phosphate ferredoxin oxidoreductase from *Methanococcus maripaludis*. *Journal of Bacteriology, 189*(20), 7281–7289. https://doi.org/10.1128/JB.00828-07.

Perli, T., van der Vorm, D. N. A., Wassink, M., van den Broek, M., Pronk, J. T., & Daran, J. M. (2021). Engineering heterologous molybdenum-cofactor-biosynthesis and nitrate-assimilation pathways enables nitrate utilization by *Saccharomyces cerevisiae*. *Metabolic Engineering, 65*, 11–29. https://doi.org/10.1016/J.YMBEN.2021.02.004.

Pouresmaeil, M., & Azizi-Dargahlou, S. (2023). Factors involved in heterologous expression of proteins in *E. coli* host. *Archives of Microbiology, 205*(5), 1–18. https://doi.org/10.1007/S00203-023-03541-9.

Rauh, D., Graentzdoerffer, A., Granderath, K., Andreesen, J. R., & Pich, A. (2004). Tungsten-containing aldehyde oxidoreductase of *Eubacterium acidaminophilum*. *European Journal of Biochemistry, 271*(1), 212–219. https://doi.org/10.1111/J.1432-1033.2004.03922.X.

Reschke, S., Duffus, B. R., Schrapers, P., Mebs, S., Teutloff, C., Dau, H., ... Leimkühler, S. (2019). Identification of YdhV as the first molybdoenzyme binding a Bis-Mo-MPT cofactor in *Escherichia coli*. *Biochemistry, 58*(17), 2228–2242. https://doi.org/10.1021/acs.biochem.9b00078.

Reschke, S., Sigfridsson, K. G. V., Kaufmann, P., Leidel, N., Horn, S., Gast, K., ... Leimkühler, S. (2013). Identification of a bis-molybdopterin intermediate in molybdenum cofactor biosynthesis in *Escherichia coli*. *Journal of Biological Chemistry, 288*(41), 29736–29745. https://doi.org/10.1074/jbc.M113.497453.

Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology, 5*, 79503. https://doi.org/10.3389/fmicb.2014.00172.

Roy, R., Menon, A. L., & Adams, M. W. W. (2001). [11] Aldehyde oxidoreductases from *Pyrococcus furiosus*. *Methods in Enzymology, 331*, 132–144. https://doi.org/10.1016/S0076-6879(01)31052-2.

Schut, G. J., Thorgersen, M. P., Poole, F. L., Haja, D. K., Putumbaka, S., & Adams, M. W. W. (2021). Tungsten enzymes play a role in detoxifying food and antimicrobial aldehydes in the human gut microbiome. *Proceedings of the National Academy of Sciences of the United States of America, 118*(43), https://doi.org/10.1073/pnas.2109008118.

Schwarz, G., Schulze, J., Bittner, F., Eilers, T., Kuper, J., Bollmann, G., ... Mendel, R. R. (2000). The molybdenum cofactor biosynthetic protein Cnx1 complements molybdate-repairable mutants, transfers molybdenum to the metal binding pterin, and is associated with the cytoskeleton. *The Plant Cell, 12*(12), 2455–2471. https://doi.org/10.1105/TPC.12.12.2455.

Seelmann, C. S., Willistein, M., Heider, J., & Boll, M. (2020). Tungstoenzymes: Occurrence, catalytic diversity and cofactor synthesis. *Inorganics, 8*(8), 44. https://doi.org/10.3390/INORGANICS8080044.

Stewart, L. J., Bailey, S., Bennett, B., Charnock, J. M., Garner, C. D., & McAlpine, A. S. (2000). Dimethylsulfoxide reductase: An enzyme capable of catalysis with either molybdenum or tungsten at the active site. *Journal of Molecular Biology, 299*(3), 593–600. https://doi.org/10.1006/JMBI.2000.3702.

tenBrink, F., Schink, B., & Kroneck, P. M. H. (2011). Exploring the active site of the tungsten, iron-sulfur enzyme acetylene hydratase. *Journal of Bacteriology, 193*(5), 1229–1236. https://doi.org/10.1128/JB.01057-10.

Venkitasubramanian, P., Daniels, L., Das, S., Lamm, A. S., & Rosazza, J. P. N. (2008). Aldehyde oxidoreductase as a biocatalyst: Reductions of vanillic acid. *Enzyme and Microbial Technology, 42*(2), 130–137. https://doi.org/10.1016/J.ENZMICTEC.2007.08.009.

Verhagen, M. F. J. M., Menon, A. L., Schut, G. J., & Adams, M. W. W. (2001). *Pyrococcus furiosus*: Large-scale cultivation and enzyme purification. *Methods in Enzymology, 330*, 25–30. https://doi.org/10.1016/S0076-6879(01)30368-3.

White, H., Strobl, G., Feicht, R., & Simon, H. (1989). Carboxylic acid reductase: A new tungsten enzyme catalyses the reduction of non-activated carboxylic acids to aldehydes. *European Journal of Biochemistry, 184*(1), 89–96. https://doi.org/10.1111/J.1432-1033.1989.TB14993.X.

Widdel, F., & Pfennig, N. (1981). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Archives of Microbiology, 129*(5), 395–400. https://doi.org/10.1007/BF00406470.

Winiarska, A., Ramírez-Amador, F., Hege, D., Gemmecker, Y., Prinz, S., Hochberg, G., ... Schuller, J. M. (2023). A bacterial tungsten-containing aldehyde oxidoreductase forms an enzymatic decorated protein nanowire. *Science Advances, 9*(22), https://doi.org/10.1126/sciadv.adg6689.