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# Protocols for the insertion of the bis-molybdopterin guanine dinucleotide cofactor into apo-molybdoenzymes from the DMSO reductase family

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## **Abstract**

Molybdoenzymes are ubiquitous, and play important roles in all kingdoms of life. The enzymes' cofactors comprise the metal molybdenum, a special organic ligand system called molybdopterin (MPT), additional small ligands like water, hydroxide, oxo-, sulfido- or selenido- functions and, in some enzymes, a coordination to the peptide chain of the protein via an amino acid ligand (e.g. serine, aspartate, cysteine or selenosysteine). The so-called molybdenum cofactor (Moco) is deeply buried in the protein at the end of a narrow funnel giving access only to the substrate. In 1974 an assay was developed by Nason and coworkers using the pleotrophic *Neurospora crassa* mutant *nit-1* for the reconstitution of molybdoenzyme activities from crude extracts. These studies lead to the understanding that Moco is the common element in all molybdoenzymes from different organisms. The assay has been further developed since using specific molybdenum enzymes as source of Moco for the reconstitution of diverse purified apo-molybdoenzymes. Alternatively, the molybdenum cofactor can be synthesized *in vitro* from stable intermediates and can be inserted into apo-molybdoenzymes by the aid of specific Moco-binding chaperones. A general working protocol is described here for the insertion of the bis-molybdopterin guanine dinucleotide cofactor (bis-MGD) into its target molybdoenzyme using the example of *Escherichia coli* TMAO reductase.

# 1. Introduction

Molybdenum is an essential trace element for bacteria, archaea and eukaryotes. In the cell, molybdenum is coordinated to the dithiolene group of the molybdenum cofactor (Moco) in almost all organisms (1). Only bacteria which are able to fix nitrogen coordinate molybdenum additionally in the iron molybdenum cofactor (FeMoco) which is exclusively found in nitrogenase (2). In contrast, more than 60 different enzymes binding the Moco were identified in the past (3). The vast majority of molybdoenzymes is found in bacteria and archaea while in animals and plants only four or five different enzymes, respectively, are present (4). The different Moco-containing enzymes are categorized on the basis of their molybdenum ligation sphere, dividing them into three different enzyme families (5): the xanthine oxidase (XO) family, the sulfite oxidase (SO) family and the dimethylsulfoxide (DMSO) reductase family (Figure 1). Enzymes of the XO family contain in general a Moco with a MPT-Mo<sup>VI</sup>OS(O<sup>-</sup>) ligand sphere, however, in bacteria the cofactor-core can also be present as molybdopterin cytosine dinucleotide cofactor (MCD) variant. Enzymes of the SO family contain a Moco with a MPT-Mo<sup>VI</sup>O<sub>2</sub>Cys core, while enzymes of the DMSO reductase family comprise a Moco with a MGD<sub>2</sub>-Mo<sup>VI</sup>XY core with X being either a sulfido-, selenido- or oxo-ligand and Y either being a hydroxo or amino acid ligand (Ser, Cys, Sec, or Asp). While eukaryotes produce only enzymes belonging to the SO and XO families, enzymes of all three families are present in prokaryotes with enzymes of the DMSO reductase family being the predominant ones.

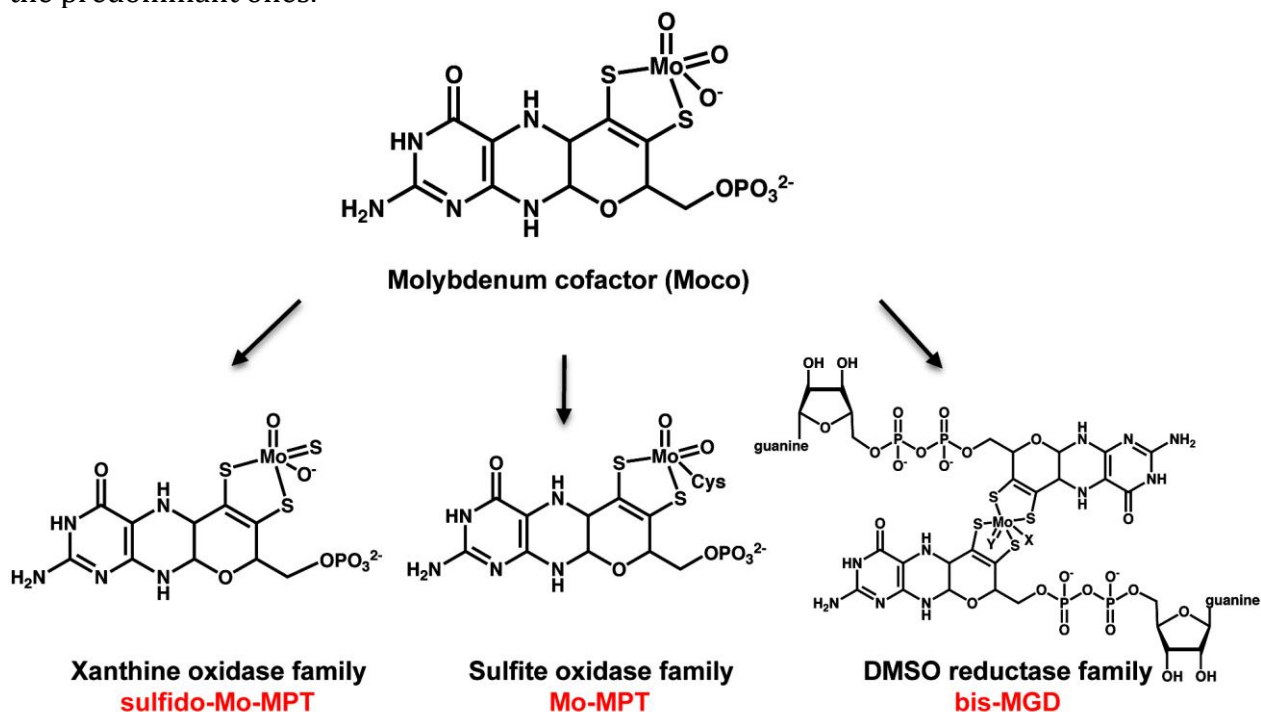


Figure 1: **The families of molybdoenzymes.** Shown are the different forms of the molybdenum cofactor (Moco, shown in the tri-oxo-form(6)) categorized into three different enzyme families are classified according to their coordination at the molybdenum atom: the xanthine oxidase, sulfite oxidase, and DMSO reductase families. The sulfite oxidase family is characterized by a MPT-Mo<sup>VI</sup>O<sub>2</sub>Cys ligand sphere. The xanthine oxidase family contains a MPT-Mo<sup>VI</sup>OS(O<sup>-</sup>) core (7). Here, the MPT core can be modified by an additional CMP nucleotide at the phosphate group, forming MCD. The DMSO reductase family contains a MGD<sub>2</sub>-Mo<sup>VI</sup>XY core with Y being either a sulfido, selenido- or oxo- ligand and X either being a hydroxo or amino acid ligand (Ser, Cys, Sec, or Asp).

Nason et al. provided in 1974 the first biochemical evidence for a common cofactor to all Moco-containing enzymes, which was removed as a low molecular weight fraction from denatured Mo-enzymes of mammalian, plant or bacterial origin (8). After separation, the protein-free Moco fraction was subsequently incorporated into a cofactor-free apo-nitrate reductase from eukaryotes (*nit-1* extract), thereby activating the enzyme. The totality of these studies led to the understanding that with the exception of nitrogenase, Moco is the common element in all molybdoenzymes from different organisms. In addition to demonstrating the universality of Moco, the *nit-1* extract assays also demonstrated that Moco is very labile with a lifetime of only a few minutes after release from molybdoenzymes, making chemical characterization of active Moco difficult. Therefore, structural characterization of Moco was achieved through the analysis of stable degradation products (9). The chemical nature of Moco has been determined by Rajagopalan and coworkers in 1982 which revealed a reduced pterin with an unusual 6-alkyl side chain consisting of four carbons, a terminal phosphate ester and a unique dithiolene group critical for metal ligation (10). Later crystal structures of Mo-enzymes have demonstrated the existence of a third pyrano ring between the OH-group at C3' of the side chain and the pterin C7 atom (11-13). Once the pyrano ring is closed, a fully reduced hydrogenated pterin is formed. Because of the unique nature of the pterin in Moco, the metal-free form of the cofactor is called molybdopterin or metal-binding pterin (MPT), the latter reflects the fact that not only Mo but also W can be coordinated by this pterin scaffold.

The biosynthesis of Moco has been revealed to be highly conserved in all organisms, which can be divided into three general steps, according to the stable biosynthetic intermediates that can be isolated (14) (Figure 2):

1. the synthesis of cyclic pyranopterin monophosphate (cPMP)
2. conversion of cPMP into MPT by introduction of two sulfur atoms
3. insertion of molybdate to form Moco

In prokaryotes, a fourth step involves the further modification of Moco by the addition of nucleotide monophosphates to the phosphate group of MPT, forming the bis-molybdopterin-guanine dinucleotide (bis-MGD) or MCD cofactor variants of the DMSO reductase or XO families, respectively (15).

While Nason and coworkers studied the reconstitution of apo-nitrate reductase from *N. crassa*, which belongs to the SO family of molybdoenzymes binding Mo-MPT (8), the first *in vitro* reconstitution of a bacterial enzyme of the DMSO reductase family with bis-MGD was reported in 1987 for nitrate reductase from *Escherichia coli* and was further optimized in the following decades showing that the activity of MobA is required for the assembly of the bis-MGD cofactor of nitrate reductase (16).

Studies on the molybdoenzyme TMAO reductase (TorA) from *E. coli* showed, that MobA acts in conjunction with the specific chaperone TorD in bis-MGD insertion into apo-TorA (17). By binding to the core of the apo-molybdoenzyme partner TorA, TorD induces a conformational change of apo-TorA that becomes competent for Moco insertion (18). Further, TorD plays a role also in the last step of the Moco maturation by interacting with the MobA protein involved in bis-MGD formation (19). Thus, the chaperone acts as a facilitator to insert the synthesized bis-MGD cofactor into the binding site of apo-TorA (20).

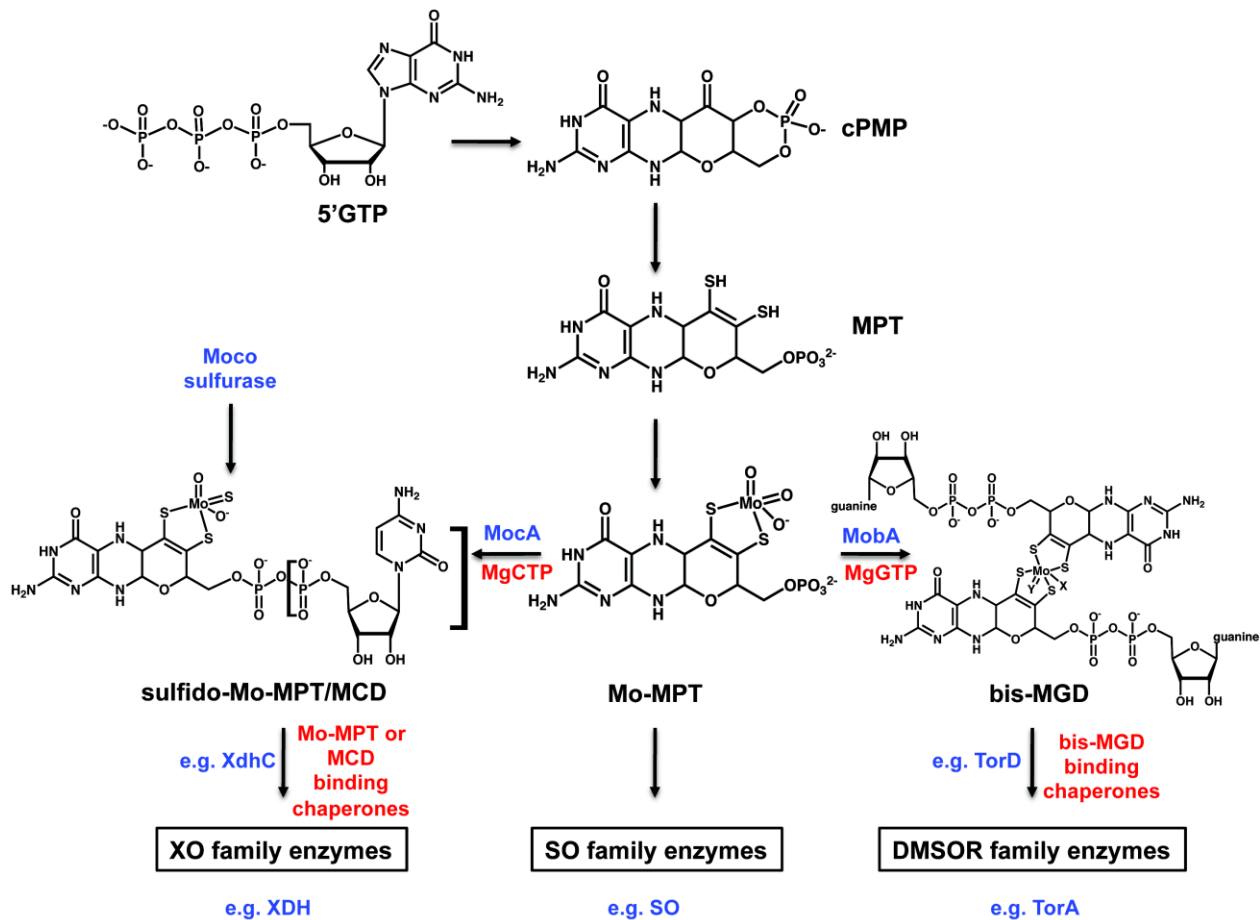


Figure 2: **The biosynthesis of the molybdenum cofactors.** Shown is a scheme of the biosynthetic pathway of Moco. The general pathway occurs via three conserved steps present in all organisms, the formation of cPMP, MPT and Mo-MPT, which are stable intermediates that can be isolated. Bacteria and Archaea contain a 4<sup>th</sup> step of Moco modification in which Mo-MPT is further modified by the addition of nucleotides, either GMP or CMP. Additionally, Moco can be further modified by the replacement of one oxo-ligand by a sulfido-ligand, forming the sulfido-containing Moco present in the xanthine oxidase family of molybdoenzymes. After synthesis, the different forms of Moco are specifically inserted into the target enzymes of each family, either directly (SO family) or by the aid of molecular chaperones (examples are gives in blue).

After bis-MGD insertion into apo-TorA, final folding of TorA is accomplished by the release of TorD from the complex. TorD-like proteins accordingly were considered to be system-specific chaperones dedicated to the respective molybdoenzyme. In total, studies on the biosynthesis of Moco and the requirement of specific chaperones for the insertion of the various Moco derivatives after their synthesis suggested that Moco is a labile molecule that does not exist in a protein-free form in the cell (9).

Therefore, it was very surprising that recent studies demonstrated that the complex bis-MGD cofactor can be extracted from the protein and reinserted into an apo-molybdoenzyme without the help of a chaperone (to be accepted). The investigations showed that under anoxic conditions bis-MGD is stable for 90 min in its protein-free form. Further, extracted bis-MGD can be inserted into an apo-molybdoenzyme without the help of a chaperone which facilitates folding after insertion.

Since the reconstitution of the *N. crassa nit-1* extract with molybdenum cofactors from the SO and XO family of molybdoenzymes have been described in detail, we are focusing on the reconstitution of isolated molybdoenzymes from the DMSO reductase family with bis-MGD

cofactors from cellular extracts, synthesized *in vitro* using MobA and TorD or direct insertion of intact bis-MGD by using apo-TorA. The major challenge for the insertion of bis-MGD into apo-molybdoenzymes lies in the fact that bis-MGD is labile towards O<sub>2</sub>, thus, all reconstitution steps must be performed under strictly anaerobic conditions.

## 2. Materials

### 2.1 Purified proteins required

- A. Apo-TorA is expressed in a *E. coli* strain incapable of Moco biosynthesis (e.g. RK5200,  $\Delta moaA$  (21,22)). A His<sub>6</sub>-tagged variant of TorA can be used to ensure simple purification (22). The purified protein is stored in 100 mM Tris-HCl, pH 7.2 at -80°C until usage. The concentration of the protein should be at least 200  $\mu$ M.
- A. The *E. coli* MobA protein is expressed as described previously (23) as a His<sub>6</sub>-tagged variant and is stored in 100 mM Tris-HCl, pH 7.2 at -80°C until usage. The concentration of the MobA protein should be at least 100  $\mu$ M
- B. The *E. coli* TorD protein is expressed as a His<sub>6</sub>-tagged variant and is stored in 100 mM Tris-HCl, pH 7.2 at -80°C until usage (22). The concentration of the TorD protein should be at least 100  $\mu$ M
- C. The protein used as bis-MGD source should be in a concentration of at least 300  $\mu$ M in e.g. 100 mM Tris-HCl, pH 7.2 (or a buffer in which the protein is stable). *Rhodobacter capsulatus* formate dehydrogenase (*RcFDH*) turned out to be an excellent bis-MGD donor protein which is used for the description of the method here. *RcFDH* is expressed as described previously as a His<sub>6</sub>-tagged protein and stored in 75 mM phosphate buffer, pH 7.2 at -80°C until usage (24). The protein concentration should be around 600  $\mu$ M, since the protein generally is only 50% loaded with bis-MGD.

### 2.2 *In vitro* reconstitution of apo-TorA from soluble extracts

1. Anoxic phosphate buffer 20 mM, pH 7
2. Purified *E. coli* TorD protein in 20 mM phosphate buffer, pH 7
3. Purified *E. coli* apo-TorA in 20 mM phosphate buffer, pH 7
4. 40 mg/ml soluble extract of an *E. coli* strain with a deletion in the genes for TMAO reductase and DMSO reductase (e.g. LCB504), in 100 $\mu$ l in phosphate buffer, pH7
5. Heat-block for Eppendorf tubes, electrophoresis system, a centrifuge and N<sub>2</sub>.

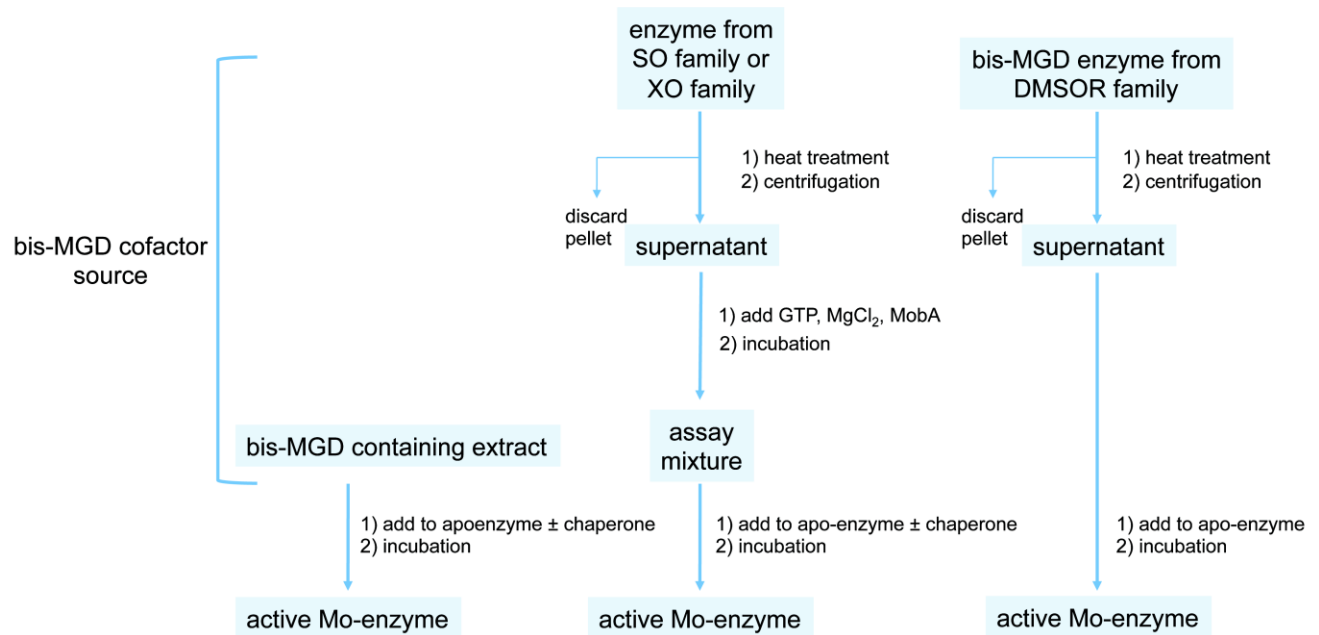
### 2.2 Reconstitution of apo-TorA with *in vitro* synthesized bis-MGD

1. Anoxic 100 mM Tris-HCl, pH 7.2
2. Anoxic 50 mM GTP
3. Anoxic 50 mM MgCl<sub>2</sub>
4. Purified *E. coli* TorD protein in 100 mM Tris-HCl, pH 7.2
5. Purified *E. coli* MobA protein in 100 mM Tris-HCl, pH 7.2
6. Purified *E. coli* apo-TorA in 100 mM Tris-HCl, pH 7.2
7. 300  $\mu$ M purified Moco-donor molybdoenzyme from the SO (e.g. human or chicken sulfite oxidase) or XO family (e.g. bovine XO purchased from sigma), in 100 mM Tris-HCl, pH 7.2
8. Heat-block for Eppendorf tubes, a centrifuge and an anaerobic chamber (Coy chamber or comparable) suitable for work under an oxygen-free atmosphere

### 2.3 Reconstitution of apo-TorA with bis-MGD isolated from a donor protein

1. Anoxic 100 mM Tris-HCl, pH 7.2
2. Purified bis-MGD containing enzyme in a concentration of 300  $\mu\text{M}$  (e.g. RcFDH)
3. Heat-block for Eppendorf tubes, a centrifuge and an anaerobic chamber (Coy chamber or comparable) suitable for work under an oxygen-free atmosphere

## 3. Methods



Scheme 1

### 3.1 *In vitro* reconstitution of apo-TorA with soluble extracts

O<sub>2</sub> free atmosphere can be obtained by bubbling N<sub>2</sub> gas in Eppendorf tubes containing *E. coli* extracts or purified protein. However, the yield of reconstitution is lower as compared to using an anaerobic chamber.

1. *E. coli* cells from a strain LCB 440 (18) containing a deletion of the *tor* and *dms* operons grown anaerobically until the late exponential phase at 37°C
2. After centrifugation, the cells are resuspended in 20 mM phosphate buffer, pH 7 to 5 ml per g of cells
3. Cell lysis by French press or sonication, centrifugation for 20 min at 15,000 rpm
4. Discard the pellet, the supernatant is centrifuged in an ultracentrifuge for 90 min 45,000 rpm
5. Adjust the protein concentration to 30 - 40 mg/ml with 20 mM phosphate buffer, pH 7
6. Keep the supernatant at 4 °C under nitrogen atmosphere
7. Mix in an Eppendorf tube:
  - 0.5  $\mu\text{M}$  apo-TorA,
  - 100  $\mu\text{l}$  soluble extract (as bis-MGD source)
  - 2.3  $\mu\text{M}$  TorD
8. Adjust to a final volume of 150  $\mu\text{l}$  with 20 mM phosphate buffer, pH 7
9. Keep for 2 hours at 37 °C under nitrogen atmosphere
10. The efficiency of the reconstitution can be checked by measuring the TorA activity in a spectrophotometric assay using benzyl viologen as electron donor

### **3.2 Reconstitution of apo-TorA in native gels**

1. Mix in an Eppendorf tube:
  - 1.5  $\mu$ M apo-TorA
  - 26.6  $\mu$ M TorD
2. Adjust the final volume to 20  $\mu$ l with 20 mM phosphate buffer, pH 7
3. Incubate for 1.5 hours at 37 °C, under nitrogen atmosphere

#### Native PAGE:

- Add 10  $\mu$ l of non-denaturing loading buffer (200 mM Tris-HCl, pH 8.8, 5 mM EDTA, 1M sucrose, 0.1% bromophenol blue), do not boil the samples
- Separate proteins on native 10% polyacrylamide gel
- After separation, the polyacrylamide gel is incubated for 60 min at 37°C under nitrogen atmosphere, in 20 ml phosphate buffer containing 1 ml of bis-MGD source (1 ml soluble extract with a protein concentration of 40 mg/ml, preparation described above)
- Transfer the polyacrylamide gel into 100 mM phosphate buffer, pH 6.5, containing 2 mM methyl viologen and 50 mM Na-dithionite in an anoxic atmosphere
- When the gel turns dark blue, add 2 mM TMAO; active TorA will turn white in the gel, corresponding to the oxidation of reduced methyl viologen

### **3.3 *In vitro* reconstitution of apo-TorA with direct bis-MGD cofactor sources**

*In vitro* reconstitution of apo-TMAO reductase with *in vitro* synthesized bis-MGD requires strict anaerobic conditions since Moco and its final product bis-MGD are highly sensitive to oxygen. All buffers, chemicals and proteins used need to be oxygen-free. We describe the preparation of samples i.e. in a Coy Chamber under a Nitrogen/Hydrogen atmosphere (95%/5%).

#### **3.3.1 *In vitro* reconstitution of apo-TorA with *in vitro* synthesized bis-MGD using MobA and TorD**

1. Anoxic 100 mM Tris-HCl, pH 7.2 buffer is prepared at least one day before the reconstitution procedure. The buffer is sterile filtered and degassed for at least 1 hour followed by incubation in the Coy chamber for at least 12 hours to remove all oxygen.
2. For enzymatic hydrolysis of GTP magnesium chloride is required. A 50 mM MgCl<sub>2</sub> solution is prepared, degassed as described in step 1 and incubated in the Coy chamber for at least 12 hours to remove residual oxygen.
3. On the day of reconstitution, 200  $\mu$ M apo-TorA, 100  $\mu$ M MobA protein in addition to the Moco donor protein in a concentration of 300  $\mu$ M are incubated in the Coy chamber for at least 2 hours at 4 °C. In 2 ml Eppendorf tubes, GTP powder for a 50 mM stock solution is transferred to the Coy Chamber and incubated for at least 2 hours at 4 °C.
4. Extraction of Moco requires heat-denaturation of the donor protein. The donor protein needs to be diluted to 30  $\mu$ M with the anoxic 100 mM Tris-HCl buffer, pH 7.2 in a total volume of 400  $\mu$ l. The denaturation of the protein is carried out at 95°C for 4 minutes followed by centrifugation of the samples at 13,000 g for a minimum of 90 seconds. The supernatant is removed carefully and should not be stored for



longer than 30 minutes under anoxic conditions. Long-term storage of isolated Moco is not recommended and will decrease the reconstitution efficiency.

5. The *in vitro* biosynthesis and simultaneous incorporation of bis-MGD into apo-TorA is started by mixing of 200  $\mu$ l Moco-containing supernatant with 6  $\mu$ l 50 mM GTP and 6  $\mu$ l 50 mM  $MgCl_2$  (final concentration = 1 mM), 6  $\mu$ l 100  $\mu$ M MobA protein (2  $\mu$ M final concentration), 6  $\mu$ l 100  $\mu$ M TorD protein and 20  $\mu$ l 20  $\mu$ M apo-TorA. Final volume of 300  $\mu$ l is adjusted by addition of 62  $\mu$ l of anoxic 100 mM Tris-HCl, pH 7.2. The reconstitution mixture needs to be incubated at 37°C for 7.5 hours under anoxic conditions. When higher end-concentrations of reconstituted TorA are required, the volume of the reaction can be generally scaled up, but it is important to keep the concentrations of all compounds as described above. An increase of the protein concentrations will result in a decreased reconstitution efficiency.
6. The reconstitution can be performed in the absence of TorD. The efficiency of the reconstitution is about 40% more effective when TorD protein is present in the assay.
7. After reconstitution the reconstituted active TMAO reductase needs to be purified from the reaction mixture to remove all low molecular weight compounds in addition to TorD and MobA. Gel filtration on a Superdex 200 or Superose 12 column, depending on the amount of sample, proved to be the method of choice.

### **3.3.2 *In vitro* reconstitution of apo-TMAO reductase with bis-MGD extracted from a DMSO-reductase family donor protein.**

For reconstitution of apo-TorA with bis-MGD isolated from a bis-MGD containing donor protein, the MobA or TorD proteins are not required.

1. Anoxic 100 mM Tris-HCl, pH 7.2 buffer are prepared at least one day before the reconstitution procedure. After preparation, the buffer is sterile filtered and degassed for at least 1 hour followed by incubation in the Coy chamber for at least 12 hours to remove all oxygen.
2. On the day of reconstitution procedure 200  $\mu$ M apo-TorA and 600  $\mu$ M R<sub>c</sub>FDH are incubated in the Coy chamber for at least 2 hours at 4 °C.
3. Extraction of Moco requires heat-denaturation of the donor protein. The donor protein R<sub>c</sub>FDH is diluted to 30  $\mu$ M with the anoxic 100 mM Tris-HCl buffer in a total volume of 400  $\mu$ l (1:20). The denaturation of the protein is carried out at 95 °C for 4 minutes followed by centrifugation of the samples at 13,000 g for a minimum of 90 seconds. The supernatant is removed carefully and should not be stored for longer than 30 minutes under anoxic conditions. Long term storage of isolated Moco is not recommended and will decrease the reconstitution efficiency. The pellet of denatured R<sub>c</sub>FDH appears black, whereas the supernatant has a yellowish color.
4. The *in vitro* reconstitution of apo-TorA with bis-MGD is started by mixing 280  $\mu$ l of the bis-MGD-containing supernatant with 20  $\mu$ l of 20  $\mu$ M apo-TorA. The reconstitution mixture is incubated at 37 °C for 7.5 hours under anoxic conditions.
5. After reconstitution, the activated TMAO reductase needs to be purified from the reaction mixture to remove all low molecular weight compounds. Gel filtration on a Superdex 200 or Superose 12 column, depending on the amount of sample, proved to be the method of choice.

6. TorD is not required for the insertion of bis-MGD into apo-TorA, therefore the reconstitution with extracted bis-MGD is described without TorD.

#### 4. Notes

- A. From preparation to preparation, and especially for TorA reconstituted with Moco from different Moco donors, the amount of incorporated bis-MGD into apo-TorA reductase will vary. To make all samples comparable, the relative FormA-GMP (stable and fluorescent oxidation product of MGD) has to be determined for each sample. Specific activities of reconstituted TorA can be correlated to FormA-GMP content per mg of enzyme to result in specific activities per unit of MGD (U/MGD)
- B. TMAO reductase of *E. coli* is highly stable in its holo form, whereas the apo form has moderate stability. Thus, removal of unreconstituted protein can be achieved by incubation of the reconstituted protein at 65 °C for 90 seconds with subsequent centrifugation at 13,000 g for 10 minutes. Apo-TorA will precipitate, whereas the holo-protein remains stable.

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