Transfer of the Molybdenum Cofactor Synthesized by Rhodobacter capsulatus MoeA to XdhC and MobA

Meina Neumann, Walter Stöcklein, and Silke Leimkuhler

From the Departments of 5Protein Analytics and 6Analytical Biochemistry, Institute of Biochemistry and Biology, University of Potsdam, 14476 Potsdam, Germany

The molybdenum cofactor (Moco) exists in different variants in the cell and can be directly inserted into molybdoenzymes utilizing the molybdopterin (MPT) form of Moco. In bacteria such as Rhodobacter capsulatus and Escherichia coli, MPT is further modified by attachment of a GMP nucleotide, forming MPT guanine dinucleotide (MGD). In this work, we analyzed the distribution and targeting of different forms of Moco to their respective user enzymes by proteins that bind Moco and are involved in its further modification. The R. capsulatus proteins MogA, MoeA, MobA, and XdhC were purified, and their specific interactions were analyzed. Interactions between the protein pairs MogA-MoeA, MoeA-XdhC, MoeA-MobA, and XdhC-MobA were identified by surface plasmon resonance measurements. In addition, the transfer of Moco produced by the MogA-MoeA complex to XdhC was investigated. A direct competition of MobA and XdhC for Moco binding was determined. In vitro analyses showed that XdhC bound to MobA, prevented the binding of Moco to MobA, and thereby inhibited MGD biosynthesis. The data were confirmed by in vivo studies in R. capsulatus cells showing that overproduction of XdhC resulted in a 50% decrease in the activity of bis-MGD-containing Me2SO reductase. We propose that, in bacteria, the distribution of Moco in the cell and targeting to the respective user enzymes are accomplished by specific proteins involved in Moco binding and modification.

The molybdenum cofactor (Moco) is an essential component of a diverse group of enzymes involved in important redox reactions in the global carbon, nitrogen, and sulfur cycles. Moco consists of a molybdenum atom coordinated to the dithiolene group of a tricyclic pyranopterin referred to as molybdopterin (MPT) (1). The biosynthesis of Moco is highly conserved in eukaryotes and prokaryotes (1) and can be divided into three general steps. In the first step, GTP is converted to GMP, which is further modified by attachment of a GMP nucleotide, forming the bis-MPT guanine dinucleotide (bis-MGD). In the second step, the bis-MGD is transformed to MPT by generation of its characteristic dithiolene group (4, 5). In the third step, molybdenum is inserted into MPT by generation of its characteristic dithiolene group (4, 5). Precursor Z is further transformed by MPT synthase into the meta-stable intermediate Precursor Z (2, 3). In the second step, Precursor Z is further transformed by MPT synthase into MPT by generation of its characteristic dithiolene group (4, 5). In the third step, molybdate is inserted into the MPT dithiolene sulfurs, a reaction catalyzed by MogA and MoeA in Escherichia coli (6–8). MoeA mediates molybdenum ligation, whereas MogA helps to facilitate this step in an ATP-dependent manner (8). Recently, studies with the homologous Arabidopsis thaliana CNX1 protein G and E domains identified the formation of an MPT-AMP intermediate before the ligation of molybdate to the MPT moiety (9, 10). An unexpected observation in the crystal structure of the A. thaliana CNX1 protein G domain was the identification of copper bound to the MPT-AMP dithiolene sulfurs (11). Up to now, the function of this novel MPT ligand has been unknown, but it was speculated that copper might play a role in sulfur transfer to Precursor Z, in protection of the MPT dithiolene from oxidation, and/or in presentation of a suitable leaving group for molybdenum insertion (12). To date, copper-MPT-AMP has not been identified as an intermediate in the biosynthesis of Moco in E. coli (8).

After the insertion of molybdenum into MPT in E. coli, Moco either can be directly inserted into molybdoenzymes (such as YedY) binding the MPT form of Moco (13) or is further modified by attachment of GMP (14, 15), forming the bis-MPT guanine dinucleotide (bis-MGD) form of Moco found in enzymes of the Me2SO reductase family (16). In E. coli, the GMP attachment to Moco is catalyzed by the MobA and MobB proteins (17). Whereas MobB was shown to be essential for this reaction (18), the role of MobB still remains uncertain. From the crystal structure, it was postulated that MobB is an adapter protein that acts in concert with MobA to achieve the efficient biosynthesis and utilization of MGD (19).

Enzymes containing the MPT form of Moco belong to either the sulfite oxidase or xanthine oxidase family, whereas enzymes binding the bis-MGD form of Moco belong to the Me2SO reductase family of molybdoenzymes. In Rhodobacter capsulatus, xanthine dehydrogenase (XDH; EC 1.17.1.4) is the only identified enzyme harboring the MPT form of the cofactor, whereas all other known molybdoenzymes bind the bis-MGD form of the cofactor (20). An essential role for the XdhC protein in the maturation of R. capsulatus XDH has been described, which entails binding of Moco and its insertion into the XdhB subunit of XDH (21). For all members of the xanthine oxidase family, the sulfurred form of Moco is essential for catalysis, whereas the equatorial of the two oxygen ligands of nascent Moco is replaced by sulfur (22). Previous work showed that XdhC specifically promotes the exchange of this ligand by interaction of the meta-stable intermediate Precursor Z (2, 3). In the second step, Precursor Z is further transformed by MPT synthase into MPT by generation of its characteristic dithiolene group (4, 5). In the third step, molybdate is inserted into the MPT dithiolene sulfurs, a reaction catalyzed by MogA and MoeA in Escherichia coli (6–8). MoeA mediates molybdenum ligation, whereas MogA helps to facilitate this step in an ATP-dependent manner (8). Recently, studies with the homologous Arabidopsis thaliana CNX1 protein G and E domains identified the formation of an MPT-AMP intermediate before the ligation of molybdate to the MPT moiety (9, 10). An unexpected observation in the crystal structure of the A. thaliana CNX1 protein G domain was the identification of copper bound to the MPT-AMP dithiolene sulfurs (11). Up to now, the function of this novel MPT ligand has been unknown, but it was speculated that copper might play a role in sulfur transfer to Precursor Z, in protection of the MPT dithiolene from oxidation, and/or in presentation of a suitable leaving group for molybdenum insertion (12). To date, copper-MPT-AMP has not been identified as an intermediate in the biosynthesis of Moco in E. coli (8).

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XdhC and MobA Bind Moco Produced by MoeA

with the l-cysteine desulfurase NifS4, which transfers the sulfur to Moco bound to XdhC (23). It has remained so far unclear which protein of the Moco biosynthesis pathway acts as the direct Moco donor for XdhC. So far, R. capsulatus XdhC is the only protein identified in bacteria shown to be involved in the modification of Moco by exchange of an o xo ligand of Moco with sulfur. To investigate the question of targeting, distribution, and insertion of different forms of Moco into the specific molybdoenzymes in R. capsulatus, we cloned and purified the MogA, MoeA, and MobA proteins from R. capsulatus for the investigation of protein-protein interactions in the homologous system. A MobB homolog seems not to be present in R. capsulatus (24).

In this study, we show for the first time that the amounts of sulforated Moco and bis-MGD produced in the cell are regulated at the protein level by protein-protein interactions. We show that both MobA and XdhC receive Moco from MoeA; however, by binding to MobA, XdhC prevents the Moco transfer to MobA, thus inhibiting MGD formation. This regulation ensures that enough Moco is abstracted from the major route of bis-MGD biosynthesis for further modification to the sulforated form of Moco and thus ensures that enough of the MPT form of Moco is provided to produce an active XDH, an enzyme involved in the purine degradation pathway.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Media, and Growth Conditions—E. coli BL21(DE3) cells were used for heterologous expression of the R. capsulatus proteins MobA, MoeA, and MogA. R. capsulatus XdhC was expressed in E. coli ER2566(DE3) cells and purified as described previously (21). The human sulfite oxidase pSL143 harboring only the tor from plasmid pPHU231 (28–30). For control experiments, the plasmids, (NH4)2SO4 was replaced with 1 g/liter serine as to obtain Moco-containing hSO-MD or in E. coli expressing only the XdhC was expressed in R. capsulatus strains for induction of XdhC or MobA expression from E. coli cultures were grown in LB medium under aerobic conditions at room temperature in a total volume of 400 μl of 100 mM Tris (pH 7.2) before the protein was denatured and analyzed for the presence of Moco or MGD by conversion to Form A (as described below).

MPT and MobA Binding by MogA, MoeA, and MobA—K_D values were determined by ultrafiltration as described previously (21). Samples contained 6 μM MogA, MoeA, or MobA and 0–24 μM Moco or MPT.

Moco, MPT, and MGD Analysis—The Moco/MPT content of the purified proteins was quantified after conversion to Form A as described previously (21). To separate Form A obtained from Moco/MPT and Form A-GMP obtained from MGD, the protocol originally described by Joshi and Rajagopalan (32) was used with some modifications. MGD was converted to Form A-GMP and Moco/MPT to Form A by overnight treatment with acidic iodine at room temperature (33). Form A was separated from Form A-GMP by chromatography on Q-Sepharose (GE Healthcare). 400 μl of Q-Sepharose was equilibrated with H2O; the oxidized samples were loaded; and Form A was eluted with 10 mM acetic acid. Form A-GMP was eluted with 50 mM HCl and converted to Form A by the addition of MgCl2, nucleotide pyrophosphatase, and alkaline phosphatase at pH 8.0. The

Cloning, Expression, and Purification of R. capsulatus MobA, MoeA, and MogA—DNA fragments containing the coding regions for R. capsulatus mobA, moeA, and mogA were amplified by PCR, and flanking restriction sites were introduced. The moeA and mogA genes were cloned into the Ndel-XhoI sites and mobA into the Ndel-Sall sites of pET28a (Novagen), resulting in plasmids pMN32, pMN53, and pMN56, respectively.

For expression of MoeA, MogA, and MobA, E. coli BL21(DE3) cells were transformed with plasmids pMN32, pMN53, and pMN56, respectively, and cell growth was started with 10 ml of overnight culture/liter of LB medium. The cells were grown at 30 °C, and expression of MgoA and MobA was induced at A_600 = 0.3–0.5 with 100 μM isopropyl β-D-thiogalactopyranoside. Cell growth was continued for 5 h, and cells were harvested and resuspended in 50 mM Na2PO4 and 300 mM NaCl (pH 8.0). After cell lysis, the soluble fraction was transferred onto a column with nickel-nitrilotriacetic acid (Ni-NTA; Qiagen Inc.). The resin was washed with 20 column volumes of phosphate buffer containing 10 mM and then 20 mM imidazole. The proteins were eluted with phosphate buffer containing 250 mM imidazole and dialyzed against 100 mM Tris (pH 7.2). For MoeA, expression was induced with 300 μM isopropyl β-D-thiogalactopyranoside; the shaking rate was increased to 210 rpm; and cells were harvested 3 h after induction of gene expression. After cell lysis, the soluble fraction was transferred onto nickel-tris(carboxymethyl)ethylenediamine (Macherey-Nagel). The column was washed with 40 column volumes of phosphate buffer containing 10 mM imidazole, and MoeA was eluted with the same buffer containing 250 mM imidazole and dialyzed against 100 mM Tris (pH 7.2).

MGD Formation by MobA—Moco was obtained after heat treatment of purified hSO-MD expressed in TP1000 cells, and MPT was obtained after heat treatment of purified hSO-MD expressed in RK5202 cells as described previously by Temple et al. (25) and Neumann et al. (21). 100 μM MobA was incubated with 160 μM Moco or MPT before excess Moco/MPT was removed by gel filtration. 1 mM Na2MoO4 was included in incubation mixtures containing Moco. 1 mM MgCl2, and 1 mM GTP were added, and the mixtures were incubated for 60 min at room temperature in a total volume of 400 μl of 100 mM Tris (pH 7.2) before the protein was denatured and analyzed for the presence of Moco or MGD by conversion to Form A (as described below).

MOP and MPT content of the purified proteins was quantified after conversion to Form A as described previously (21). To separate Form A obtained from Moco/MPT and Form A-GMP obtained from MGD, the protocol originally described by Joshi and Rajagopalan (32) was used with some modifications. MGD was converted to Form A-GMP and Moco/MPT to Form A by overnight treatment with acidic iodine at room temperature (33). Form A was separated from Form A-GMP by chromatography on Q-Sepharose (GE Healthcare). 400 μl of Q-Sepharose was equilibrated with H2O; the oxidized samples were loaded; and Form A was eluted with 10 mM acetic acid. Form A-GMP was eluted with 50 mM HCl and converted to Form A by the addition of MgCl2, nucleo-
pH of the samples was adjusted to pH 5.3 by the addition of 10 μl of 50% acetic acid before application to a C18 reversed-phase high pressure liquid chromatography column (4.6 × 250-mm Hypersil ODS, 5-μm particle size) equilibrated in 5 mM ammonium acetate and 15% methanol. In-line fluorescence was monitored by an Agilent 1100 series detector with excitation at 383 nm and emission at 450 nm.

Surface Plasmon Resonance (SPR) Measurements—All binding experiments were performed with the SPR-based instrument BiacoreTM 2000 on CM5 sensor chips at 25 °C and a flow rate of 10 μl/min using BioControl 2.1 and BioEvaluation 3.0 software (Biacore AB) as described previously (23). The proteins were immobilized after dilution in 10 mM acetate buffer at pH 4 (bovine serum albumin, MoeA, and MogA) or pH 5 (XdhC, XDH, and MobA). For control experiments, the N-terminal His6 tags of MoeA, MogA, and MobA were cleaved using the thrombin CleanCleave kit (Sigma). Cleavage was controlled by SDS-PAGE.

In Vitro Transfer of Moco from MoeA to XdhC—For production of Moco, 15 μM MPT obtained from hSO-MD was incubated with 30 mM MgoA, 30 mM MoeA, 37.5 mM Na2MoO4, 1 mM MgCl2, and 1 mM ATP in a volume of 200 μl of 100 mM Tris (pH 7.2). After 15 min of incubation at room temperature, 8 μM XdhC was added, and the mixture was further incubated for 20 min before MoeA and MogA were removed by Ni-NTA chromatography. Single components were left out for control experiments. Free Moco in the XdhC fraction was removed by an additional gel filtration step using NICK columns (GE Healthcare). 400 μl of the XdhC fraction was treated with acidic iodine, and bound Moco was quantified as Form A fluorescence (Sigma). Cleavage was controlled by SDS-PAGE.

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The same setup as described above was used to analyze the competition of Moco transfer from MoeA to XdhC in the presence of MobA. To avoid free Moco in the assay, these mixtures contained 15 μM XdhC and either 15 μM or 150 μM MobA in addition to 1 mM GTP.

In Vitro Transfer of Moco from MobA to XdhC—25 μM MobA was incubated with 100 μM Moco and 1 mM Na2MoO4 for 10 min at room temperature, and excess Moco was removed by gel filtration. Moco bound to MobA was quantified after conversion to Form A and Form A-GMP, respectively. Subsequently, Form A-GMP was converted to Form A, and Form A fluorescence was quantified in MobA samples incubated with MPT (bar I) or MPT that was converted to MGD (bar II), Moco (bar III), and Moco that was converted to MGD (bar IV). ND, no Form A detected.

Analysis of XDH and Me2SO Reductase Activities in R. capsulatus Crude Extracts—Plasmids were mobilized from E. coli S17-1 into R. capsulatus KS36 by filter mating as described previously (31). Crude extracts were obtained after cell lysis by sonification and subsequent removal of cell debris by centrifugation. Protein concentrations were determined following the method of Bradford (34). XDH activity was measured as described previously (24). The specific XDH activity (units/mg) is defined as the reduction of 1 μmol of NAD+/min/mg of enzyme. Me2SO reductase activity was measured as described by McEwan et al. (35) with dithionite-reduced benzyl viologen as the electron donor. Me2SO reductase activity (units/mg) is defined as the reduction of 1 μmol of Me2SO/min/mg of protein.

RESULTS

Purification and Analysis of the Functional Activities of R. capsulatus MobA, MoeA, and MogA—For purification of R. capsulatus MobA, MoeA, and MogA, fusion proteins were generated each containing an N-terminal His6 tag (see “Experimental Procedures”). After heterologous expression in E. coli BL21(DE3) cells, the soluble fractions of MobA, MoeA, and MogA were purified by affinity chromatography (see “Experimental Procedures”). After elution, one major band was displayed for MobA, MoeA, and MogA on Coomassie Brilliant Blue R-stained SDS-polyacrylamide gels, corresponding to molecular masses of 22.1, 44.6, and 20.3 kDa, respectively (Fig. 1A). This procedure yielded ~6.5 mg of MobA/liter of E. coli culture, 1.5 mg of MoeA/liter of E. coli culture, and 13.3 mg of MogA/liter of E. coli culture.

To show the functionality of R. capsulatus MoeA and MogA, Moco was produced from MPT in vitro and inserted into
human apousulfite oxidase following the procedure described by Nichols and Rajagopalan (8) for E. coli MoeA and MogA. In contrast to the E. coli proteins, for which maximum human sulfite oxidase reconstitution was observed using a MogA/MoeA ratio of 1:1, the best human sulfite oxidase reconstitution was obtained when R. capsulatus MogA and MoeA were used in a ratio of at least 1:1.4 (data not shown). Thus, for all further assays, R. capsulatus MogA and MoeA were mixed in a 1:1 ratio.

To test the functionality of R. capsulatus MobA, its ability to produce MGD from either MPT or Moco in the presence of MgGTP was analyzed. Moco was extracted from hS0-MD expressed in E. coli TP1000 cells (25), whereas MPT was obtained from hS0-MD expressed in RSK202(modC−) cells (26). After the addition of MgGTP to the incubation mixture, MGD was formed only from Moco (Fig. 1B, bars II and IV). Bound MPT and the remaining Moco eluted in the Moco/MPT fraction (bars I and III). These results show that molybdenum insertion into MPT has to precede MGD formation. Thus, for all further experiments, Moco was used as a precursor for the formation of MGD.

**Analysis of Protein-Protein Interactions by SPR Measurements**—To identify possible protein-protein interactions between XdhC, MogA, MoeA, and MobA, SPR measurements were employed for real-time detection of specific interactions using the purified proteins. XdhC, MoeA, MogA, and MobA were immobilized via amine coupling to a CM5 chip, and interactions were analyzed with each protein partner. The results obtained by SPR measurements for the protein pairs listed in Table 1 showed the tightest interaction between MoeA and XdhC, with $K_D$ values of 1.45 and 1.15 μM depending on the immobilized protein. In contrast, no significant interaction between XdhC and MogA was obtained with either immobilized protein partner, whereas in another set of experiments, MoeA bound to MogA with $K_D$ values of 6.2 and 6.0 μM (Table 1). This also shows that XdhC preferentially interacts with MoeA and not with MogA. The interaction between MoeA and MobA was also investigated. When MoeA was immobilized, MobA interacted with a $K_D$ of 3.5 μM, whereas when MobA was immobilized, the $K_D$ was significantly higher (27 μM) (Table 1). The second value for immobilized MobA shows that the dissociation constant between both proteins was negatively influenced by the immobilization of MobA. Because both XdhC and MobA interacted with MoeA and might compete for the same binding site, a possible interaction between XdhC and MobA was also investigated. As shown in Table 1, an interaction between both proteins was identified with $K_D$ values of 1.75 and 1.04 μM for immobilized XdhC and MobA, respectively. In contrast to the MoeA-MobA interaction, the dissociation constant for this protein pair was not influenced by the immobilization of MobA, showing that MobA was immobilized in a functional form, partly impairing the binding site for MoeA.

Control experiments showed that MoeA did not interact with immobilized XDH, supporting the idea that Moco transfer to XDH is mediated by XdhC, rather than a direct transfer of Moco from MoeA to XDH. Additional control experiments were performed to exclude a possible influence of the N-terminal His$_6$ fusion, His$_6$-tagged proteins, and MobA on the dissociation constants. For this purpose, the His$_6$ tags of MogA, MoeA, and MobA were cleaved by thrombin treatment, and the interaction of these proteins with immobilized XdhC was compared with the sensograms obtained for the His$_6$-tagged proteins. Although untagged MogA also showed no interaction with XdhC (data not shown), comparison of the binding curves of His$_6$-tagged and untagged MoeA and MobA with immobilized XdhC showed no significant difference in the Biacore sensograms (supplemental Fig. S1), clearly showing that the His$_6$ tag had no influence on the protein-protein interactions or the dissociation constants.

**In Vitro Transfer of Moco from MoeA to XdhC**—To investigate whether Moco bound to MoeA can directly be transferred to XdhC, Moco was produced in vitro by incubation of MoeA and MogA with MPT, MoO$_4^{2−}$, and MgATP before XdhC was added. To ensure that the incubation mixture did not contain free MPT, which can be bound directly by XdhC, a MogA/MPT ratio of 2:1 was chosen. After incubation for 20 min, MogA and MoeA were removed by Ni-NTA chromatography, and the amount of Moco bound to XdhC was quantified after the conversion of Moco to Form A (see “Experimental Procedures”). The results in Fig. 2 show that Moco was specifically transferred to XdhC in incubation mixtures containing MoeA, MgATP, and MoO$_4^{2−}$ (bar III). The inclusion of MogA resulted in higher Moco saturation of XdhC (bar I) as a result of increased Moco production in the incubation mixture. The results in Fig. 2 also show that Moco was specifically transferred to XdhC and not MPT because the omission of MoO$_4^{2−}$ from the incubation mixtures did not give rise to Form A fluorescence (bar V).

Because an interaction between MoeA and both MobA and XdhC was seen by SPR measurements, we were interested in whether XdhC and MobA directly compete for Moco produced by MoeA. To analyze this, GTP and increasing amounts of MobA were added to an incubation mixture containing MPT, MoeA, MogA, MgATP, MoO$_4^{2−}$, and XdhC. The results in Fig. 3A show that a 10-fold excess of MobA in the presence of GTP resulted in a drastically decreased Moco saturation of XdhC (bar II versus bar I). In contrast, omission of GTP from the incubation mixtures with equimolar concentrations of XdhC

**TABLE 1**

| Immobilized protein | RU$^b$ | Protein partner | $K_D$ | $\chi^2$ |
|---------------------|-------|----------------|-------|---------|
| XdhC | 570 | MoeA | 1.45 | 0.374 |
| MoeA | 9260 | XdhC | 1.15 | 1.83 |
| XdhC | 570 | MogA | ND |  |
| MogA | 2010 | XdhC | ND |  |
| XdhC | 570 | MobA | 1.75 | 0.283 |
| MobA | 1130 | XdhC | 1.04 | 1.06 |
| XDH | 2810 | MoeA | ND |  |
| MobA | 2600 | MobA | 3.5 | 0.339 |
| MobA | 2025 | MoeA | 27 | 0.281 |
| MoeA | 9260 | MoeA | 6.0 | 0.168 |
| MogA | 2180 | MoeA | 6.2 | 0.247 |

$^a$ Proteins were immobilized via amine coupling (see “Experimental Procedures”).

$^b$ RU, resonance units; ND, none detectable.

$^c$ Proteins were injected using the RINJECT protocol, injecting samples in a concentration range of 0.1–6.4 μM. Cells were regenerated by injection of 20 ms HCl.

$^d$ $K_D$ values were obtained by global fitting procedures for a 1:1 binding.
and MobA resulted in an increase in the Moco content of XdhC (bar III versus bar I). This shows a rapid conversion of Moco to MGD when GTP is present, and thus, the Moco concentration is reduced in the incubation mixture. In the absence of GTP, Moco is not converted to MGD; thus, a 10-fold excess of MobA results in only a slightly reduced Moco content of XdhC. These results show that MobA and XdhC compete for Moco produced by MoeA and, in addition, that binding of Moco to XdhC is only slightly reduced by the concentration of MobA in the presence of GTP.

Because SPR measurements also showed an interaction between XdhC and MobA, it was of further interest to determine whether Moco can be directly transferred from MobA to XdhC. For these investigations, MobA was incubated with Moco; excess Moco was removed by gel filtration; and XdhC was added. Subsequently, MobA and XdhC were separated by Ni-NTA chromatography, and the Moco content of XdhC was quantified. The results in Fig. 3B show that Moco remained bound to MobA in this incubation mixture and was not further transferred to XdhC.

**XdhC and MobA Bind Moco Produced by MoeA**

**TABLE 2**

| Protein | $K_D$ for MPT binding$^a$ | $K_D$ for Moco binding$^b$ |
|---------|-------------------------|---------------------------|
| MobA    | 0.92 ± 0.04             | 0.54 ± 0.02               |
| MoeA    | 0.94 ± 0.03             | 0.95 ± 0.02               |
| MogA    | 0.87 ± 0.04             | 1.01 ± 0.04               |

$^a$ 6 μM MocA, MoeA, or MobA was used. $^b$ $R_{max}$ describes the maximum saturation of the respective protein revealed by a 1:1 fitting procedure following the law of mass action.

$K_D$ values were determined as described previously (21) using Moco and MPT in a concentration range of 0–24 μM.

**Dissociation Constants for MPT and Moco Binding to MobA, MoeA, and MobA**—To determine the dissociation constants for binding of Moco and MPT to MobA, MoeA, and MobA, the purified proteins were incubated with varying concentrations of Moco or MPT for 15 min at 4 °C before unbound Moco/MPT was separated by ultrafiltration using a membrane with a molecular mass cutoff of 10 kDa. The Moco/MPT concentration in the flow-through fraction was quantified after conversion to the stable, fluorescent oxidation product Form A (see “Experimental Procedures”).

Quantification of Moco and MPT after ultrafiltration in the presence and absence of MocA, MoeA, or MobA allowed the determination of $K_D$ values for Moco and MPT binding to these proteins (Table 2). The amount of Moco or MPT bound to MobA, MoeA, or MobA and the free MocA, MoeA, or MobA concentrations were calculated according to the determined free and total Moco/MPT concentrations in relation to the total protein concentration. Fitting revealed a function according to the law of mass action for a ratio of Moco/MPT to MocA, MoeA, or MobA of 1:1 (Table 2). The $K_D$ values in Table 2 show that MocA bound MPT 30 times more tightly than Moco, whereas MobA bound Moco ~4 times more tightly than MPT, and MoeA bound both cofactor forms to the same extent. The binding of Moco to MoeA appears to be weaker in comparison with the binding of Moco to MobA or XdhC (3.6 μM) (21). In total, the $K_D$ values determined for MobA and XdhC were in the same range, whereas the ones determined for MocA showed the weakest binding.

**XdhC Inhibits the Binding of Moco to MobA**—To analyze whether increasing concentrations of XdhC also influence...
Moco binding to MobA, purified MobA was incubated with XdhC prior to the addition of Moco and MgGTP. To ensure saturation of the proteins with Moco, the cofactor was added in excess to the incubation mixture. The formation of MGD was analyzed by its conversion to Form A from Form A-GMP as described under “Experimental Procedures.” The results in Fig. 4A show that MGD formation by MobA was inhibited by increasing amounts of XdhC. At equimolar concentrations of XdhC and MobA, MGD production was reduced by 70%, whereas a 10-fold excess of XdhC resulted in an 80% reduction. To elucidate the nature of the negative effect of XdhC on MGD production, we analyzed whether XdhC prevents the binding of Moco to MobA. For this purpose, MobA was preincubated with Moco; unbound Moco was removed from the mixture; and XdhC and MgGTP were subsequently added. As shown in Fig. 4B, increasing amounts of XdhC had no influence on the formation of MGD by Moco-saturated MobA. These results show that, in the course of binding to MobA, XdhC prevents the binding of Moco, so no MGD can be formed.

Analysis of the Dependence of XDH and Me₆SO Reductase Activities in R. capsulatus KS36 Cells on Overproduced XdhC—After the identification of the direct competition of XdhC and MobA for available Moco in vitro, we investigated whether the same effects occur in vivo. To examine the in vivo consequences of high XdhC concentrations on bis-MGD formation in the cell, a plasmid expressing the xdhC gene under the control of the strong nifH promoter was introduced into R. capsulatus KS36 cells by conjugation. This plasmid allows the induction of xdhC gene expression under nitrogenase-derepressing conditions in medium containing serine as the sole nitrogen source. To analyze the influence of bis-MGD formation when large amounts of XdhC are present, cells were grown in RCV minimal medium containing serine and Me₆SO, and after cell lysis, the crude extract was assayed for Me₆SO reductase and XDH activities. The results in Table 3 show that XDH activity remained unaffected by the presence of XdhC, whereas Me₆SO reductase activity was reduced by >50% when XdhC was overexpressed. The effects of high MobA concentrations on Me₆SO reductase and XDH activities were also analyzed as a control. For this purpose, a plasmid expressing the mobA gene under the control of the nifH promoter was constructed and transferred into R. capsulatus KS36 cells. The results in Table 3 show that Me₆SO reductase and XDH activities remained unaffected by high levels of MobA in the cell, showing that mono-oxo-Moco biosynthesis is not inhibited by high concentrations of MobA at physiological GTP concentrations. The data confirm the results obtained in the in vitro studies and show that high XdhC concentrations in the cell result in decreased MGD production.

**DISCUSSION**

For *R. capsulatus* XDH, the XdhC protein has been identified as a chaperone that is involved in the maturation of XDH (21, 23, 36). In contrast to proteins that bind the bis-MGD form of Moco such as *R. capsulatus* Me₆SO reductase, MobA is not essential for the generation of active XDH, which contains the MPT form of Moco (24). However, for XDH to be active, the sulfurred form of Moco has to be produced by exchange of the equatorial oxygen ligand with sulfur. This reaction is catalyzed by the l-cysteine desulfurase NifS4 while Moco is bound to XdhC (23). XdhC further protects the Mo = S group from oxidation and is involved in the insertion of sulfurred Moco into XDH. Interactions of both MobA and XdhC with MoeA were identified, and the MoeA-MobA and MoeA-XdhC pairs showed Kₐ values in the same range. Most *R. capsulatus* molybdoenzymes such as Me₆SO reductase bind bis-MGD, and under derepressing conditions, Me₆SO reductase is present in large amounts in the cell (37, 38). In contrast, XDH is constitutively expressed and represents only a minor fraction of the total cell proteins (20). We present here a fully defined in vitro system for studying the mechanism of Moco biosynthesis and targeting. Our in vitro experiments have shown that MobA and XdhC obtain Moco produced by MoeA; however, most of the Moco supplied by MoeA is converted to MGD when MobA is present (Fig. 5). In addition, MobA is unable to transfer Moco to XdhC by binding Moco tightly and thereby enabling the biosynthesis of MGD to ensure the supply for all bis-MGD-containing enzymes. To prevent all available Moco in the cell from being converted to MGD, XdhC has to ensure that at least some Moco is converted to its sulfurred form for further transfer to XDH. We have shown that XdhC not only binds to MoeA but also interacts
with MobA and thereby prevents the binding of Moco to MobA, simultaneously inhibiting MGD formation. Our results have shown that XdhC has a crucial role to guarantee the Moco supply for *R. capsulatus* XDH. The *in vitro* studies were confirmed by *in vivo* studies in *R. capsulatus* showing that overproduction of XdhC results in a decrease in Me$_2$SO reductase activity. In contrast, overproduction of MobA does not influence the activities of Me$_2$SO reductase and XDH. Because the *xdhC* gene in *R. capsulatus* is coexpressed with *xdhAB*, XdhC is in general present only in low amounts in the cell. Because no induction of *xdh* gene expression under certain growth conditions has been reported so far (20), the cellular role of XdhC is to abstract a minor part of the total amount of synthesized Moco in the cell for further modification to the sulfurred form. The other part of Moco is converted to MGD by MobA. Because XDH requires only a small part of the total Moco concentration in the cell, a mutation of *R. capsulatus mobA* does not result in an increase in XDH activity, as reported previously by Leimkühler and Klipp (24). In addition, previous results also showed that *mobA* gene expression is constitutive at low levels, and no increase in *mobA* expression was observed even under conditions of high MGD demand (24). This suggests a high turnover rate of the MobA-catalyzed MGD production, but also implies that MobA is not directly involved in the insertion of produced MGD into the respective MGD-binding molybdoenzymes and that other proteins might mediate the transfer of MGD from MobA to the target enzyme (39, 40). This observation was confirmed by overproduction of MobA in *R. capsulatus*, which does not give rise to a higher activity of bis-MGD-containing Me$_2$SO reductase. In addition, the production of Moco is restricted in *R. capsulatus* by linking part of the genes for Moco biosynthesis to expression of the genes for Me$_2$SO reductase (41), explaining why Me$_2$SO reductase is not increased by the overexpression of MobA because the amount of produced Moco is the limiting factor.

In addition to the newly identified MobA-XdhC and XdhC-MobA interactions, the SPR measurements partly confirmed the interactions identified previously for *E. coli* MomA, MoeA, MobB, and MobA in a bacterial two-hybrid approach (42). Whereas the *E. coli* two-hybrid studies revealed a major role for MobB in the interactions between MoeA, MobA, and MobA, no MobB homolog was so far identified in *R. capsulatus*. In addition, in the two-hybrid system, interactions between *E. coli* MobA-MoeA and MoeA-MoeA were identified only in strains producing active Moco; however, the interaction of these protein pairs was only slightly increased. In contrast, we identified interactions between the analyzed protein pairs in the absence of Moco. Thus, differences seem to exist in the interaction of proteins in Moco biosynthesis in *E. coli* and *R. capsulatus*.

Most molybdoenzymes in *R. capsulatus* and *E. coli* contain the bis-MGD form of the cofactor; however, the formation of bis-MGD is one of the most enigmatic steps in Moco biosynthesis. It is still not known whether the two MGD molecules assemble on MobA or after insertion into target proteins such as Me$_2$SO reductase and nitrate reductase A. Here, we have shown that molybdenum ligation to MPT has to precede MGD formation. So far, XdhC is the only protein for which a role in Moco binding and insertion into its specific target protein has been identified. Because organisms like *E. coli* contain several different bis-MGD-containing enzymes, it remains to be elucidated whether factors exist that ensure the trafficking of MGD or bis-MGD into specific enzymes like trimethylamine-N-oxide reductase and nitrate reductase under certain growth conditions. It remains speculative whether chaperones like TorD for trimethylamine-N-oxide reductase and NarJ for nitrate reductase A are involved in targeting the right amount of cofactor to the acceptor protein in a manner similar to XdhC. Because an interaction between MobA and NarJ was identified using an *E. coli* two-hybrid assay (40), this implies that NarJ might be involved in a similar reaction.

**FIGURE 5. Model for the role of XdhC and MobA in the biosynthesis of Moco and MGD.** Moco is produced from MPT by the MogA-MoeA complex, catalyzing the ATP-dependent ligation of the molybdenum atom to MPT. Synthesized Moco can subsequently be transferred either to MobA, converting it into bis-MGD (which is inserted into enzymes of the Me$_2$SO (DMSO) reductase family), or to XdhC, forming the sulfurred form of Moco by exchange of an oxo ligand with sulfur (which is inserted into XDH). XdhC interacts with MobA and thereby inhibits the transfer of Moco to MobA. Direct Moco transfer from MobA to XdhC was not detected. Modification of Moco and trafficking to the specific target proteins are tightly regulated by the direct binding of Moco to both XdhC and MobA.
XdhC and MobA Bind Moco Produced by MoeA

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