Mechanism of Assembly of the Bis(Molybdopterin Guanine Dinucleotide)Molybdenum Cofactor in Rhodobacter sphaeroides Dimethyl Sulfoxide Reductase*

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A fully defined in vitro system has been developed for studying the mechanism of assembly of the bis(molybdopterin guanine dinucleotide)molybdenum cofactor in Rhodobacter sphaeroides dimethyl sulfoxide reductase (DMSOR). R. sphaeroides DMSOR expressed in a mobA Escherichia coli strain lacks molybdopterin and molybdenum but contains a full complement of guanine in the form of GMP and GDP. Escherichia coli MobA, molybdopterin-Mo, GTP, and MgCl₂ are required and sufficient for the in vitro activation of purified DMSOR expressed in the absence of MobA. High levels of MobA inhibit the in vitro activation. A chaperone is not required for the in vitro activation process. The reconstituted DMSOR can exhibit up to 73% of the activity observed in recombinant DMSOR purified from a wild-type strain. The use of radiolabeled GTP has demonstrated incorporation of the guanine moiety from the GTP into the activated DMSOR. No role was observed for E. coli MobB in the in vitro activation of apo-DMSOR. This work also represents the first time that the MobA-mediated conversion of molybdopterin to molybdopterin guanine dinucleotide has been demonstrated directly without using the activation of a molybdoenzyme as an indicator for cofactor formation.

In all molybdenum-containing enzymes except nitrogenase, the metal is coordinated to the organic cofactor molybdopterin (MPT), which also serves as the cofactor for tungsten-containing enzymes (1). The MPT molecule is a substituted pterin ring that coordinates the metal through a diethionite linkage (Fig. 1A). Additional variability of the molybdenum cofactor is found in bacteria with the attachment of GMP and GDP. Escherichia coli MobA, molybdopterin-Mo, GTP, and MgCl₂ are required and sufficient for the in vitro activation of purified DMSOR expressed in the absence of MobA. High levels of MobA inhibit the in vitro activation. A chaperone is not required for the in vitro activation process. The reconstituted DMSOR can exhibit up to 73% of the activity observed in recombinant DMSOR purified from a wild-type strain. The use of radiolabeled GTP has demonstrated incorporation of the guanine moiety from the GTP into the activated DMSOR. No role was observed for E. coli MobB in the in vitro activation of apo-DMSOR. This work also represents the first time that the MobA-mediated conversion of molybdopterin to molybdopterin guanine dinucleotide has been demonstrated directly without using the activation of a molybdoenzyme as an indicator for cofactor formation.

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tion of the MGD cofactor, it is difficult to separate the NR-specific role of NarJ as a chaperone and the more general cofactor biosynthesis role of the mob proteins, since both functions are required to form the active NR enzyme in these experiments.

In view of the difficulties described above, the need was apparent for a new system for studying the conversion of MPT to MGD. The use of R. sphaeroides DMSOR eliminated many of the complications associated with NR. DMSOR is a single subunit, soluble enzyme that contains bis(MGD)Mo as the sole prosthetic group (9), whereas NR is a membrane-bound, heterotrimeric complex that contains [Fe-S] clusters and a heme group in addition to the molybdenum cofactor. Recombinant DMSOR can also be heterologously expressed in E. coli (19), and both the recombinant enzyme and that purified from Rhodobacter have been extensively characterized (4, 19–21). One possible complication is the presence in the R. sphaeroides operon of a gene called dmsB (22) or dorB (23) that has been proposed to encode a chaperone for the DMSOR protein. However, heterologously expressed, recombinant DMSOR is active and incorporates the molybdenum cofactor in the absence of this proposed chaperone, indicating that a R. sphaeroides chaperone is not essential for successful expression of R. sphaeroides DMSOR in E. coli.

In this paper, we present a completely defined in vitro system for studying the mechanism of assembly of the bis(MGD)Mo cofactor in R. sphaeroides DMSOR. The Rhodobacter enzyme has been expressed in E. coli in the absence of one or both of the mob gene products and activated using only purified components in a process that requires MobA, GTP, MgCl₂, and MPT. After this in vitro activation, DMSOR from a mob⁻ strain exhibited a maximum of 73% of the activity observed in recombinant DMSOR purified from a wild-type strain. The conversion of MPT to MGD by MobA has also been shown to occur in the absence of DMSOR.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids**—The DMSOR expression plasmid used in this work was created by digesting the pHJ720 construct (19) (Table I) with NcoI and HindIII to release the complete mature R. sphaeroides DMSOR coding sequence including an N-terminal His₆ tag. This insert was subsequently ligated into similarly digested pTrc 99 A (Amerham Pharmacia Biotech) to form pHJ820.

For creation of the MobA expression constructs, an oligonucleotide primer was designed that covered the translational start region of the mobA gene. This primer contained mismatches creating two restriction sites, an upstream BamHI site and an NcoI site that changed the start codon for mobA from GTG to ATG and the second codon from AAT to GCT, converting the second amino acid from Asn to Ala. A second oligonucleotide primer complementary to the translation stop site of the mobA gene with mismatches creating a downstream SphI site was also designed. These two oligonucleotides were used to prime PCRs with Elongase (Life Technologies) using E. coli MC4100 (Table I) genomic DNA as the template, and a fragment of the expected size (620 base pairs) was amplified. This PCR product was digested with BamHI and SphI and subsequently ligated with similarly digested pLysE to create pCT300A (Table I). To create a high yield expression vector for MobB, pCT300A was digested with NcoI and SalI to release the mobB gene, which was subsequently ligated into pTrc 99 A to form pCT800A.

A similar procedure was used for the mobB gene as well. The oligonucleotide covering the translation start site contained mismatches creating an upstream BglII site and a NcoI site at the ATG start codon. The primer covering the translation stop site contained mismatches creating a downstream SphI site. A PCR fragment of the expected size (569 base pairs) was amplified, digested with BglII and SphI, and ligated with pLysE digested with BamHI and SphI to create pCT300B (Table I). To create a high yield expression vector for MobB, pCT300B was digested with NcoI and SalI to release the mobB gene, which was subsequently ligated into pTrc 99 A to form pCT800B. Oligonucleotides were synthesized by Life Technologies Custom Primers, and automated sequencing was performed at the Duke University DNA Analysis Facility.

**Protein Expression and Purification**—Wild type DMSOR was purified from R. sphaeroides as described previously (3). Recombinant DMSOR was purified from MC4100 cells (Table I) containing pHJ820. Cells were grown aerobically at 37 °C overnight in LB supplemented with 100 μg/ml ampicillin. This culture was then diluted 1:25 into LB supplemented with ampicillin and 0.5 mM Na₂MoO₄ and subsequently grown at 30 °C until A₆₀₀ = 1. This culture was diluted 1:20 into M9ZB medium supplemented as described previously (19). except Na₂MoO₄ was present at 1.0 mM, isopropyl-β-D-galactopyranoside was added to 5 μM, and ampicillin replaced all other antibiotics. The cells

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**Fig. 1.** Structures of molybdopterin-molybdobium (A), MGD (B), and bis(MGD)Mo (C).
were then grown anaerobically for 20–24 h at room temperature before harvesting.

TP1000 cells (Table I) containing pH820 and grown as described above were used to purify mobAB DSMR. TP1000 cells containing pH820 and either pCT800A or pCT300B were grown as described above with the addition of 34 µM chloramphenicol and were used to purify mobB and mobA DSMR, respectively. All forms of recombinant DSMR were purified as described previously (19) except that cell lysis was achieved using a Microfluidics M110L Microfluidizer Processor, and a 0.5 M NaCl wash step was added to the Cibacron blue affinity column during the purification of MobA. After purification, MobA was dialyzed into 50 mM Hepes, 10 mM mercaptoethanol, 0.1 M NaCl, pH 7.6.

**Protein Analysis—** Purified DSMR was quantitated spectrophotometrically using an extinction coefficient at 280 nm of 200,000 M⁻¹ cm⁻¹ (3). Comparisons using the Pierce BCA assay indicated that the mobAB forms of DSMR have the same extinction coefficient at 280 nm as the wild-type protein. The concentrations of purified MobA and MobB were calculated using molar extinction coefficients at 280 nm of 26,000 and 28,220 M⁻¹ cm⁻¹, respectively (13).

**DSMR activity and the total guanine and molybdenum content of**

**TABLE I**

| Strains | Genotype | Reference/source |
|---------|----------|-----------------|
| MC4100  | F− lacU169 araD139 rpsL150 relA1 ptsF rpsB 45b  | M. Casadauban |
| TP1000  | MC4100 (mobAB) | Palmer et al. (15) |
| Plasmids | | |
| pH720   | Mature CDS R. sphaeroides DSMR in pET-28 | This work |
| pTrc 99 | Expression vector with a trc promoter | This work |
| pBH820  | CDS of His-tagged DSMR from pH720 in pTrc 99 A | This work |
| pLyIE   | TT lysozyme gene inserted into pACYC184 | Novalen |
| pCT300A | TT lysozyme gene of pLyIE replaced by the CDS of MobA | This work |
| pCT800A | CDS of MobA from pCT300A ligated into pTrc 99 A | This work |
| pCT800B | CDS of MobB from pCT800A ligated into pTrc 99 A | This work |
| pTG918  | CDS of mature human SO in pTrc 99 A | Temple et al. (27) |

The concentrations of purified MobA and MobB were calculated using molar extinction coefficients at 280 nm of 26,000 and 28,220 M⁻¹ cm⁻¹, respectively (13).

**Activation of DMSOR Purified from a mob**—In a standard assay, 10–11 µg of DSMR was combined with MobA in a 50:1 molar ratio in a round cuvette (Fisher). Sufficient GTP and MgCl₂ were added to produce a final concentration of 1 mM each, and 50 mM Tris/HCl, pH 7.5, was added to bring the total volume to 150 µl. This mixture was placed in the Coyer chamber for a minimum of 15 min before 50 µl of heat-denatured SO was added. The tube was then capped securely before removal from the Coyer chamber and incubation at 37 °C for 1.5 h. To monitor the extent of activation, the MeSO reductase activity of the reconstituted mixture was assayed as described previously (19). Measurements were corrected for the activity observed under the same conditions in the absence of DSMR, and at least two assays were performed for each data point.

**For the time course experiment, 5.5 µg of DSMR was used per assay, and the incubation period varied from 0 to 675 min. To determine the extent of activation of MobA concentration on the extent of activation, the amount of DSMR was maintained at 10 µg while the molar ratio of MobA to DSMR was varied from 10:1 to 0.002:1. In both of these cases, the procedure described above was followed in all other aspects.**

**Activation of mob**—**DMSR Using Radiolabeled GTP**—For this activation, 0.11 mg of mobAB DSMR was combined with MgCl₂ and MobA in a 2.50 molar ratio of MobA to DSMR in a total volume of 180 µl. In two separate controls, MobA was left out of the mixture, and wild-type DSMR was substituted for the protein purified from a mob stain. An aliquot of 400 µl of heat-denatured SO was added to each sample before it was sealed and removed from the Coyer chamber. Using an airtight syringe, [α-32P]GTP (PerkinElmer Life Sciences) was added to a sealed, anaerobic solution of GTP, and 20 µl of this solution was then injected into each reaction mixture. The final concentration of GTP and MgCl₂ was 0.1 mM, and the mixture contained 0.14 µCi/ml. Each sample was then incubated at 37 °C for 3 h before the addition of 1.2 ml of 50 mM Tris/HCl, pH 7.5, to each sample to permit easy transfer to a Slide-A-Lyzer (Pierce). All three samples were then dialyzed two times against 2 liters of 50 mM Tris/HCl, pH 7.5, before measurement of bound radioactivity using a Beckman LS 1801 scintillation counter.

**Form A-GMP Analysis—** MobA (126 µg) was mixed with a 1 µM concentration each of GTP and MgCl₂ and 200 µl of heat-denatured SO in a total volume of 600 µl under anaerobic conditions. The mixture was then incubated at 37 °C for 3 h. Tubes containing no MobA or 0.5 mg of wild-type DSMR substituted for MobA were treated in an identical manner. The volume of the samples was then increased to 9 ml by the addition of 8.4 ml of 10 mM sodium phosphate buffer, pH 7.

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run. Each fraction was neutralized by the addition of 200 μl of 1 M Tris/HCl, pH 8.5, and NaCl and MgCl₂ were added to a final concentration of 0.1 M and 15 mM, respectively, obtaining a final volume of 2 ml. Using a J4–8960 Aminco-Bowman Spectrophotofluorometer, the fluorescence of each fraction was measured with an excitation wavelength of 370 nm and emission wavelength of 450 nm. The 50 mM HCl fluorescence of each fraction was measured.

### RESULTS

**Expression of DMSOR in mob⁻ Cells**—The previous construct used to express recombinant *R. sphaeroides* DMSOR, pJH720, required the presence of T7 RNA polymerase within the host cells (19), thus limiting the number of suitable expression cell types. To avoid this complication, the coding sequence used to express recombinant DMSOR including the N-terminal His₆ tag was transferred for DMSOR into the pTrc99 A vector to form pJH820 (Table I). Using this new vector in *E. coli* MC4100 cells (solid line) and the mobAB⁻ strain TP1000 (dashed line) was treated to form the fluorescent MPT derivative form A, which elutes at 12 min under the conditions described under "Experimental Procedures."

### Table II

**Effect of the mobAB locus on recombinant *R. sphaeroides* DMSOR**

| Expression strain | Phenotype | Activity | μM guanine/μM protein | μM Mo/μM protein | Molybdopterin* |
|-------------------|-----------|----------|-----------------------|------------------|---------------|
| MC4100            | Wild type | 24       | 1.74                  | 0.83             | Yes           |
| TP1000, pCT300A   | ΔmobB     | 22       | 1.82                  | 0.84             | Yes           |
| TP1000, pCT300B   | ΔmobA     | <0.3     | 2.20                  | ND\(^a\)         | ND            |
| TP1000            | ΔmobAB    | <0.3     | 2.15                  | ND\(^a\)         | ND            |

\(^a\) Detected by form A analysis.

\(^b\) ND, none detected.

**Fig. 2.** HPLC elution profile for form A. DMSOR purified from wild-type MC4100 cells (solid line) and the mobAB⁻ strain TP1000 (dashed line) was treated to form the fluorescent MPT derivative form A, which elutes at 12 min under the conditions described under "Experimental Procedures."
the mobB<sup>−</sup> DMSOR had the typical color characteristic of rDM-
SOR and was fully active. This is in accordance with work by
Palmer <em>et al.</em> (15) demonstrating that MobB is not required for
the expression of active NR, trimethylamine N-oxide reductase,
or formate dehydrogenase lyase.

The guanine moieties present in mobAB<sup>−</sup> and mobA<sup>−</sup> DS-
SOR were extracted from the purified proteins by boiling in an
aqueous SDS solution and subsequently identified by HPLC
analysis. In both cases, approximately 70% of the guanine was
present as GMP and 30% as GDP; no GTP was detected. There
was no evidence of dGDP, but we were unable to separate GMP
dGMP under the conditions used.

**Analysis of DMSOR Expressed in the Absence of Molybdate—**
Previously, it was observed that recombinant expression of
R. sphaeroides DMSOR in the BL21(DE3) E. coli strain
required supplemental molybdate in the medium (19). To in-
vestigate the role of molybdate in bis(MGD)Mo biosynthesis,
recombinant DMSOR was purified from BL21(DE3) cells
grown in the absence of supplemental molybdate. While it had
been previously shown that DMSOR expressed under the same
conditions in the presence of supplemental molybdate is active
and contains approximately 74% molybdenum in addition to
MPT and a full complement of guanine (19), in the absence of
exogenous molybdenum, the protein contained 2.1 mol of gua-
nine/mol of protein, and no MPT or molybdenum was detected.

**Activation of DMSOR Purified from an mobA<sup>−</sup> Strain—**
The in vitro activation of DMSOR from a mobA<sup>−</sup> strain presented
itself as a sensitive and convenient assay for production of the
bis(MGD)Mo cofactor. Since R. sphaeroides DMSOR has been
successfully expressed in E. coli (19), no problems were antic-
ipated with the use of the E. coli cofactor biosynthesis proteins
in this in vitro assay. To assess the roles of E. coli MobA and
MobB in the in vitro activation, the two proteins were ex-
pressed in TP1000 cells using pCT800A and pCT800B, respec-
tively (Table I). Both proteins were purified using the method
of Eaves <em>et al.</em> (13) with only minor modifications.

In view of the total lack of pterin in mobAB<sup>−</sup> and mobA<sup>−</sup>
DMSOR, it was clear that a source of MPT had to be included
in any in vitro activation attempt. Since sulfite oxidase (SO)
contains the MPT form of the cofactor and can be purified in
relatively large quantities (27), heat-denatured, recombinant
human SO was chosen as a source of MPT. Any risk of MGD
contamination was avoided by expressing the recombinant SO
in the mobAB<sup>−</sup> TP1000 cells. Anaerobic conditions were used
for the release of MPT from SO and subsequent activation of
DMSOR due to the extreme sensitivity of the cofactor to oxygen
(1).

The assay components were combined with mobAB<sup>−</sup> or
mobA<sup>−</sup> DMSOR under anaerobic conditions and incubated at
37 °C for 1.5 h. The extent of activation was determined by
measuring the DMSOR activity generated in the sample. Using
this method, it was determined that in vitro activation of
mobAB<sup>−</sup> or mobA<sup>−</sup> DMSOR required the presence of MobA,
GTP, MgCl<sub>2</sub> and heat-denatured SO as a source of MPT (Table
III). GTP and MgCl<sub>2</sub> were present at 1 mM each, and MobA was
present in a 1:50 molar ratio with DMSOR. No activation was
observed in the absence of any one of these components.

GMP and ATP were unable to substitute for GTP under the
conditions used. The addition of 50 mM Na<sub>2</sub>MoO<sub>4</sub> also did not
affect the extent of activation; nor did the addition of MobB in
a 1:50 molar ratio with DMSOR (Table III). To examine the
possible requirement for an additional component for the
proper functioning of MobB, activation was carried out in the
presence of 20 μl of TP1000 lysate in addition to the standard
assay components. Even under these conditions, there was no
difference in the extent of activation seen with the addition of

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**TABLE III**

| Assay components | Activity<sup>a</sup> units/mg DMSOR | mobAB<sup>−</sup> DMSOR | mobA<sup>−</sup> DMSOR |
|------------------|-----------------------------------|----------------------|----------------------|
| Standard Mixture | 8.33                              | 8.98                 |
| (GTP, MgCl<sub>2</sub>, MPT, and MobA) | | | |
| Minus MPT        | ≤0.3                              | ≤0.3                 |
| Minus GTP        | ≤0.3                              | ≤0.3                 |
| Minus MobA       | ≤0.3                              | 0.53                 |
| Minus MgCl<sub>2</sub> | ≤0.3                        | ≤0.3                 |
| Plus MobB        | 8.08                              |                      |
| Plus 50 mM Na<sub>2</sub>MoO<sub>4</sub> | 7.82                         | 8.67                 |
| Replacement of GTP with GMP | ≤0.3                          | ≤0.3                 |
| Replacement of GTP with ATP | ≤0.3                          | ≤0.3                 |

<sup>a</sup> All values corrected for the activity observed under the same con-
ditions in the absence of DMSOR.

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**FIG. 3.** Time course for the in vitro activation of mobAB<sup>−</sup> DMSOR. DMSOR
activity was measured as a function of incubation time.

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MobB alone. Additionally, no significant differences were observed between the activation of mobAB$^{2-}$ and mobA$^{2-}$ DMSOR (Table III)

The crystal structure of the E. coli MobA protein is presented in the accompanying article (28). In attempts at obtaining the structure of the MobA-GTP complex, it was found that the nucleotide was much better defined as a complex with Mn$^{2+}$ than when Mg$^{2+}$ was used as the cation. In the reconstitution procedure described here, no significant changes were observed in the level of activation when 1 mM MnCl$_2$ was substituted for 1 mM MgCl$_2$, showing that the MobA-GTP-Mn$^{2+}$ complex is a catalytically relevant species.

**Time Course for in Vitro Activation**—To determine the highest level of in vitro activation possible, Me$_2$SO reductase activity was measured as a function of incubation time (Fig. 3). Maximum activation required at least 7.5 h under the conditions used, at which time the reconstituted enzyme exhibited an average of 17.4 units of activity/mg of DMSOR. This represents about 73% of the activity observed in rDMSOR purified from the wild-type, MC4100 strain. Doubling the MPT concentration, increasing the molar ratio of MobA to DMSOR to 1:10, or doing both simultaneously did not significantly change the final specific activity. Preincubating MobA with GTP, MgCl$_2$, and MPT for 2 h at 37 °C prior to the addition of mobAB$^{-}$ DMSOR also did not change the extent of activation during the first 90 min.

**Excess MobA Inhibits in Vitro Activation of mobA$^{2-}$ DMSOR**—To determine the effect of MobA concentration on the extent of activation, the concentration of mobAB$^{2-}$ DMSOR was maintained at 10 μg while the molar ratio of MobA to DMSOR was varied from 10:1 to 0.002:1. No significant differences were observed in the level of activation after a 1.5-h incubation period when MobA was present at or below an equimolar ratio with mobAB$^{2-}$ DMSOR (Fig. 4, top). However, when the molar concentration of MobA exceeded that of mobAB$^{2-}$ DMSOR, the extent of activation was attenuated. In fact, almost no activation occurred when the molar ratio of MobA to DMSOR reached 4:1 (Fig. 4, bottom). The presence of MobB did not inhibit activation under the conditions used, even when the molar concentration of MobB was 5 times that of mobAB$^{-}$ DMSOR.

**Activation of mob$^{-}$ DMSOR Using Radiolabeled GTP**—The requirement for GTP in the activation of mobAB$^{-}$ DMSOR could indicate that the guanine moieties of the newly formed MGD were derived from the added GTP. Alternatively, it was possible that the guanine moieties present in the purified mobAB$^{-}$ DMSOR prior to activation were joined to the MPT to form MGD. To investigate the latter possibility, mobAB$^{2-}$ DMSOR was reconstituted in the presence of α-$^{32}$P-labeled GTP...
followed by dialysis to remove any unincorporated nucleotides. For this experiment, the concentrations of GTP and MgCl₂ were decreased to 0.1 mM each, and the molar ratio of MobA to DMSOR was changed to 1:250. Neither of these changes significantly lowered the extent of activation from that observed under the standard conditions.

Following dialysis, the sample of mobAB⁻ DMSOR activated in the presence of the radiolabeled GTP contained 8,590 cpm. In the presence of MobA, substitution of active DMSOR purified from R. sphaeroides for mobAB⁻ DMSOR yielded only 688 cpm after dialysis. Similarly, when MobA was excluded from a sample containing mobAB⁻ DMSOR, only 611 cpm were found after dialysis. These data indicate that the guanine moiety in the final form of the DMSOR cofactor is derived from the GTP added to the activation mix rather than the guanine moieties already present on the purified protein.

**Direct Evidence for the in Vitro Formation of MGD—**Up to this time, the formation of MGD by MobA has been demonstrated by indirect means such as the absence of MGD in a mob⁻ cell strain (10) or the requirement for MobA to activate NR (14, 15) or DMSOR purified from a mob⁻ cell strain. The procedure developed here, however, offered the chance to directly demonstrate the production of MGD from MPT by MobA. To that affect, MobA was incubated for 3 h in the presence of MPT (supplied in the form of heat-denatured SO) and a 1 mM concentration each of GTP and MgCl₂. This mixture was then incubated overnight with SDS and iodine according to the method of Johnson et al. (10) to convert MPT to the form A derivative and MGD to form A-GMP. A QAE-Sephadex column was then used to separate the two fluorescent derivatives.

As seen in the top of Fig. 5, in the absence of MobA, the form A peak generated from MPT was present, but there was no significant form A-GMP peak, indicating that no appreciable MGD was formed in the absence of MobA. When MobA was present in the incubation mixture, the form A peak was considerably smaller, and a form A-GMP peak was detected (Fig. 5, middle). To confirm the identity of form A-GMP, the fractions containing this derivative were treated with alkaline phosphatase and pyrophosphatase. After this treatment, the fluorescence of these fractions was measured again, and a large increase in the fluorescence was observed (Fig. 5, dashed lines). This fluorescence increase is associated with the scission of the pyrophosphate bond, thereby eliminating the quenching effects of the ribonucleotide on the inherent fluorescence of A (10). The fluorescence spectrum of the species present after phosphatase treatment was identical to that of form A. The amount of form A-GMP produced under these conditions was comparable with that present in the control reaction containing 0.5 mg of R. sphaeroides DMSOR (Fig. 5, bottom). The addition of mobAB⁻ DMSOR to the MobA reaction mixture did not appreciably alter the size of the form A-GMP peak produced.

**DISCUSSION**

We present here a fully defined, in vitro system for studying the mechanism of assembly of the bis(MGD)/Mo cofactor. The in vitro generation of activity in R. sphaeroides DMSOR purified from a mob⁻ strain serves as a sensitive and convenient assay of bis(MGD)/Mo assembly and insertion into the apoprotein. Using this assay, it has been demonstrated that MobA, MPT, GTP, and MgCl₂ are required and sufficient for the activation of mobAB⁻ and mobA⁻ DMSOR. Clearly, activation of mobA⁻ DMSOR, unlike that of E. coli NR, can proceed in the absence of a chaperone. Therefore, the specific roles of the mob proteins in the assembly of the DMSOR cofactor can be examined without the complication of the enzyme-specific role of a chaperone. In addition, the MobA-mediated conversion of MPT to MGD has been demonstrated directly for the first time without using

![Fig. 5. Direct demonstration of the MobA-mediated conversion of MPT to MGD.](image)

FIG. 5. Direct demonstration of the MobA-mediated conversion of MPT to MGD. MPT, GTP, and MgCl₂ were incubated in the absence of MobA (top) and in the presence of 128 µg of MobA (middle) or with 0.5 mg of active R. sphaeroides DMSOR (bottom). After incubation, MPT and MGD were converted to the fluorescent derivatives, form A and form A-GMP, respectively, which were subsequently separated using a QAE-Sephadex column. The fluorescence of the eluting fractions was measured before (solid line) and after (dashed line) phosphatase treatment.

The activation of a molybdooenzyme to indicate cofactor formation. This has also demonstrated that MGD formation can occur in the absence of a molybdooenzyme apoprotein.

The Rhodobacter mobAB⁻ and mobA⁻ DMSOR proteins used in this activation assay were devoid of any detectable MPT or molybdenum. It was demonstrated previously that E. coli NR (29) and DMSOR (30) also do not contain any MPT or molybdenum when expressed in a mob⁻ strain. Therefore, the guanine moiety of the cofactors appears to play an essential role for binding of that cofactor in many MGD-containing proteins. Alternatively, the MPT-binding site could be latent in apo-DMSOR, although the binding sites for the two guanine moieties are obviously present as indicated by the presence of a full complement of guanine in mobA⁻ DMSOR. Hänelmann et al. (24) have purified CO dehydrogenase, a molybdopterin cy-
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It is unclear whether the guanine moieties present in mobA–
DMSOR play any role in cofactor biosynthesis and insertion in vivo. However, the absence of MPT in apo-DMSOR shows that occupancy of the guanine nucleotide binding site does not by itself create the MPT binding site. The guanine moieties incorporated into the activated DMSOR originate from the GTP added to the activation mixture, as demonstrated by the use of radiolabeled GTP. Although the GMP and GDP are bound to apo-DMSOR strongly enough to be present stoichiometrically in the purified protein, the data presented here show that they can be replaced in the process of inserting the assembled bis(MGD)Mo cofactor into the protein. It may be that the guanine moieties found in mobAB and mobA–DMSOR serve to stabilize the protein as a prelude to cofactor insertion, and one possible role for the proposed DMSOR chaperone is to catalyze the rapid exchange of the bound nucleotides for bis(MGD)Mo. It is also possible that, in vivo, the apoprotein interacts with a chaperone to prevent intermediate binding of GMP and GDP. Even with the guanine moieties present, heat sensitivity and overall low yield indicate that mobA–DMSOR is less stable than the holoenzyme.

As shown here, MPT is an essential component in the activation of DMSOR from a mobA– strain. MPT was not included in earlier studies on the activation of E. coli NR because inactive NR from a mob– strain was incorrectly believed to contain MPT (16). This has led to speculation on the source of the pterin component of the cofactor and to the suggestion that the lack of sufficient MPT may explain the low level of activation once mobile NR from a E. coli DMSOR was added. It is likely that the rate-limiting step is the replacement of bound guanine nucleotides with the bis(MGD)Mo cofactor. In view of the requirement for the NAR chaperone in the activation of mob–NR (15, 17), it would be interesting to determine if the addition of the proposed R. sphaeroides DMSOR chaperone to the reactivation mixture increases the rate of enzyme activation. The role of a chaperone in cofactor insertion is especially intriguing, since, in holo-DMSOR, the bis(MGD)Mo cofactor is deeply buried in the interior of the protein (9). Due to the larger size, multisubunit structure, and additional prosthetic groups present in NR, it is conceivable that such a chaperone is essential for cofactor insertion in NR while not required in the simpler DMSOR protein.

E. coli MobB appears to play no role in the in vitro or in vitro activation of R. sphaeroides DMSOR. In contrast, MobB appears to enhance NR activation, although it is not required for the process (15). Like the chaperone, MobB may not play as critical a role in cofactor production and insertion for the simpler molybdoenzymes. It may also be that E. coli MobB may not be able to substitute for the Rhodobacter counterpart in the reconstitution of R. sphaeroides DMSOR. Although the first 160 amino acids of R. sphaeroides MobB are highly similar to the entire sequence of E. coli MobB, the R. sphaeroides protein contains 292 additional amino acids not found in the E. coli protein (31). E. coli and R. sphaeroides MobA are much more closely related, with 39% identity in their amino acid sequences. Whereas E. coli MobB is obviously not required for activation of mobA–DMSOR, it would be interesting to see whether the addition of R. sphaeroides MobB to reconstitution assays would affect the rate or extent of DMSOR activation.

Although this study does not focus on metal chelation to the MPT cofactor, the lack of MPT and molybdenum in recombinant DMSOR expressed in BL21(DE3) cells in the absence of supplemental molybdenum does seem to imply that metal chelation precedes dinucleotide attachment. This is especially likely in view of the inability of H. pseudoflava to produce MCD in the absence of molybdenum despite the presence of MPT in the cells (24). Therefore, the MobA protein in E. coli, and the analogous MCD-synthesizing protein in H. pseudoflava are probably specific for Mo-MPT and are unable to attach the guanine or cytosine nucleotide to molybdenum-free MPT. Under the in vitro conditions described here, the pterin component acted upon by MobA is clearly Mo-MPT, since the cofactor is supplied by heat-denatured SO. This is supported by the inability of added molybdate to increase the extent of activation. A more complete model for cofactor biosynthesis and insertion can now be proposed for R. sphaeroides DMSOR. Molybdenum chelation to the pterin appears to precede dinucleotide formation. MobA then links the GMP moiety supplied by Mg-
GTP to Mo-MPT to form Mo-MGD. Formation of Mo-MGD can proceed in the absence of an acceptor apoprotein. Subsequently, the two Mo-MGD molecules are assembled to form bis(MGD)Mo, either on MobA or on the acceptor apoprotein. If both MGD moieties are already coordinated to molybdenum, the formation of bis(MGD)Mo would require elimination of one of these metal atoms. In the in vitro reconstitution procedure, neither MobB nor a DMSOR-specific chaperone is required in cofactor formation or insertion, although the presence of a chaperone may accelerate cofactor insertion.

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Mechanism of Assembly of the Bis(Molybdopterin Guanine Dinucleotide)Molybdenum Cofactor in *Rhodobacter sphaeroides* Dimethyl Sulfoxide Reductase

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