In Vitro Molybdenum Ligation to Molybdopterin Using Purified Components*

Received for publication, December 7, 2004, and in revised form, December 22, 2004
Published, JBC Papers in Press, January 4, 2005, DOI 10.1074/jbc.M413783200

Jason D. Nichols and K. V. Rajagopalan‡

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

We have previously shown that Escherichia coli MoeA and MogA are required in vivo for the final step of molybdenum cofactor biosynthesis, the addition of the molybdenum atom to the dithiolene of molybdopterin. MoeA was also shown to facilitate the addition of molybdenum in an assay using crude extracts from E. coli moeA cells. The experiments detailed in this report utilized an in vitro assay for MoeA-mediated molybdenum ligation to de novo synthesized molybdopterin using only purified components and monitoring the reconstitution of human aposulfite oxidase. In this assay, maximum activation was achieved by delaying the addition of aposulfite oxidase to allow for adequate molybdenum coordination to occur. Tungsten, which substitutes for molybdenum in hyperthermophilic organisms, could also be ligated to molybdopterin using this system, though not as efficiently as molybdenum. Addition of thiol compounds to the assay inhibited activity. Addition of MogA also inhibited the reaction. However, in the presence of ATP and magnesium, addition of MogA to the assay increased the level of aposulfite oxidase reconstitution beyond that observed with MoeA alone. This effect was not observed in the absence of MoeA. The results presented here demonstrate that MoeA is responsible for mediating molybdenum ligation to molybdopterin, whereas MogA stimulates this activity in an ATP-dependent manner.

In all molybdenum-containing enzymes, with the exception of nitrogenase, the molybdenum cofactor (Moco) consists of a mononuclear Mo atom coordinated to the cis-dithiolene moiety of molybdopterin (MPT). In Escherichia coli, biosynthesis of Moco begins with the conversion of GTP to a pterin intermediate termed precursor Z, catalyzed by the MoaA and MoaC proteins (1–4). MPT is synthesized from precursor Z by the MoaD and MoaE proteins, which together compose the MPT synthase complex (5, 6). The MoB protein is involved in reactivating the MoaD subunit of MPT synthase between rounds of MPT synthesis (7, 8). Ligation of the Mo atom to MPT requires the MoeA and MogA proteins along with the ModABC molybdocyte transporter system (9, 10). In E. coli, the cofactor is further modified by the covalent addition of GMP to the MPT phosphate, a reaction catalyzed by the MoB protein (11, 12).

Previous results from our laboratory verified that E. coli MoeA and MogA are both required for in vivo Mo ligation to the MPT dithiolene. Analysis of crude extracts produced from E. coli moeA and mogA cells showed the presence of MPT but the absence of MPT-guanine dinucleotide, the biosynthesis of which was found to require prior attachment of Mo to MPT. Recombinantly expressed human sulfite oxidase (SO) purified from both strains contained MPT but was devoid of Mo. In vitro experiments demonstrated the ability of MoeA to activate the recombinant Mo-free, MPT-containing human SO in moeA crude extract. MogA was incapable of supporting the same type of activity in mogA crude extract. Thus, MoeA appeared to be the protein directly responsible for Mo ligation, whereas MogA had an alternative, unknown function in metal ligation (9).

Although the mogA crude extract assay demonstrated that MoeA can facilitate Mo incorporation into MPT-containing SO, it did not define the mechanism of Mo ligation to de novo formed MPT. Therefore, the development of an assay for Mo ligation that utilized only purified components was undertaken. Our laboratory has previously developed fully defined systems for assaying both MPT and MPT-guanine dinucleotide biosynthesis. The in vitro synthesis of MPT-guanine dinucleotide using only MoeA, Mg2+, GTP, and Moco from denatured SO is quantitated by monitoring the activation of Rhodobacter sphaeroides apo-DMSO reductase (12), whereas de novo synthesized MPT is quantitated by the reconstitution of aposulfite oxidase (apoSO) or by conversion to the stable, fluorescent derivative Form A (6, 13). The results presented in this report delineate the development and optimization of a fully defined in vitro assay for MoeA-mediated Mo ligation to de novo synthesized MPT. The assay contains only purified components (activated MPT synthase, precursor Z, molybdate, MoeA, and apoSO) and monitors the activation of MPT-free human apoSO. Efficient apoSO activation required preincubation of the nascent MPT with MoeA and molybdate prior to apoSO addition, and metal ligation was not as efficient if MoeA were not present during this first step.

As tungsten is just below Mo on the periodic table and many Moco-containing enzymes utilize tungsten in place of Mo (14, 15), experiments were performed to determine whether tungsten could be ligated to MPT in this system. Contrary to previous reports (16, 17), the presence of thiol compounds was not necessary for activation and actually inhibited the assay. Initial studies with MogA in the defined assay found that its presence inhibited apoSO reconstitution. However, because recent reports indicated the possible requirement for ATP in MogA function (18, 19), further studies in the presence of ATP were performed. Here we have shown that, in the presence of ATP and Mg2+, MogA augments the level of apoSO reconstitution obtained with MoeA alone. MoeA was required for this stimulatory effect as apoSO reconstitution was not observed in its absence.

* This work was supported by National Institutes of Health Grant GM00091. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.
† To whom correspondence should be addressed: Box 3711, Duke University Medical Center, Durham, NC 27710. Tel.: 919-681-8845; Fax: 919-684-8919; E-mail: raj@biochem.duke.edu.
‡ The abbreviations used are: Moco, molybdenum cofactor; MPT, molybdopterin; SO, sulfite oxidase; apoSO, His-tagged MPT-free sulfite oxidase; MoaD-SH, thiacarboxylated MoaD; ICP-MS, inductively coupled plasma emission mass spectrometry.

This paper is available on line at http://www.jbc.org
MATERIALS AND METHODS

Protein Expression and Purification—E. coli MoeA (20), MogA (21), MoaE, and active (thiocarboxylated) MoaD (MoaD-SH) (22) were expressed and purified from BL21(DE3) cells as described previously. His-tagged, MPT-free human apoSO was expressed and purified from E. coli cells in the absence of supplemental Na₂MoO₄, as described previously (9). Following purification, MoeA, MoaD-SH, and apoSO were exchanged into 100 mM Hepes, pH 7.5, using a PD-10 gel filtration column (GE Healthcare) and stored in aliquots at −80 °C until needed.

Purified Component Assay—Precursor Z was purified as described previously (23). Assays using purified components were performed aerobically in 100 mM Hepes, pH 7.5, at room temperature, and the concentrations below represent those in the final reaction volume after apoSO addition. Unless otherwise indicated, precursor Z (2 μM) was added to a mixture of MPT synthase (2 μM MoeA and 6 μM MoaD-SH), MoeA (20 μM), and Na₂MoO₄ (50 μM). After a specified time (tₙ) of generally 15 min, apoSO (2 μM) was added. Reactions were incubated for a further specified time (tₙ) of generally 20 min and then assayed for SO activity by quantitating the reduction of cytochrome c at 550 nm as described previously (24). To determine the extent of MPT synthesis during the prereaction step, MPT was converted to the fluorescent Form A (dephospho) derivative at various tₙ. For this conversion, 50 μl of 1% I₂/2% KI and 38 μl of 10% HCl were added to 400 μl of the prereaction mixture, and the samples were boiled for 20 min (25). The samples were brought to pH ~8.3 by the addition of 17 μl of 10% NaOH and incubated overnight in the presence of 15 mM MgCl₂ and 1 μl (1 unit) of calf intestine alkaline phosphatase (Roche Applied Science). Samples were passed through a 0.45-μm Spin X microcentrifuge filter (Corning) before being injected onto a C-18 HPLC column. Dephospho-Form A was quantitated using an Agilent 1100 Series fluorescence detector with an excitation wavelength of 295 nm and emission of 448 nm.

When assay components included MogA, ATP-Mg²⁺, tustgante, Na₂S, glutathione, or cysteine, these were added prior to precursor Z unless indicated. To determine the extent of MPT synthesis in the presence of cysteine, 400-μl reactions containing various concentrations of cysteine and final concentrations of 2 μM MoeA, 4 μM MoaD-SH, and 2 μM precursor Z in 100 mM Hepes, pH 7.5, were incubated for 20 min at room temperature prior to the addition of 200 μl of 1% I₂/2% KI and 38 μl of 10% HCl. MPT conversion to Form A was accomplished as described above.

To determine the extent of W incorporation, SO was purified from assays containing 20 μM MoeA and 5 μM tustgante (without molybdate). At tₙ = 20, these reactions were applied to 1 ml of nickel-nitrilotriacetic acid (Qiagen) columns equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, containing 10 mM imidazole. Columns were washed with 2 ml of equilibration buffer. Following elution with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, containing 200 mM imidazole, samples were exchanged into 10 mM Tris, pH 7.5, using a PD-10 gel filtration column and concentrated to less than 500 μl with a Centricon 30 filtration device (Amicon). Proteins were analyzed for W content using inductively coupled plasma emission mass spectrometry (ICP-MS) performed by Dr. Mark Rudnicki at the Nicholas School of Environment and Earth Sciences, Division of Earth and Ocean Sciences, Duke University. SO purified from similar reactions devoid of precursor Z was used for background subtraction.

Moco Transfer to ApoSO—The transfer of Moco to apoSO was monitored using a modification of the method described by Leimkühler et al. (6). Treatment of MPT or Moco with iodoacetamide produces a non-fluorescent derivative termed carboxamidomethyl MPT (camMPT). However, under non-denaturing conditions, SO-bound Moco is inaccessible to iodoacetamide (6) and can be quantitated by conversion to Form A following iodoacetamide treatment of the sample. For this set of experiments, activation assays with a final volume of 100 μl were used. After tₙ, iodoacetamide was added to a final concentration of 250 mM in 200 μl, and the reaction mixture was incubated for 30 min at room temperature. The volume was increased to 800 μl by the addition of 600 μl of 100 mM Hepes, pH 7.5, and the remaining underivatized Moco and MPT were converted to dephospho-Form A as described above.

RESULTS

In Vitro Mo Ligation Using Purified Components—We have previously demonstrated that purified E. coli MoeA is able to reconstitute SO activity in a moeA cell lysate in the presence of Na₂MoO₄ (9). To better examine the process of Mo ligation, a fully defined assay for this step of Moco biosynthesis was developed utilizing purified E. coli proteins and precursor Z to make Moco, the formation of which was monitored by the activation of MPT-free recombinant human SO. In this assay, MPT was synthesized de novo from precursor Z by E. coli MoeA and MoaD-SH (the active, thiocarboxylated form of MoaD), which together constitute active MPT synthase (6, 26). In this two-stage assay, the first step was initiated by the addition of precursor Z to reaction mixtures containing MoeA, MoaD-SH, MoeA, and MoO₄²⁻. After a specified incubation time (designated t₁), apoSO was added to begin the second step, with the incubation time between this addition and the determination of SO activity being designated as t₂. As seen in Fig. 1A, low levels of nonspecific apoSO activation were observed in this defined assay system in the absence of MoeA. However, a much greater level of activation was achieved in the presence of 20 μM MoeA with ~10-fold higher activity at 50 μM MoO₄²⁻ and 5-fold higher activity at 500 μM MoO₄²⁻. SO activity continued to increase with increasing molybdate concentrations above 500 μM both in the presence and absence of MoeA (data not shown). As can be seen in Fig. 1B, when varying amounts of MoeA were added to the assay in the presence of 50 μM molybdate, maximal activation was typically achieved at a concentration of ~20 μM.

Reconstitution of SO activity in the defined assay was maximized using final concentrations of 2 μM precursor Z, 2 μM MoaE, 6 μM MoaD-SH, and 2 μM apoSO (Fig. 2A). Theoretically, because the conversion of one molecule of precursor Z to MPT requires two molecules of MoaD-SH to donate one sulfur each to form the MPT dithiolene, 2 μM precursor Z would only require 4 μM 100% active MoaD-SH for complete conversion to
MPT. However, an excess of MoaD-SH was used to assure complete MPT production. In the previously published examination of MoA-mediated Mo ligation in moeA− crude extracts, an optimal reaction temperature of 37 °C was identified (9). In contrast, Fig. 2B shows that despite an initial burst at 37 °C, Mo ligation in this fully defined assay system is more efficient at room temperature than at 37 °C when $t_1$ was greater than 5 min.

**Efficiency of In Vitro Mo Ligation**—Under standard conditions, when $t_2 = 20$ min, maximum SO reconstitution was achieved at a $t_1$ of −15 min as seen in Fig. 3A. To determine whether this lag time represented the time required for MPT synthesis, the time frame for MPT production in the preincubation reactions was examined by conversion of MPT to the stable, fluorescent derivative Form A at various $t_1$ times. As seen in Fig. 3A, MPT synthesis was complete in ~2 min. Therefore, the 15-min $t_1$ prereaction time must represent another aspect of Moco production, such as ligation of the Mo atom to MPT. To explore whether $t_1$ does indeed represent the time required for MoA-mediated Mo ligation to the newly formed MPT, apoSO activation was quantitated at various concentrations of molybdate with MoA either present during the prereaction or added with apoSO. Fig. 3B shows that activation was less efficient if MoA were absent during the prereaction, even when $t_2 = 40$ min. Further, the incubation time for the prereaction ($t_1$) greatly affected the maximum level of SO activity achievable. As seen in Fig. 3C, the extent of SO activation observed with $t_1 = 0$ in the presence of MoA was only slightly higher than that observed with $t_1 = 15$ min in the absence of MoA. In contrast, the rate (as opposed to the maximum level) of apoSO reconstitution was only mildly affected by $t_1$. When the half time for $t_2$ (or the time required to reach 50% of maximal activity following apoSO addition) was calculated for the data in Fig. 3C, a decrease of only 17.6–12.5 min was observed as $t_1$ increased from 0 to 15 min. These data indicate that for maximal reconstitution of apoSO, incubation of MoA with MPT and MoO$_4^{2−}$ is required for sufficient MoA-mediated Mo ligation to occur prior to apoSO addition.

As the prereaction step of the assay appeared to be required for adequate Mo ligation, it was thought that the incubation period following apoSO addition was primarily the time required for insertion of Moco after apoSO was added to the assay. To explore this possibility, experiments were designed to monitor the fate of Moco following apoSO addition. A previous report from this laboratory demonstrated that SO-bound MPT was protected from iodoacetamide inactivation (6). For the experiments described here, iodoacetamide was added at various times subsequent to apoSO addition to convert any accessible.
After 30 min, the Moco remaining was converted to Form A. Calculated using the equations generated for the best fit lines of the data. The results indicate that Mo ligation was strongly inhibited when tungstate was present during the prereaction. This is compared with only mild inhibition when tungstate was added with the apoSO and with a lack of significant inhibition when tungstate was added at $t_1 = 20$ min (followed by a further 20-min incubation prior to determination of SO activity). In the presence of 50 μM molybdate, inhibition of 50% of activation required $\approx 75$ times more tungstate (3.8 mM), demonstrating a higher specificity for molybdate. ICP-MS analysis of SO purified from assays containing 5 mM tungstate (in the absence of molybdate) showed that W was incorporated into MPT/Moco bound MPT/Moco in the sample was then assayed by conversion to Form A. As seen in Fig. 4, with a $t_{1/2}$ of $\approx 3$ min, the rate for pterin insertion appears to be much faster than the observed rate for apoSO reconstitution ($t_{1/2} = 12.5$ min). These data indicate that the observed rate for apoSO activation reflects more than just the rate for Moco insertion into apoSO.

**Activation in the Presence of Tungsten and Sulfur Compounds**—Organisms that thrive in extreme environments typically utilize W in place of Mo (27), and there are multiple reports detailing the purification and analysis of W-substituted molybdenum enzymes, including SO (14, 28–32). Thus, it was of interest to examine whether W would be ligated to MPT under the conditions described here. The results from these experiments, shown in Fig. 5A, indicate that Mo ligation was strongly inhibited when tungstate was present during the prereaction. This is compared with only mild inhibition when tungstate was added with the apoSO and with a lack of significant inhibition when tungstate was added at $t_1 = 20$ min (followed by a further 20-min incubation prior to determination of SO activity). In the presence of 50 μM molybdate, inhibition of 50% of activation required $\approx 75$ times more tungstate (3.8 mM), demonstrating a higher specificity for molybdate. ICP-MS analysis of SO purified from assays containing 5 mM tungstate (in the absence of molybdate) showed that W was incorporated into $\approx 23\%$ of the total SO. These data provide further evidence that the majority of Mo ligation occurs during the prereaction, as inhibition was much more severe if tungstate was present during this step. Once ligated to MPT, significant replacement of Mo with W did not appear to occur.

FIG. 4. Pterin insertion during incubation following apoSO addition. Extent of pterin transfer to apoSO (○) compared with reconstitution of apoSO activity (△) versus $t_1$. Activation assays contained 20 μM MoeA and 50 μM Na$_2$MoO$_4$. For Moco transfer experiments, iodoacetamide was added at various $t_1$ times to a concentration of 250 mM. After 30 min, the Moco remaining was converted to Form A. $t_{1/2}$ were calculated using the equations generated for the best fit lines of the data.

**Effects of tungstate and thiol reagents on apoSO activation.** A: extent of apoSO activation versus tungstate in the presence of 20 μM MoeA and 50 μM molybdate. Tungstate was either present during the prereaction (△), added concomitantly with apoSO (○), or added at $t_1 = 20$ min with a further 20 min incubation (▲). Data sets were normalized to the maximum specific activity obtained in that set. B, extent of apoSO activation versus Na$_2$S (○), glutathione (△), or L-cysteine (▲) in the presence of 20 μM MoeA and 50 μM Na$_2$MoO$_4$ and versus L-cysteine in the presence of 1 mM Na$_2$MoO$_4$ and the absence of MoeA (◇). In all cases, Na$_2$S, glutathione, and cysteine were present during the prereaction. Each data set has been normalized to the specific activity obtained for that set in the absence of thiol. Activity due to nonspecific reduction of cytochrome c was subtracted prior to normalizing. For all experiments, $t_1 = 15$ min (except for cysteine where $t_1$ was 20 min) and $t_2 = 20$ min unless otherwise indicated. The concentration required to reach 50% of maximum activation was calculated using the equations generated for the best-fit line of the data.

The observations by Hasona et al. (16) that the moeA$^-$ phenotype could be suppressed in the presence of sulfide led to the hypothesis that MoeA might catalyze the formation of a thiomolybdate compound. A later report indicated that MoeA-mediated Mo ligation might require the presence of either cysteine or glutathione (17). Based upon these observations, experiments to analyze the effects of thiol reagents on the defined assay were performed. Fig. 5B shows the effects of various concentrations of Na$_2$S, cysteine, or glutathione on apoSO activation. All three thiol reagents were found to inhibit apoSO activation, though at different concentrations. In the presence of 20 μM MoeA and 50 μM molybdate, 50% inhibition of activation was achieved at $\approx 1.3$ mM Na$_2$S (650-fold excess versus [precursor Z]), 26-fold versus [MoO$_4^{2−}$]) or 0.4 mM glutathione (200-fold excess versus [precursor Z], 8-fold versus [MoO$_4^{2−}$]). Cysteine was found to be inhibitory at much lower concentrations, beginning at $\approx 1$ μM. Because it inhibited at such low concentrations compared with sulfide and glutathione, further experiments were performed with cysteine. Interestingly, the presence of cysteine also inhibited nonspecific apoSO reconstitution (Fig. 5B), indicating that the observed inhibition is not a direct inhibition of MoeA. Additional experiments showed that MPT synthesis, assayed by conversion to dephospho-Form A, was unaffected by the presence of cysteine (data not shown). These data indicate that the presence of these sulfur compounds nonspecifically inhibits either Mo ligation to MPT or Moco insertion into apoSO. The results from these experiments indicate that thiomolybdate formation is not part of MoeA function.

**Effects of MogA on in Vitro Mo Ligation**—Previous studies from our laboratory found that although MogA was required for in vivo Mo ligation in E. coli, it was not required for similar activity in vitro. Thus, the role of this protein in Moco formation remained unclear (9). To address this, the effect of MogA on the purified component assay was examined. Our initial
studies, shown in Fig. 6A, found that the addition of MogA had no enhancing effect and, in fact, strongly inhibited activation of apoSO. In the presence of 20 μM MoeA and 50 μM molybdate, apoSO activation was reduced 50% at −1.2 μM MogA, a concentration comparable to half of the theoretical maximum concentration of MPT produced in the reactions (2 μM from 2 μM precursor Z). MogA demonstrated similar inhibition of the non-specific (MoeA-independent) activation. It is likely that these results reflect an ability of MogA to tightly bind and sequester the cofactor as described previously for its eukaryotic homologues (33, 34). Consistent with this hypothesis, experiments with iodoacetamide treatment showed a significant drop in the amount of cofactor incorporated into apoSO with increasing concentrations of MogA (Fig. 6A).

Recent reports have suggested that ATP may be involved in the as yet undetermined in vivo activity of MogA (18, 19). To explore this possibility, the effect of the presence of ATP on the MogA inhibition of apoSO reconstitution in the defined assay was examined. Although the presence of ATP-Mg2+ alone had no significant effect on activity in the absence of MogA, the addition of MogA along with 1 mM ATP and 1 mM MgCl2 greatly increased the level of SO activity beyond that observed with the standard assay (Fig. 6B). This effect reached its maximum at 2 μM MogA, where the level of apoSO activation was nearly twice that obtained in the absence of MogA. The increase in apoSO reconstitution was only observed in the presence of MoeA because ATP-Mg2+ had no effect on MogA inhibition of non-specific apoSO reconstitution (Fig. 6B). The stimulatory effect of MogA and ATP on apoSO reconstitution was also dependent upon the presence of Mg2+ as seen in Fig. 6C. Further experiments showed that the nonhydrolyzable ATP analog γ-S-ATP could not substitute for ATP (data not shown), indicating that MogA function involves ATP hydrolysis. Despite the absolute increase in SO activity generated by the addition of MogA with ATP and Mg2+, their presence had no discernable effect upon the rate of increase in apoSO activation observed with increasing concentrations of MoeA (Fig. 6D).

**DISCUSSION**

Our laboratory previously demonstrated that de novo synthesized MPT could be converted to active Moco in the presence of high concentrations of molybdate. Formation of Moco was much less efficient, however, at lower Mo concentrations likely to be present under physiological conditions (6). Though there are no available estimates of intracellular Mo concentrations, the association of molybdate with the ModE repressor protein has a reported K_d of 0.8 μM, indicating that under normal conditions intracellular Mo concentrations are likely to be in the low μM range (35). The results presented in this report describe the development of a fully defined in vitro assay for monitoring the MoeA-mediated ligation of Mo to de novo synthesized MPT and demonstrate that MoeA enhances the rate and extent of Moco formation at low molybdate levels.

Tungsten ligation to MPT appeared to be much less efficient than ligation of Mo. As tungsten-dependent organisms are anaerobic and usually hyperthermophilic (15), it has been proposed that W is utilized in nature to catalyze reactions under extreme conditions (14). It is possible that anaerobic, and potentially higher temperature, conditions favor W rather than Mo attachment to MPT. It would be interesting to explore
whether W would be incorporated more efficiently than Mo in *in vitro* metal ligation assays using the Moco biosynthesis homologues from some of the extremophiles that utilize W. Additionally, in all tungstenoyl enzymes studied to date, W is bound by either a bis-MPT or bis(MPT-dinucleotide) form of the cofactor where the metal is coordinated by the dithiolenes of two MPT molecules (14, 15). Therefore, it is entirely possible that the single MPT-W form of Moco assayed here may not be as stable as MPT-Mo.

Based upon the results presented here, it seems highly unlikely that *in vivo* Mo ligation involves a thiomolybdylate species as has been previously proposed (16). The thiol reagents examined here were all either inhibitory or relatively ineffectual at concentrations up to 10-fold the concentration of molybdate (50 \( \mu M \)) in the assays, and none was required for activity. Additionally, thiomolybdylate formation would likely require the utilization of a cysteine desulforase (36), and there is no evidence indicating the involvement of such a protein in Mo ligation or Moco insertion. The observed inhibition implies that thiols compete with the MPT dithiolene for binding molybdate. It is unclear why cysteine inhibits at such low concentrations compared with sulfide and glutathione. Because cysteine is the side chain ligand to Mo in the SO family of Moco enzymes (15), supplemental cysteine may interfere with this ligation. With both cysteine and glutathione, lower concentrations inhibited SO reconstitution, whereas SO activity began to increase at higher concentrations (>1 mM) (data not shown). This observation helps explain why Sandu et al. (17) saw a stimulatory effect in assays containing 5 mM cysteine or glutathione as this is well past the concentration where the above effect was observed. Higher concentrations of cysteine or glutathione may stabilize Moco or nonspecifically (as MoeA was not required for this effect) aid in Moco formation.

Although Mo ligation to MPT can occur in the absence of either MoeA or MogA, this non-specific reaction requires molybdate concentrations so high as to be physiologically irrelevant. Therefore, the most probable function of the MoeA and MogA proteins is to collectively facilitate efficient metal ligation to the newly formed MPT at the low molybdate concentrations likely encountered in the cell.

The results presented here demonstrate that the MoeA protein facilitates the ligation of the Mo atom to MPT in the absence of ATP or MogA and that the presence of MogA enhances this reaction in an ATP-dependent manner. Future experiments are directed at utilizing this assay system for the functional analysis of mutations in MoeA in order to gain a better understanding of its role in mediating metal ligation to MPT.

**Acknowledgments**—We thank Dr. Mark Rudnicki at the Nicholas School of Environment and Earth Sciences, Division of Earth and Ocean Sciences at Duke University for performing the ICP-MS analysis. We thank Susan Stager for help in protein purification and Dr. Margot Wuebbens and Dr. Heather Wilson for critical reading of the manuscript.

**REFERENCES**

1. Wuebbens, M. M., and Rajagopalan, K. V. (1995) *J. Biol. Chem.* 270, 1082–1087
2. Reider, C., Eisenreich, W., O’Brien, J., Richter, G., Goets, E., Boyle, P., Blanchard, S., Bacher, A., and Simon, H. (1998) *Eur. J. Biochem.* 255, 24–36
3. Hanzelmann, P., Hernandez, H. L., Menzel, C., Garcia-Serres, R., Huyhn, B. H., Johnson, M. K., Mendel, R. B., and Schindelin, H. (2004) *J. Biol. Chem.* 279, 34741–34748
4. Hanzelmann, P., and Schindelin, H. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 12670–12675
5. Pitterle, D. M., and Rajagopalan, K. V. (1999) *J. Bacteriol.* 171, 3372–3378
6. Leimkuhler, S., and Rajagopalan, K. V. (2001) *J. Biol. Chem.* 276, 1837–1844
7. Leimkuhler, S., Wuebbens, M., and Rajagopalan, K. V. (2001) *J. Biol. Chem.* 276, 24085–24091
8. Leimkuhler, S., and Rajagopalan, K. V. (2001) *J. Biol. Chem.* 276, 22024–22031
9. Nichols, J., and Rajagopalan, K. V. (2002) *J. Biol. Chem.* 277, 24995–25000
10. Gronnow, A. M., and Shammugam, K. T. (1997) *Arch. Microbiol.* 168, 345–354
11. Johnson, J. L., Indermaur, L. W., and Rajagopalan, K. V. (1993) *J. Biol. Chem.* 266, 12140–12145
12. Temple, C. A., and Rajagopalan, K. V. (2000) *J. Biol. Chem.* 275, 40202–40210
13. Pitterle, D. M., Johnson, J. L., and Rajagopalan, K. V. (1993) *J. Biol. Chem.* 268, 13506–13509
14. Johnson, M., Rees, D., and Adams, M. (1996) *Chem. Rev.* 96, 2817–2839
15. Hille, R. (2002) *Trends Biochem. Sci.* 27, 360–367
16. Hasona, A., Ray, R. M., and Shammugam, K. T. (1998) *J. Bacteriol.* 180, 1466–1472
17. Sandu, C., and Brandtsch, R. (2002) *Arch. Microbiol.* 178, 465–470
18. Kuper, J., Llamas, A., Hecht, H. J., Mendel, R. B., and Schwarz, G. (2004) *Nature* 430, 803–806
19. Llamas, A., Mendel, R. B., and Schwarz, G. (2004) *J. Biol. Chem.* 280, 77348–77351
20. Wuebbens, M. M., and Rajagopalan, K. V. (1995) *Eur. J. Biochem.* 230, 606–610
21. Liu, M. T. W., Wuebbens, M. M., and Schindelin, H. (2000) *FEBS Lett.* 503, 168–172
22. Pitterle, D. M., and Rajagopalan, K. V. (1993) *J. Biol. Chem.* 268, 13493–13496
23. Garrett, R. M., and Rajagopalan, K. V. (1996) *J. Biol. Chem.* 271, 7387–7391
24. Johnson, J. L., and Rajagopalan, K. V. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 6856–6860
25. Pitterle, D. M., and Rajagopalan, K. V. (1993) *J. Biol. Chem.* 268, 13493–13505
26. Pilato, R., and Siegel, E. (1999) in *Bioinorganic Catalysis* (Reedijk, J., and Bouwman, R., eds), 2nd Ed, pp. 81–152, Dekker, New York
27. Hagedorn, P. L., Hagen, W. R., Stewart, L. J., Docrat, A., Bailey, S., and Garner, C. D. (2003) *FEBS Lett.* 555, 606–610
28. Jones, H. P., Johnson, J. L., and Rajagopalan, K. V. (1977) *J. Biol. Chem.* 252, 4988–4993
29. Temple, C. A., Graf, T. N., and Rajagopalan, K. V. (2000) *Arch. Biochem. Biophys.* 383, 261–267
30. Schmitz, R. A., Albracht, S. P., and Thauer, R. K. (1992) *FEBS Lett.* 309, 78–81
31. Bue, J., Santini, C. L., Giordani, R., Czajek, M., Wu, L. F., and Giordano, G. (1999) *Trends Biochem. Sci.* 24, 156–160
32. Schmitz, R. A., Albracht, S. P., and Thauer, R. K. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 7788–7792
33. Anderson, L. A., Palmer, T., Price, N. C., Bornemann, S., Boxer, D. H., and Pau, R. N. (1997) *Eur. J. Biochem.* 246, 119–126
34. Mihara, H., and Esaaki, N. (2002) *Appl. Microbiol. Biotechnol.* 60, 12–23

*Purified Component Assay for Molybdenum Ligation*
