In Vitro Incorporation of Nascent Molybdenum Cofactor into Human Sulfite Oxidase*

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Silke Leimkühler and K. V. Rajagopalan‡
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

We were able to reconstitute molybdopterin (MPT)-free sulfite oxidase in vitro with the molybdenum cofactor (Moco) synthesized de novo from precursor Z and molybdate. MPT-free human sulfite oxidase apoprotein was obtained by heterologous expression in an Escherichia coli mutant with a defect in the early steps of MPT biosynthesis. In vitro reconstitution of the purified apoprotein was achieved using an incubation mixture containing purified precursor Z, purified MPT synthase, and sodium molybdate. In vitro synthesized MPT generated from precursor Z by MPT synthase remains bound to the synthase. Surprisingly, MPT synthase was found capable of donating bound MPT to MPT-free sulfite oxidase. MPT was not released from MPT synthase when either bovine serum albumin or Moco-containing sulfite oxidase was used in place of aposulfite oxidase. After the inclusion of sodium molybdate in the reconstitution mixture, active sulfite oxidase was obtained, revealing that in vitro MPT synthase and aposulfite oxidase are sufficient for the insertion of MPT into sulfite oxidase and the conversion of MPT into Moco in the presence of high concentrations of molybdate. The conversion of MPT into Moco by molybdate chelation apparently occurs concomitantly with the insertion of MPT into sulfite oxidase.

Sulfite oxidase belongs to the family of molybdenum cofactor (Moco)1-containing enzymes characterized by the presence of a mononuclear molybdenum coordinated to the unique diholene group of a pterin derivative named molybdopterin (MPT). The overall reaction catalyzed by most Moco-containing enzymes, including sulfite oxidase, involves the transfer of an oxygen atom between the substrate and water in a two-electron oxidation-reduction reaction. Sulfite oxidase is ubiquitous among animals and catalyzes the physiologically vital oxidation of sulfur-containing amino acids cysteine and methionine. The enzyme is a homodimer located in the mitochondrial intermembrane space. Each 52-kDa subunit of the human enzyme contains a small N-terminal heme domain and a large C-terminal Moco-binding domain (1). Genetic deficiency of sulfite oxidase, which can be caused by either a mutation in its structural gene (isolated sulfite oxidase deficiency) or a defect in the synthesis of Moco (molybdenum cofactor deficiency), results in neurological abnormalities and often leads to death at an early age (2).

The basic structure of Moco has been shown to be identical in all organisms, and its biosynthetic pathway seems to be conserved because genes encoding highly homologous proteins for Moco biosynthesis have been found in archaea, bacteria, higher plants, Drosophila, and higher animals including humans. Biosynthesis of Moco has been studied most extensively in Escherichia coli, and several genetic loci (moe, mob, mod, moe, and mog) have been implicated in the pleiotrophy of the molybdenum enzymes, most of them being involved in the biosynthesis of Moco (3). The reactions of the Moco biosynthetic pathway comprise three stages that are identical in all organisms using molybdoenzymes: (i) conversion of a guanine nucleotide into the meta-stable precursor Z, (ii) conversion of precursor Z into MPT, and (iii) insertion of molybdenum into MPT, thus forming Moco. However, in most bacteria an additional stage in the biosynthetic pathway involves further modification of Moco leading to the formation of dinucleotide variants of MPT containing GMP, CMP, AMP, or IMP (4). Conversion of precursor Z to MPT requires the opening of a cyclic phosphate to produce a terminal monoester as well as the transfer of sulfur to generate the diholene group essential for molybdenum ligation (5). Pitterle et al. (6) demonstrated that incubation of purified precursor Z with MPT synthase resulted in the formation of MPT. MPT synthase forms a heterotetramer composed of two small MoaD subunits (8.5 kDa each) and two large MoaE subunits (16.8 kDa each). In the activated form of MPT synthase, the C terminus of the small subunit is converted to a glycine thiocarboxylate that acts as the sulfur donor for the conversion of precursor Z to MPT. In turn, MPT synthase is resulfurated by the MoeB protein designated as MPT synthase sulfurase. The involvement of proteins analogous to MoaD and MoeB in pathways leading to the biosynthesis of thiamine and ferredoxin led to the proposal that those pathways also operate by the thiocarboxylate mechanism (3). In the case of the ThiS protein, mass spectrometric evidence for a C-terminal glycine thiocarboxylate has been presented (7). Based on the observation that high concentrations of molybdate in the growth medium can partially rescue a mogA mutant, the MogA protein has been suggested to catalyze the in vivo insertion of molybdenum into MPT (8).

Free Moco is extremely unstable (9) and thus cannot be used in studies on the reconstitution of Moco-free proteins. In this paper, we describe an in vitro system for the incorporation of nascent Moco into Moco-free human sulfite oxidase expressed and purified from an E. coli strain with a mutation in the early steps of Moco biosynthesis. We show that MPT, produced in vitro from precursor Z by MPT synthase and stabilized by tight
binding to the synthase, can be incorporated into apoprotein oxidase in the absence of molybdate and without the requirement of any other proteins. We were also able to reconstitute sulfite oxidase activity by the inclusion of molybdate in the *in vitro* system. These results reveal that no other proteins are required for the *in vitro* insertion of MPT into sulfite oxidase or for the conversion of MPT into Moco under the conditions used in the reconstitution mixture.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—*E. coli* chlAI, chlN, and chlM strains, now designated *moaA*, *moeB*, and *moaD*, are isogenic mutants that were described previously (10). *E. coli* BL21(DE3) cells were obtained from Novagen. The ΔDE3 lysogenization kit from Novagen was used to integrate the gene for T7 RNA polymerase into the chromosome of the *E. coli* moeB strain.

**Gel Electrophoresis**—Analytical polyacrylamide gel electrophoresis was carried out in a discontinuous gel system (11) using 4–20% nondenaturing gradient polyacrylamide gels (Bio-Rad). Gel filtration molecular mass standards were obtained from Bio-Rad.

**Purification of the Reaction Components**—Precursor Z was isolated from *E. coli* mooa− cells using high-performance liquid chromatography (HPLC) with reverse phase and anion exchange columns (12). Cloned MPT synthase was expressed in a pET15b vector (Novagen) in *E. coli* BL21(DE3) and moeB−(DE3) cells, and the protein was purified by ammonium sulfate precipitation and gel filtration according to the procedure described by Rudolph et al. (13). The human sulfite oxidase gene was cloned into a pTrec-His vector resulting in an N-terminal fusion to a 6xHis-tag, expressed in *E. coli* mooa− cells and purified by Ni-NTA chromatography (14). For comparison, non-His-tagged human sulfite oxidase was used, which was expressed and purified from *E. coli* as described by Temple et al. (14).

**Enzyme Assays**—Sulfite oxidase activity was assayed by the method described by Pitterle et al. (6) using purified precursor Z in 10 mM sodium citrate buffer, pH 3, that was adjusted to pH 7.2 with NaOH, and MPT synthase in 100 mM Tris, pH 7.2. One unit of sulfite oxidase activity is defined as an absorbance change (ΔA) of 1/min.

**In Vitro Reconstitution of Sulfite Oxidase Activity**—MPT was produced in vitro by the method described by Pitterle et al. (6) using purified precursor Z in 10 mM sodium citrate buffer, pH 3, that was adjusted to pH 7.2 with NaOH, and MPT synthase in 100 mM Tris, pH 7.2. For a standard reconstitution assay, 30 nmol of precursor Z and 100 μg of MPT synthase were allowed to react at room temperature in the presence or absence of 20 mM sodium molybdate. After 15 min, 12 μg of sulfite oxidase in 100 mM Tris, pH 7.2, was added, and the reaction mixture, in a total volume of 80 μl, was incubated at room temperature for 30 min before aliquots were taken and either analyzed for MPT content or assayed in appropriate dilutions for sulfite oxidase activity.

**MPT Analysis by Generation of Form A (Dephospho)**—To analyze the amount of MPT present in sulfite oxidase or MPT synthase, the purified protein was subjected to a procedure that converts MPT to the stable oxidized fluorescent degradation product form A by treatment of the protein at pH 2.5 in the presence of iodine at room temperature, as originally described by Johnson et al. (17). After treatment with alkaline phosphatase, dephospho form A was identified by HPLC analysis at room temperature at a flow rate of 1 ml/min with an Alltech C18 HPLC column equilibrated in 50 mM ammonium acetate, 10% methanol. Form A was assayed by monitoring its fluorescence with an excitation at 297 nm and emission at 440 nm. All HPLC analyses were performed using the Hewlett Packard 1090 solvent delivery system, and eluting material was monitored either for absorbance using a Hewlett Packard 1040A diode array detector or for fluorescence using a Hewlett Packard 1046 fluorescence detector.

**RESULTS**

**Purification of Recombinant Human Sulfite Oxidase from an *E. coli* mooa− Mutant**—To obtain human sulfite oxidase without added Moco, the enzyme was expressed heterologously in an *E. coli* chlAI purif strain. This strain carries a point mutation in the *mooa* gene, which is required for the first step of Moco biosynthesis, namely the conversion of a guanine nucleotide into precursor Z. The enzyme was expressed as an N-terminal fusion to a His-tag and purified by Ni-NTA chromatography as described by Temple et al. (14). The absorption spectrum of the purified inactive protein showed the typical spectrum of a cytochrome b, representing the N-terminal part of the protein (data not shown). Native polyacrylamide gel electrophoresis revealed that the inactive enzyme, without the molybdenum cofactor bound to its C-terminal part, retained its ability to form a homodimer with a molecular mass of ~110 kDa (Fig. 1).

**In Vitro Synthesis of MPT from Precursor Z Using Active MPT Synthase**—To synthesize MPT in vitro, purified precursor Z and purified MPT synthase were incubated at room temperature as described by Pitterle et al. (6). Purified precursor Z was obtained from cells that contain a mutation in *moaD* and thus accumulate the precursor (12). Active MPT synthase was purified after expression in BL21 cells (13). Inactive recombinant MPT synthase was purified from a moeB− strain deficient in MPT synthase sulfurylase activity (5). In agreement with the results obtained by Pitterle et al. (6), MPT formed by incubation of active MPT synthase with precursor Z remains bound to MPT synthase (Fig. 2). For these experiments, active or inactive MPT synthase was incubated with precursor Z at room temperature and dialyzed afterward to remove excess precursor Z. Subsequent heat treatment in the presence of acidic iodine released form A, the oxidized fluorescent degradation product of MPT. HPLC analysis revealed that form A could be obtained from active MPT synthase (Fig. 2A), whereas no form A was obtained from inactive MPT synthase (Fig. 2B). The fluorescence peak eluting at 3.8 min from the C18 reverse phase HPLC column after iodine treatment of inactive MPT synthase was identified as compound Z, the oxidized product of precursor Z (data not shown). Therefore, MPT remains tightly bound to active MPT synthase after dialysis, whereas inactive MPT synthase binds precursor Z tightly but is unable to convert it to MPT because of the absence of the glycine thiocarbamate at the C-terminus of the small MoaD subunit.

**In Vitro Reconstitution of Human Sulfite Oxidase**—*E. coli* MPT synthase was previously shown capable of donating bound MPT to the inactive apoprotein reductase present in *Neurospora crassa nit-I* extracts (6). To test whether MPT synthase is also capable of donating MPT to human sulfite oxidase, purified apoprotein oxidase was incubated with active MPT synthase and precursor Z under aerobic conditions. MPT synthase and precursor Z were incubated for 15 min at room temperature prior to the addition of apoprotein oxidase. Sulfite oxidase activity was analyzed after a 30-min incubation of the enzyme with the mixture of MPT synthase/precursor Z. Somewhat unexpectedly, the inclusion of sodium molybdate in the *in vitro* incubation mixture prior to the addition of sulfite oxidase gave rise to sulfite oxidase activity (Table I). The specific ac-
tivity of reconstituted sulfite oxidase was about 50% that of native sulfite oxidase (Table I). In contrast, incubation of apo-sulfite oxidase with MPT synthase and precursor Z without the addition of molybdate or with inactive MPT synthase and precursor Z did not reconstitute sulfite oxidase activity.

To determine whether the maximum of 50% reconstitution of sulfite oxidase activity was due to limited incorporation of MPT into the enzyme or limited molybdate insertion into MPT, an analysis of the amount of cofactor present in reconstituted sulfite oxidase was carried out. Purified His-tagged apoprotein was added to the reconstitution mixtures in the presence or absence of sodium molybdate. After incubation as described above, sulfite oxidase was isolated by Ni-NTA chromatography and dialyzed against 100 mM Tris, pH 7.2. His-tagged sulfite oxidase was purified from the reactivation mixtures by Ni-NTA chromatography and dialyzed against 100 mM Tris, pH 7.2. MPT or Moco was converted into form A as described previously.

**Table I**

Activity of native sulfite oxidase or in vitro reconstituted apoprotein oxidase using cytochrome c or ferricyanide as electron acceptors

| Treatment | Sulfite oxidase activity | Cytochrome c<sup>a</sup> | Fe(CN)<sub>6</sub><sup>a</sup> | units/mg |
|-----------|--------------------------|-------------------------|------------------------|---------|
| Native sulfite oxidase | None | 1062 | 103 | |
| Aposulfite oxidase | None | —<sup>b</sup> | —<sup>b</sup> | |
| Aposulfite oxidase | + Inactive MPT synthase | —<sup>b</sup> | —<sup>b</sup> | |
| Aposulfite oxidase | + Precursor Z | —<sup>b</sup> | —<sup>b</sup> | |
| Aposulfite oxidase | + Active MPT synthase | 552 | 49 | |
| Aposulfite oxidase | + Precursor Z | — | — | |

<sup>a</sup> Sulfite oxidase activity was assayed at room temperature by monitoring the reduction of cytochrome c at 550 nm or ferricyanide at 420 nm. One unit of sulfite oxidase activity is defined under “Experimental Procedures.” For the reactivation of 12 μg of apoprotein oxidase, 100 μg of MPT synthase, 30 nmol of precursor Z or 20 mM sodium molybdate were added. Appropriate aliquots of the reactivation mixtures were assayed for sulfite oxidase activity. Mean values were calculated from at least three independent measurements.

<sup>b</sup> —, amount was below the limit of detection.

**Fig. 2.** Analysis of fluorescent derivatives obtained from active and inactive MPT synthase after incubation with precursor Z. HPLC elution profiles of form A isolated from 300 μg of active MPT synthase incubated with precursor Z (A) or 300 μg of inactive MPT synthase incubated with precursor Z (B). Incubation mixtures contained 1.5 mg of active/inactive MPT synthase and 300 nmol of precursor Z. After a 30-min incubation, MPT synthase was desalted over a PD10 column equilibrated in 100 mM Tris, pH 7.2, and bound MPT was then converted into form A as described under “Experimental Procedures.”

**Fig. 3.** Analysis of fluorescent derivatives of the MPT cofactor from reconstituted sulfite oxidase. HPLC elution profiles of form A isolated from 82 μg of native sulfite oxidase (A), 82 μg of apoprotein oxidase incubated with inactive MPT synthase and precursor Z (B), 82 μg of apoprotein oxidase incubated with active MPT synthase, precursor Z, and 30 mM sodium molybdate (D). Incubation mixtures originally contained 360 μg of sulfite oxidase, 1.5 mg of MPT synthase, and 300 nmol of precursor Z. All incubations were carried out as described under “Experimental Procedures” at room temperature in a total volume of 1 ml in 100 mM Tris, pH 7.2. His-tagged sulfite oxidase was purified from the reactivation mixtures by Ni-NTA chromatography and dialyzed against 100 mM Tris, pH 7.2. MPT or Moco was converted into form A as described previously.
could be obtained from aposulfite oxidase treated with inactive MPT synthase and precursor Z (Fig. 3B). Because the enzyme activity obtained from reconstituted sulfite oxidase correlated with the amount of cofactor present in the enzyme, it can be concluded that the less than maximal reconstitution of activity is due to the correspondingly lesser incorporation of MPT and that the molybdenum content of the reconstituted enzyme corresponds to the MPT content.

**Time-dependent Reconstitution of Sulfite Oxidase Activity**—To determine the effect of incubation time on the reconstitution of sulfite oxidase with Moco, a time course experiment was carried out. Sulfite oxidase was added under aerobic conditions to a mixture of MPT synthase, precursor Z, and sodium molybdate, which were preincubated for 15 min at room temperature. Aliquots of the aerobic incubation mixture were assayed at specific intervals for sulfite oxidase activity. Following the addition of sulfite oxidase (Fig. 4), maximum sulfite oxidase activity was achieved 10 min after the addition of sulfite oxidase to the *in vitro* system. Longer incubation times failed to yield a higher than 50% reconstitution of enzyme activity compared with native sulfite oxidase (Table I). Because Moco is known to be very sensitive to aerobic oxidation, the effect of anaerobiosis on the *in vitro* reconstitution assays was examined. Surprisingly, generation of MPT and insertion of Moco into sulfite oxidase under anaerobic conditions did not increase the extent of sulfite oxidase reconstitution (data not shown). Under anaerobic as well as aerobic conditions, maximum activation of sulfite oxidase was achieved after an incubation time of 10 min and corresponded to reconstitution of about 50–60% of the enzyme molecules.

**Effect of the Concentration of Precursor Z, MPT Synthase, Molybdate, and Sulfite Oxidase on the *in Vitro* Reconstitution of Sulfite Oxidase with Moco**—To determine whether any components of the *in vitro* activation of sulfite oxidase were limiting for obtaining complete reconstitution of sulfite oxidase, the dependence of activation on the concentrations of the reactants was investigated. In general, the reactivation mixtures consisted of 100 μg of MPT synthase, 30 nmol of precursor Z, 12 μg of aposulfite oxidase, and 20 mM sodium molybdate. When the sodium molybdate concentration was varied over a range of 0–50 mM, maximal sulfite oxidase activity was attained at a concentration of 5 mM (Fig. 5A). When precursor Z concentration was varied from 0 to 50 nmol, a maximum of sulfite oxidase activity was obtained at a concentration of 6–8 nmol (Fig. 5B). When MPT synthase concentration was varied over a range of 0–250 μg, maximum reconstitution of sulfite oxidase was achieved at a concentration of ~50 μg of the enzyme (Fig. 5C). Collectively, for the reconstitution of 12 μg of sulfite oxidase present in the *in vitro* activation system, none of the components were limiting under the standard conditions. As shown in Fig. 5D, sulfite oxidase concentrations greater than 50 μg/ reactivation mixture were required to produce a decrease in specific activity. Because this is approximately 4 times the amount of sulfite oxidase used in the standard reconstitution assay, it can be concluded that all components required for the *in vitro* synthesis of Moco are present in optimum amounts in
the in vitro assay and that the extent of reconstitution cannot be increased with higher concentrations of any of the components of the reconstitution mixture.

**Analysis of the Mechanism of MPT Incorporation into Sulfite Oxidase**—With the knowledge that MPT can be inserted into sulfite oxidase in vitro, it was of further interest to study the mechanism of insertion of MPT and molybdate into apoprotein sulfite oxidase. To determine whether molybdenum is incorporated into MPT at a nonprotein bound stage before the insertion of MPT into sulfite oxidase, the stability of MPT produced by MPT synthase over time after the addition of sodium molybdate was investigated. MPT synthase, precursor Z, and sodium molybdate were preincubated under aerobic conditions for 15–75 min before the addition of sulfite oxidase. After sulfite oxidase addition, the activation mixtures in 100 mM Tris, pH 7.2, were incubated for 30 min at room temperature before sulfite oxidase activity was determined.

Tagged sulfite oxidase. The flow-through from the Ni-NTA column, containing MPT synthase, was collected, dialyzed, and concentrated. Form A was generated from equal amounts of MPT synthase after incubation with His-tagged MPT-containing sulfite oxidase (A) and 112 μg of MPT synthase after incubation with His-tagged MPT-free sulfite oxidase (B). Incubation mixtures contained 300 μg of MPT synthase, 150 nmol of precursor Z, 2 mM sodium molybdate, and 1 mg of sulfite oxidase. After a 30-min incubation, the reactivation mixture was dialyzed against 50 mM NaPi and 300 mM NaCl, pH 8.0, and applied to a Ni-NTA column to bind His-tagged sulfite oxidase. The flow-through from the column was collected and dialyzed against 100 mM Tris, pH 7.2. MPT synthase was concentrated to 280 μg/ml and then treated with acidic iodine to convert MPT to form A.

**Time-dependent Molybdenum and MPT Incorporation into Sulfite Oxidase**—The data presented above demonstrate that MPT exists only in a protein-bound stage and is transferred specifically to MPT-free sulfite oxidase. To determine whether molybdenum is inserted into MPT bound to MPT synthase before being transferred to sulfite oxidase, precursor Z, MPT synthase, and sodium molybdate were incubated for 15 min at room temperature, and MPT synthase was purified afterward over a gel filtration column to remove free molybdate. Sulfite oxidase was added to the gel-filtered MPT synthase, and the ability of MPT synthase to reconstitute sulfite oxidase activity without the addition of extra molybdate was analyzed. No sulfite oxidase activity was reconstituted under these conditions. However, inclusion of molybdate in the reconstitution mixture did produce the expected level of activity (data not shown). From these data it can be concluded that MPT synthase is unable to bind molybdate or convert MPT to Motoc.

The ability to reconstitute demolybdo-sulfite oxidase with molybdate in vitro was already observed by Jones et al. (15), who reported that molybdate could reconstitute a maximum of 30% of the demolybdo-sulfite oxidase isolated from tungsten-treated rats. To examine the conditions for molybdenum insertion into sulfite oxidase-bound MPT, MPT synthase was prein...
cubated with precursor Z for 15 min at room temperature, and molybdate was added either before or at different time points after the addition of sulfite oxidase. After molybdate was added, the reactivation mixtures were incubated for 30 min at room temperature. Aliquots were then removed and assayed for sulfite oxidase activity. As shown in Fig. 8, the ability to reconstitute sulfite oxidase activity decreased steadily with increasing time before molybdate addition to the reconstitution mixture. The extent of reconstitution of sulfite oxidase activity reached its minimum at 10 min and did not decrease further. These data show that optimal reconstitution of sulfite oxidase is achieved when molybdate is added to the reconstitution mixture either at the same time or prior to the addition of sulfite oxidase. It may also be concluded that molybdate is inserted into MPT as soon as the latter is bound to sulfite oxidase, but perhaps because a conformational change of sulfite oxidase after the insertion of MPT, a less than maximal reconstitution with molybdate is achieved when its addition is delayed from time of MPT transfer.

After showing that the molybdenum atom is inserted into MPT concomitant with or prior to MPT insertion into sulfite oxidase, it was of further interest to analyze whether time courses of cofactor handoff from MPT synthase to sulfite oxidase differ in the presence and absence of molybdate in the reconstitution mixture. For this purpose, MPT synthase and precursor Z were preincubated for 15 min at room temperature, and after the addition of sulfite oxidase to the reconstitution mixture, the transfer of MPT from MPT synthase to sulfite oxidase was inhibited at different time points by the addition of 200 mM iodoacetamide. After a further 30-min interval, MPT or Moco was converted into form A as described under “Experimental Procedures.” Each point demonstrates the relative fluorescence of form A obtained from the peak area after HPLC analysis.

These findings are in agreement with the data obtained for the time-dependent reconstitution of sulfite oxidase activity shown in Fig. 4. It can therefore be concluded that molybdate does not affect the kinetics of MPT handoff from MPT synthase to MPT-free sulfite oxidase.

**DISCUSSION**

In most reconstitution studies on cofactor-containing proteins, the standard procedure is to obtain the apoprotein by a resolution procedure to remove the cofactor and use a source of the cofactor to reconstitute the apoprotein. In the case of sulfite oxidase, this approach is not possible because of the size of the protein, the presence of two prosthetic groups, and the extreme instability of released Moco.

To obtain Moco-free sulfite oxidase, the cloned gene of human sulfite oxidase was expressed in the Moco deficient *moxA* mutant of *E. coli* as a His-tagged protein. The purified apoprotein contained stoichiometric amounts of heme, showing that the absence of Moco does not prevent heme binding to the N-terminal domain of sulfite oxidase. The data presented in Fig. 1 also showed that the apoprotein is a dimer just like native sulfite oxidase. The x-ray structure of the highly homologous chicken sulfite oxidase revealed that the sulfite oxidase monomer actually folds into three domains, the N-terminal heme domain, the central domain containing Moco, and the C-terminal domain entirely responsible for the dimeric structure of sulfite oxidase (18). The fact that the apoprotein of human sulfite oxidase is a dimer demonstrates that, despite the absence of Moco in the central domain, the C-terminal domain folds in the correct manner to generate the dimer interface. The data presented in this paper have shown that the central domain of the apoprotein is sufficiently unfolded to be able to bind MPT and form Moco in *vitro*. These findings support the conclusion that each of the three domains of sulfite oxidase can attain its folded structure independently of the other two domains.
As for a source of the extremely unstable cofactor, the data presented here demonstrate that Moco can be synthesized in vitro by ligation of molybdate to MPT generated de novo from precursor Z and MPT synthase. This was a somewhat surprising observation because genetic and biochemical studies in E. coli have implied the involvement of the MogA and MoeA gene products in cofactor biosynthesis. It has been proposed that molybdenum, which enters the cell as the stable oxyanion molybdate by a high affinity molybdate transport system (19), undergoes some type of modification prior to incorporation into MPT within the cell. For example, the role of the MoeA protein in generating a thiomolybdenum species that might be used in Moco biosynthesis has been suggested (20). Our results show that in vitro, molybdate can be inserted into MPT and reconstitute sulfite oxidase activity without further modification by any other proteins. So far, E. coli MogA has been proposed to act as a molybdochelatase incorporating molybdenum into MPT in vivo (21), an observation based on the finding that high concentrations of molybdate can partially rescue a moga mutant. Although MogA binds MPT tightly and a putative binding pocket for MPT has been identified in the crystal structure of E. coli MogA, binding of molybdate could not be demonstrated for MogA to date (22, 23). Because the MogA protein is not required for in vitro insertion of molybdate into MPT, its function in the cell still remains unclear. It can only be speculated that MogA might act as an MPT carrier or storage protein because MogA has a high affinity for MPT or that MogA might be required for the insertion of molybdate into MPT under low molybdate concentrations, a function not required in vitro with concentrations of sodium molybdate of 5 mM and higher.

The data presented above indicate that the in vitro reconstitution of apoprotein oxidase proceeds as shown in Fig. 10. As already reported by Pitterle et al. (6), MPT synthase is required for the conversion of precursor Z to MPT, a reaction that can be carried out in vitro with the purified components. MPT produced in vitro by active MPT synthase remains bound to the system, whereas inactive MPT synthase, lacking the thioacaboxylate at the terminal glycine of the small subunit, is unable to convert precursor Z to MPT but does bind precursor Z tightly. Because MPT synthase was shown to be incapable of binding molybdate or forming Moco, MPT must be released from MPT synthase for the conversion of MPT into Moco. Free MPT has previously been shown to be highly sensitive to aerobic oxidation. Because all reactions for the successful in vitro reconstitution of apoprotein oxidase were performed under aerobic conditions, it would appear that MPT is transferred directly to MPT-free sulfite oxidase so that MPT stays protein bound and is therefore protected from aerobic oxidation. In our system, 50% reconstitution of sulfite oxidase was achieved when molybdate was added to the in vitro reconstitution mixture prior to or at the same time as the addition of sulfite oxidase. After the insertion of MPT into sulfite oxidase, the ability to reconstitute sulfite oxidase activity with added molybdate decreased with time. This might indicate that after MPT is incorporated into sulfite oxidase, the structure of the enzyme changes such that conditions are no longer optimal for insertion of molybdate into sulfite oxidase. Because molybdate is very similar to sulfate, it is possible that molybdate can be bound to the substrate-binding site present in sulfite oxidase, (18) which enables the insertion of molybdate into MPT even after a structural change of the enzyme. Even though we could show that the cofactor handoff from MPT synthase to sulfite oxidase is a specific reaction in which the cofactor is released from MPT synthase only after incubation with MPT-free sulfite oxidase, it still remains unclear whether this reaction will occur in the same manner in vivo. In the cell, it is likely that molybdate is inserted into MPT before its incorporation into sulfite oxidase, a reaction that might be carried out by the MogA protein. We could show that MPT synthase is also able to pass MPT to the MogA protein (data not shown) so that in vivo a possible pathway for cofactor handoff might be that MPT synthase interacts with MogA, the molybdenum atom is inserted into MogA-bound MPT, and the molybdenum-containing cofactor is inserted into sulfite oxidase by the MogA protein.

Overall, the maximum activation of sulfite oxidase obtained in our in vitro system corresponds to reconstitution of about 50% of the enzyme molecules with Moco. It is likely that the isolated sulfite oxidase contains improperly folded molecules and only about 50% of enzyme molecules can be reconstituted. This finding is in agreement with the observation that apoprotein oxidase is extremely unstable, and maximum reconstitution of apoprotein oxidase was achieved only with freshly prepared enzyme.

In summary, the work presented above describes for the first time the in vitro reconstitution of apoprotein oxidase with MPT and molybdate. Sulfite oxidase deficiency in humans, which can be caused by either a mutation in the gene encoding sulfite oxidase alone or a mutation in one of the genes involved in Moco biosynthesis, results in neurological abnormalities and often leads to death at an early age. It is an autosomal recessive disease and no therapy is known to date. The in vitro reconstitution of sulfite oxidase, with Moco yielding in an active enzyme, might present a starting point for the reconstitution of sulfite oxidase activity in patients with mutations in the biosynthetic pathway of Moco.

A search of completed and incomplete genomes shows that the Moco biosynthetic pathway has widespread phylogenetic existence and is present even in pathogens such as Helicobacter pylori, Vibrio cholerae, Mycobacterium tuberculosis, and several others. The roles of molybdooenzymes in these pathogens are unknown, but one possibility is that the ability of molybdooenzymes to hydroxylate or dehydroxylate certain compounds enables these pathogens to detoxify such compounds. Indeed, recently Kozmin et al. (24) have shown that the extreme sensitivity of the E. coli mutant Δ(uvrB-bio) to 6-hydroxylaminopurine and other base analogs is due to mutations in the Moco biosynthetic pathway. This finding raises the possibility...
that inhibition of the Moco biosynthetic pathway could provide an avenue for developing bacteriocidal compounds to counter the effects of infection by bacteria including pathogens. The reaction catalyzed by MPT synthase, i.e. the conversion of precursor Z to MPT, is the best defined step in the Moco biosynthetic pathway and, as demonstrated here, can be accomplished in vitro. The present studies have enabled the coupling of the reaction catalyzed by MPT synthase to the activation of aposulfite oxidase, providing an extremely sensitive assay for the activity of MPT synthase. In the future, this procedure should prove valuable for assessing the potential pharmacological usefulness of putative inhibitors of MPT synthase.

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Silke Leimkühler and K. V. Rajagopalan

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