Biochemical and Structural Analysis of the Molybdenum Cofactor Biosynthesis Protein MobA*

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Molybdopterin guanine dinucleotide (MGD) is the form of the molybdenum cofactor that is required for the activity of most bacterial molybdoenzymes. MGD is synthesized from molybdopterin (MPT) and GTP in a reaction catalyzed by the MobA protein. Here we report that wild type MobA can be copurified along with bound MPT and MGD, demonstrating a tight binding of both its substrate and product. To study structure-function relationships, we have constructed a number of site-specific mutations of the most highly conserved amino acid residues of the MobA protein family. Variant MobA proteins were characterized for their ability to support the synthesis of active molybdenum enzymes, to bind MPT and MGD, to interact with the molybdenum cofactor biosynthesis proteins MobB and MoeA. They were also characterized by x-ray structural analysis. Our results suggest an essential role for glycine 15 of MobA, either for GTP binding and/or catalysis, and an involvement of glycine 82 in the stabilization of the product-bound form of the enzyme. Surprisingly, the individual and double substitution of asparagines 180 and 182 to aspartate did not affect MPT binding, catalysis, and product stabilization.

The transition metal molybdenum is an essential element for most living organisms. It is required for the activities of a range of enzymes that catalyze two electron redox reactions (1). Molybdenum in these enzymes is always found coordinated to an organic cofactor, which in its most simple form is molybdopterin (MPT). The biosynthesis of MPT is by an evolutionarily conserved multistep pathway. The molecular structures of most of the proteins involved in MPT synthesis have now been determined, and the biochemistry of its assembly and insertion into molybdoenzymes is an area of intense study (2–7).

The biosynthesis of the molybdenum cofactor (Moco) can be divided into three stages. The first step is the rearrangement of a guanine nucleotide to form precursor Z (8). The second stage is the introduction of two sulfur atoms into the pyran ring of precursor Z to give MPT (9–11). The third step is the chelation of molybdenum and the formation of active Moco. Whereas in eukaryotes this step is catalyzed by two domain proteins (Cnx1 in plants, gephyrin in mammals), in bacteria the separate MogA and MoeA proteins have been implicated in this latter step (5, 6, 12, 13). Unique to prokaryotes, a further modification of the basic MPT structure by attachment of a mononucleotide to the terminal phosphate of MPT occurs (14). This modification is essential for the activity of most, but not all, bacterial Moco-containing enzymes. In Escherichia coli, most of the molybdoenzymes require the GMP-modified form of molybdopterin, molybdopterin guanine dinucleotide (MGD) for their activity, although enzymes from other bacteria that utilize molybdopterin cytosine dinucleotide have also been reported (14, 15). In E. coli, the GMP attachment step is catalyzed by the cellular protein MobA (16). Mutants in mobA fail to synthesize MGD and accumulate elevated quantities of MPT (14). The E. coli mobA gene is cotranscribed with a further gene, mobB, encoding a nucleotide-binding protein, which is not absolutely required for MGD synthesis (17, 18).

Recent work has suggested that in vivo, molybdenum cofactor biosynthesis most probably occurs on protein complexes rather than by the separate action of the biosynthetic enzymes (19). In particular it seems that molybdenum insertion and nucleotide attachment to form MGD in E. coli are intimately linked. Using a two-hybrid approach, it has been shown that the MobA protein can interact with the MoeA and MobB proteins. Furthermore, the interaction of MobA with MoeA is only observed in cells that can synthesize MPT-molybdenum, suggesting that a protein-bound form of MPT-molybdenum is delivered to MobA for GMP attachment (19).

Recently, we and others (20, 21) have reported the crystal structure of MobA. Consistent with its known function, the protein shares striking homology with sugar-nucleotide phosphotransferases. The MobA enzyme, which is active as a monomer, has an overall α/β architecture where the N-terminal half of the molecule adopts a Rossman fold. Soaking with high concentrations of GTP and divalent metal ions has revealed that this N-terminal region contains the GTP binding site (21). Using a fully defined in vitro system, it has been demonstrated...
that MobA alone, when incubated with GTP, Mg²⁺ and a source of MPT catalyzes the formation of MGD, indicating that it is both necessary and sufficient for GMP attachment (22). In this study we have sought to further explore the biochemical properties of MobA. We show that the wild type protein, as purified, contains bound MPT and MGD in the ratio of 1:3. Based on sequence comparisons and the high resolution x-ray structure, we have constructed a number of site-directed variants of the *E. coli* MobA protein. Our biochemical and structural analyses indicate an essential role for glycine 15 in the conversion of MPT to MGD and of glycine 82 in stabilizing the product-bound form of the enzyme. Inactive variant forms of MobA do not lose the ability to interact with MobB and MoeA in the two-hybrid system, indicating that the area of interaction is distinct from the catalytic site.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals used were from the highest grade available. Xanthine oxidase (EC 1.1.3.22) from buttermilk grade I was obtained from Sigma. Nickel-nitrilotriacetic acid superflow matrix was prepared after centrifugation (26), was loaded onto a 1 ml of nickel-(MgO) and lysed by sonication. The crude cell extract, which was stored at 4°C, was applied immediately onto a 10-ml PD10 desalting column that had been previously pre-equilibrated with 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 10% (v/v) glycerol. The resultant protein was determined after room temperature oxidation with acid residues are completely conserved across the MobA family of proteins. Many of these highly conserved residues fall in the putative substrate binding pocket of the *E. coli* MobA protein that has been identified previously (20, 21). To probe the functions of these residues, we have constructed a number of site-directed mutations in the *mobA* gene (Fig. 1). Conservation of residues Arg, Lys, Asp, and Asn was studied (25). Lys and Asn were substituted by leucine, and the two invariant asparagines (Asn and Asn) were replaced singly and in combination by aspartate.

To test the effect of the mutations on the ability of the cell to synthesize active molybdendum cofactor in *vivo*, we measured the specific nitrate reductase activity of the *mobAB* mutant with plasmin encoding the histidine-tagged wild type or variant proteins. As shown in Fig. 2, only two mutations led to a complete loss of MobA function. Substitution of glycine 15 for leucine (G15L), or aspartate 101 for alanine (D101A), totally abolished MobA activity. However, further analysis of the D101A mutation indicated that most of the protein was present in the cells in the form of inclusion bodies, which could account for the lack of activity. Therefore we made a further substitution of aspartate 101 for asparagine (D101N). The *mob* mutant expressing D101N showed reduced, but still detectable, nitrate reductase activity. Of the other point mutations, the *mob* mutant expressing the K25A-substituted protein repeatedly showed lower nitrate reductase activity. The other mutations gave essentially similar levels of active nitrate reductase as the wild type protein.

**Copurification of Molybdendnum Cofactor Forms with His-tagged MobA**—We next wanted to study individual activities of the purified proteins to understand which of the functional properties of MobA were affected by the mutations. As a consequence of the reaction catalyzed by MobA it should interact with its substrates (MPT, GTP) as well as with the reaction prod-
Biochemical and Structural Analysis of MobA

Table I

Summary of x-ray data and model parameters for MobA mutants

<table>
<thead>
<tr>
<th>Variant</th>
<th>R19A</th>
<th>G22L</th>
<th>D101N</th>
<th>N180D</th>
<th>N182D</th>
</tr>
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<tbody>
<tr>
<td>Data collection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beaml ine</td>
<td>PX9.5</td>
<td>PX9.5</td>
<td>PX9.6</td>
<td>PX9.5</td>
<td>PX9.6</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.20</td>
<td>1.00</td>
<td>0.87</td>
<td>1.20</td>
<td>0.87</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.65</td>
<td>1.75</td>
<td>1.65</td>
<td>1.65</td>
<td>2.00</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>21329</td>
<td>18116</td>
<td>21570</td>
<td>21218</td>
<td>12166</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.4 (83.8)</td>
<td>98.6 (96.7)</td>
<td>99.8 (99.9)</td>
<td>98.7 (88.0)</td>
<td>98.1 (86.9)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.4</td>
<td>4.9</td>
<td>3.6</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>0.034 (0.141)</td>
<td>0.068 (0.234)</td>
<td>0.060 (0.186)</td>
<td>0.062 (0.266)</td>
<td>0.059 (0.163)</td>
</tr>
<tr>
<td>(I)/σ(I)</td>
<td>36.9 (6.2)</td>
<td>21.1 (3.8)</td>
<td>19.0 (6.2)</td>
<td>20.9 (2.9)</td>
<td>17.4 (4.4)</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>0.061</td>
<td>0.125</td>
<td>0.171</td>
<td>0.133</td>
<td>0.117</td>
</tr>
</tbody>
</table>

Refinement

- **Rmerge** (based on 95% of data; %) = 17.6 17.5 18.5 17.9 17.1
- **Rmerge** (based on 5% of data; %) = 21.1 21.9 22.5 21.7 22.6
- **DPF** (based on **R**merge; Å) = 0.101 0.121 0.107 0.104 0.173
- **Residues with most favored φ/ψ (%)** = 88.8 89.4 88.1 90.0 88.1
- **r.m.s.d. bond distances (Å)** = 0.019 0.021 0.020 0.020 0.020
- **r.m.s.d. angles (°)** = 1.851 1.913 1.898 1.879 1.851
- **Average temperature factors (Å²)**
  - Main-chain atoms = 14.6 |
  - Side-chain atoms = 16.1 |
  - Waters = 27.2 |
  - Overall = 16.3 |
- **r.m.s.d. vs. wild type structure (Å)** = 0.089 0.472 0.225 0.199 0.186
- **PDB accession code** = 1H4C 1H4D 1H4E 1HJJ 1HJL

Notes:
- The figures in parentheses indicate the values for outer resolution shell.
- **R**merge = Σ i |Ii - <I>/Σ j |Ij|, where Ii is the intensity of an observation of reflection j and <I> is the average intensity for reflection j.
- **R**free = Σ |Fcalc - Fobs|/Σ |Fcalc|, the mean fractional isomorphous change between the wild type structure factors (Fcalc) and the variant structure factors (Fobs).
- The R-factors **R**merge and **R**free are calculated as follows: R = Σ |Fcalc - Fobs|/Σ |Fcalc| × 100, where Fcalc and Fobs are the observed and calculated structure factor amplitudes, respectively.
- Diffraction-component precision index (39), an estimate of the overall coordinate errors calculated in REFMAC (35).
- As calculated using PROCHECK (38).
- After least squares superposition based on main chain atoms.

Fig. 1. Alignment of MobA amino acid sequences from bacteria. The abbreviations used are: Eco, E. coli (17); Rsp, Rhodobacter sphaeroides (41); Ppu, Pseudomonas putida (GenBank™/EBI accession number AJ2429522); Bsu, Bacillus subtilis (GenBank™/EBI accession number AAC24900). Conserved residues that were subjected to mutagenesis in this study are indicated by asterisks.underline(s) under the sequence.

uct (MGD). We have shown previously that the G-domain of the MPT-binding protein Cnx1 (12), as purified from E. coli, contains bound MPT (13). We therefore reasoned that the purified wild type MobA protein may also contain one or more forms of bound molybdenum cofactor. Consistent with this, we were able to demonstrate that the histidine-tagged wild type protein could be copurified with up to 50 pmol of molybdenum cofactor/mg of protein (1.1 mmol Moco/mol of MobA). When the analysis was repeated under conditions of mild oxidation (that prevents cleavage of the phosphodiester bond), it was apparent that most of the bound molybdenum cofactor was in the form of the reaction product, MGD (Fig. 3B). We typically observed a ratio of MPT:MGD of 1:3 for the wild type protein.

We next expressed and purified the variant MobA proteins.

All of the His-tagged variant proteins (apart from the D101A variant) were expressed to a similar level and were isolated to elevated levels of total Moco.

When comparing the ratios of bound MPT:MGD shown in Fig. 3B, several of the substituted proteins had similar ratios to the wild type protein. These included the R19A, R156A, N180D,
N182D, and N180,182D (marked as NNDD in Fig. 3B) amino acid replacements. These results are consistent with our earlier observations that expression of these variant forms of MobA gave wild type levels of nitrate reductase activity (Fig. 2). However, a number of the substituted proteins showed differing ratios of bound substrate and product than the wild type protein. The G22L substitution routinely showed an increase in the ratio of bound MGD relative to wild type (Fig 3B). This observation suggests that the G22L form of the protein may have an increased affinity for the reaction product. The K25A, G78L, and D101N versions of MobA led to a notable increase in the ratio of bound MPT relative to MGD. Taken together with the observation that both the K25A and D101N replacements resulted in a marked decrease in the level of nitrate reductase activity (Fig. 2), this indicates that the altered MPT:MGD ratio is of functional significance.

G15L was the only modified form of MobA that showed a complete absence of nitrate reductase activity, consistent with an inability to synthesize MGD. However, the G15L variant of MobA, as purified, contained a relatively high amount of bound MPT. This may be due to the fact that there are higher levels of MPT in the cell when MobA is inactive (14), which probably accounts for the relatively high amount of MPT bound. This supports the idea that the defect in the G15L variant is not at the level of MPT binding, but must instead be at the level of binding of the other substrate (GTP) or at the catalytic step. The leucine side chain probably partially occludes the guanine binding pocket or alternatively disrupts the local structure sufficiently to prevent GTP binding. To ascertain whether this MobA variant was capable of interacting with the reaction product, we expressed the plasmid-encoded His-tagged G15L MobA protein in a strain carrying the wild type chromosomal copy of mobA. As shown in Table II, under these circumstances, the G15L protein could also interact with MGD. It is notable that this variant only binds half the wild type level of MGD. This is presumably due to a lower affinity of the variant MobA protein for the product resulting from occlusion or disruption of the guanine binding pocket.

An unexpected result was obtained with the MobA G82L variant. Despite repeated attempts, we were only able to detect MPT in the purified G82L protein (Fig. 3C). This observation is particularly surprising given the fact that when expressed in the mob mutant strain TP1000, the G82L variant of MobA was able to support a nitrate reductase activity level that was almost 60% of wild type, indicating that it was capable of synthesizing MGD. These observations imply that the binding of the reaction product, MGD, is destabilized as a result of the substitution.

**Table II**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amount of bound MPT</th>
<th>Amount of bound MGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP1000 pMobA wild type</td>
<td>19.4</td>
<td>59.5</td>
</tr>
<tr>
<td>TP1000 pMobA G15L</td>
<td>71.3</td>
<td>0</td>
</tr>
<tr>
<td>MC4100 pMobA G15L</td>
<td>17.8</td>
<td>30.4</td>
</tr>
</tbody>
</table>

Other Molybdenum Cofactor Biosynthetic Proteins—Using bacterial two-hybrid experiments it has previously been reported that *in vivo*, the MobA protein interacts with the MobB and MoeA proteins (19). It was therefore of interest to test whether the G15L form of MobA, which lacked catalytic activity, or the K25A and D101N variants, which showed reduced activity, were compromised for their ability to interact with other molybdenum cofactor biosynthetic proteins. As shown in Fig. 4, the G15L, K25A, and D101N forms of MobA were all capable of interacting with both the MoeA and MobB proteins, and these interactions were stronger than for the wild type MobA protein. We have also tested the G82L form of MobA, which we have demonstrated above to be destabilized in the binding of MGD. This form of MobA also showed enhanced interaction with MoeA and MobB proteins. These observations indicate that the catalytic site of MobA is distinct from the site(s) of protein-protein interaction.

**Structural Analysis of Amino Acid-substituted MobA Proteins**—All of the variant MobA proteins that were analyzed for Moco binding were also subjected to crystallization trials. Of these, five yielded crystals that were suitable for x-ray data collection. These were the R19A, G22L, D101N, N180D, and N182D substituted proteins, and their structures were subsequently determined to resolutions ranging from 1.65 to 2.00 Å. In all cases, significant structural changes were restricted to the vicinity of the site of substitution (Fig. 5). The largest changes were observed for the G22L variant, where the preceding five residues, which form the central part of the consensus loop, adopted a different conformation. A salt bridge, present in the wild type MobA structure between D101 and K25 was absent in the D101N variant, and both of these side chains had slightly different conformations. By contrast the R19A-substituted structure was essentially identical to that of the wild type protein. Furthermore, no significant changes were apparent for either the N180D or N182D variants, although the substituted side chains adopted slightly different configurations relative to the wild type. It is possible that those variants that did not yield crystals had significantly different structures to the wild type protein. However, this seems unlikely,
since with the exception of G15L, all were active, and even the latter was able to bind both MPT and MGD. It is more likely that subtle changes in surface charge and/or side chain conformations were sufficient to prevent crystal lattice formation under the wild type crystallization conditions.

**DISCUSSION**

In this study we have substituted 10 of the most highly conserved amino acid residues in the MobA protein family. Almost all of these conserved residues cluster around the proposed substrate binding pocket of MobA (20, 21). Residues Gly15, Arg19, and Gly22 are found in the consensus loop, most of which is poorly ordered in the MobA structure, which is proposed to wrap around the substrates and partially sequester them from solvent exposure during catalysis. This may be a mechanism whereby the enzyme prevents futile hydrolysis of GTP by water once it has bound in a catalytically favorable conformation. Arg19 has been proposed to play a crucial role in the catalytic mechanism of MobA by stabilizing the transition state. Our results do not support this contention. Substitution of Arg19 for alanine gave a MobA protein that was functionally active and that could bind MPT and MGD in the same ratio as the wild type protein. Structural analysis of this variant showed that it was virtually identical to the wild type protein. Likewise we were able to substitute glycine 22 for a bulky leucine side chain without a significant loss of MobA function. The x-ray structure of the G22L derivative of MobA showed that the substitution resulted in the loop adopting a slightly different conformation. Interestingly, we showed that this variant protein binds significantly more MGD than wild type. This might suggest that the loop is less flexible as a result of the G22L substitution, resulting in a locking in of the product. A third residue in the loop region is absolutely critical for function of MobA. Replacement of glycine 15 by leucine completely abolished the activity of MobA. Although we were unable to obtain a high resolution structure for this MobA derivative, the substitution has probably not grossly affected the fold of the enzyme, since we have shown that the protein not only interacts with MPT, but also retains the ability to interact with MobB and MoeA in vivo. Moreover, when the variant protein was overproduced in a background where the wild type chromosomal copy of mobA was present, the G15L form of MobA was also shown to bind MGD. It is most likely that this substitution affects the ability of the protein to bind the other substrate GTP; this region of the protein has been shown by crystal soaking experiments to be involved in stacking interactions with the guanine base (21). Additionally, glycine 78 is also involved in interactions with the bound GTP, and consistent with this, a MobA protein with a substitution of this residue to leucine bound significantly less MGD.

Residue Asp101 has been shown to coordinate the catalytic metal ion (Mn$^{2+}$ in the GTP-bound structure of Lake et al. (21), probably Mg$^{2+}$ under *in vivo* conditions). In the apo form of MobA, there is no bound metal ion and D101 forms a salt bridge with Lys25. This bond has been proposed to orient Asp101 for interaction with the metal (20). In the GTP-bound form, Lys25 contacts the β-phosphate of GTP (21). Substitution of Asp101 to alanine probably led to aberrant folding of the protein; the variant protein was certainly inactive and could only be recovered in the inclusion body fraction. A substitution of this residue for asparagine was well tolerated by the protein, although the x-ray structure revealed that the D101N form of MobA no longer formed a salt bridge with Lys25. The D101N variant showed significantly reduced MobA activity *in vivo*, and most of the bound Moco was in the form of MPT, consistent with an inability to effect efficient catalysis. A substitution of Lys25 for alanine also gave a significant reduction of MobA activity and a 2-fold decrease in the level of bound molybdenum cofactor, which was mainly due to a reduction of the bound MGD (Fig 3B).
Residues Asn^{180} and Asn^{182} have been postulated to form part of the MPT binding site, and to contact O4 and N5 of MPT (21). We constructed single aspartate substitutions at each of these residues as well as a doubly substituted protein. All three variant proteins were active and bound the same quantities of MPT and MGD as wild type MobA. The high resolution structures of the singly substituted proteins were essentially identical to wild type. Our experimental data suggest that these highly conserved residues are not strictly required for MPT binding, so that the position of MPT as extrapolated by Lake et al. (21) based on bound GTP might be different. This would imply a different conformation of MGD to that observed in bacterial Moco-containing enzymes, which might be possible due to the free rotation around the pyrophosphate bond between the nucleotide and molybdopterin.

One of the most surprising observations was for the G82L form of MobA. This variant was clearly capable of synthesizing MGD; however, we were not able to detect any MGD bound to bacterial Moco-containing enzymes, which might be possible due to the free rotation around the pyrophosphate bond between the nucleotide and molybdopterin.

We also acknowledge the assistance of J. Nicholson, M. Papiz, T. Clarke, and S. Davies for assistance during x-ray data collection experiments at the Synchrotron Radiation Source (Daresbury, UK).