Insight into the Role of Escherichia coli MobB in Molybdenum Cofactor Biosynthesis Based on the High Resolution Crystal Structure*

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Two proteins, which are co-transcribed in Escherichia coli (MobA and MobB), are involved in the attachment of a nucleotide moiety to the molybdenum cofactor to form active molybdopterin guanine dinucleotide. Although not essential for this process, the dimeric MobB increases the activation of molybdoenzymes, incorporating this cofactor by a mechanism that is not understood. The structure of MobB has been elucidated in two crystal forms, one of which has provided a model at 1.9-Å resolution with \( R_{\text{work}} \) and \( R_{\text{free}} \) values of 21.5 and 28.7%, respectively. The MobB subunit displays an \( \alpha/\beta \)-fold arranged into a major and minor domain, the latter of which is inserted between the major and minor domains of the partner subunit, creating an elongated dimer constructed around a 16-stranded \( \beta \)-sheet. Structural homologues have been identified, and they include a number of nucleotide-binding proteins. Comparisons indicate that although the phosphate-binding regions are highly conserved, MobB lacks the elements of structure required to interact with and efficiently bind a nucleotide base. In the present structure, a sulfate is bound to the Walker A phosphate-binding motif of MobB. The possibility that MobB forms a complex with the nucleotide-binding MobA, the protein with which it is co-transcribed, is explored, and modeling suggests that such a MobA-MobB complex is feasible. This hypothesis is supported by recent biochemical evidence indicating that MobB interacts with several proteins involved in various stages of molybdenum cofactor biosynthesis including MobA. We propose therefore that MobB is an adapter protein that acts in concert with MobA to achieve the efficient biosynthesis and utilization of molybdopterin guanine dinucleotide.

Molybdenum is an essential trace element associated with a diverse range of redox-active enzymes (1). Such molybdoenzymes catalyze basic metabolic reactions in the carbon, nitrogen, and sulfur cycles exploiting the redox chemistry of this second row transition metal. With the exception of the nitroge-

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The atomic coordinates and structure factors (code 1NP6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Selenium.
to as the phosphate binding or P-loop, or alternatively, the G₁ motif of bacterial GTPases (13) and is often predictive for nucleotide triphosphate binding, suggesting that the protein might be involved in the binding of the guanine nucleotide. MobB does display weak binding of GTP (K₁ of 2.0 μM) and low GTPase activity (Kₚ= 7.5 μM, turnover rate = 3 × 10⁻³ min⁻¹, see Ref. 14).

The crystal structures of MoaC (15), the MobB-MoaD complex (16), MoaA (17, 18), MogA (19), and MobA (20, 21) have been solved and, with the exception of MobA, all form oligomers. This, along with biochemical evidence showing that MobB and MoaD (22) and MobA, MobB, MogA, and MoaE (23) are involved in heterogeneous complex formation, indicates that protein-protein interactions are critical in molybdenum cofactor biosynthesis. The biological function of MobB is, unlike the other proteins discussed above, not clear. To investigate the role that MobB might contribute to Moco biosynthesis, we have determined the high resolution crystal structure of the E. coli protein.

MATERIALS AND METHODS

Cloning and Expression—The mobb gene from E. coli was amplified from genomic DNA by PCR. Two oligonucleotide primers were designed (5′-CTA-GCA-CAT-ATG-ATA-CCG-CCA-CTC-GGA-3′ and 5′-GCA-GCC-A-CTC-GAG-TCT-CTG-CTG-TTG-3′) to introduce unique restriction sites for NdeI and XhoI, respectively (shown in bold). The PCR product was cloned into the pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen). Positive clones were selected in E. coli BL21 (DE3) and transformed using the Blunt TOPO PCR cloning kit (Invitrogen). Positive clones were sequenced, and then following excision, the gene was ligated into the pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen). Positive clones were sequenced, and then following excision, the gene was ligated into expression vector pET15b (Novagen), which ultimately produces a protein carrying a hexahistidine tag at the N terminus. The new plasmid was heat-shock-transformed into E. coli BL21 (DE3) and selected on Luria-Bertani agar plates containing 100 μg/ml ampicillin (LB/ampicillin). Bacteria were cultured in LB/ampicillin media and grown at 37 °C to an optical density of 0.6 at 600 nm. Expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside, and the temperature was reduced to 20 °C. Cell growth continued for 16 h before cells were harvested by centrifugation (2500 × g) for 20 min at 4 °C. In addition to the expression of native MobB, selenomethionine (SeMet) MobB was expressed in E. coli B834 (DE3) cells. Cultivation of this variant was done in M9 minimal media supplemented with the usual amino acids except that SeMet (50 μg/ml) was substituted for l-methionine. Protein expression was induced, and cell growth was carried out in the same manner as for native MobB (above).

Purification—Once harvested, the cell pellets were resuspended in 50 mM Tris-Cl buffer, pH 7.5, and lysed by three passages through a French pressure cell at a pressure of 900 p.s.i. The insoluble cell fraction was separated by centrifugation (38,000 × g) for 20 min at 4 °C, and the supernatant was filtered before being applied to a Ni²⁺-resin HiTrap column (Amersham Biosciences) that had been equilibrated with 20 mM Tris-Cl buffer. Elution of the protein was achieved by running a linear gradient of 0–500 mM imidazole. Fractions were collected and analyzed by SDS-PAGE, and those containing MobB were pooled and dialyzed overnight against 50 mM Tris-HCl, pH 8.5. The N-terminal His₆ tag was cleaved with thrombin (10 units/mg) in 50 mM phosphate-buffered saline at 5 h at 20 °C. The sample was then loaded onto a Poros HP strong anion exchange column (Applied Biosystems). This column was equilibrated in 50 mM Tris-HCl, pH 9.0, and elution was achieved with a linear gradient of 0–500 mM NaCl in the same buffer. The fractions containing MobB were again pooled, analyzed, and dialyzed overnight into 50 mM Tris-HCl, pH 8.0, and then concentrated to 10 mg/ml for use in crystallization trials.

Crystallization—Two crystal forms of MobB were obtained using the hanging drop vapor diffusion method. Monoclinic blocks (0.25 mm maximum dimension) were occasionally grown from a 4-μl drop that consisted of 2 μl of protein solution containing 5 mM MgCl₂, 5 mM GDP, and 5 mM dithiothreitol as well as 2 μl of the reservoir solution comprising 150 mM (NH₄)₂SO₄, 10.5% (w/w) polyethylene glycol 4000, and 15% (w/v) glycerol. A hexagonal crystal form (rods of (0.15 × 0.15 × 0.5 mm³) proved more reproducible than the monoclinic form and was obtained using a reservoir of 100 mM NaAc and 2 μM (NH₄)₂SO₄ at pH 4.6.

Data Collection and Processing—A medium resolution multivariate anomalous dispersion data set was collected from the hexagonal crystal form of the SeMet protein at station ID29 at the European Synchrotron Radiation Facility using a CCD Area Detector Systems Corp. Q210 detector. These SeMet crystals belonged to the space group P6₁22 with unit cell dimensions a = 241.5, c = 49.2 Å. The asymmetric unit contains two monomers (subunits A and B) giving a combined molecular mass of ~39 kDa with 77% solvent content and a Matthews coefficient (24) Vₘ of 5.4 Å³/Da. High resolution diffraction data were collected from a native monomeric crystal on PX 9.6 at the Synchrotron Radiation Source, Daresbury Laboratory using a CCD ADSC Q4 detector. This crystal form has space group P2₁ with a = 92.4, b = 164 Å, c = 54.2 Å, and β = 97.7°. Here the solvent content for two molecules in the asymmetric unit was 48% (Vₘ of 2.4 Å³/Da). Prior to each data collection, crystals were transferred through a cryoprotectant consisting of 50% glycerol and 50% of the relevant reservoir solution before being flashed-cooled to −173 °C in a stream of nitrogen. The data were indexed, integrated, and scaled using either MOSFLM (25) or DENZO and SCALEPACK (26). Data manipulation was achieved using the CCP4 suite (27), and details are listed in Table 1.

### Table I

| P6₁22 | P6₁22 | P6₁22 | P2₁ native |
|-------|-------|-------|------------|
| λ irony | λ irony | λ irony | λ irony |
| Wavelength (Å) | 0.9790 | 0.9791 | 0.9150 | 0.87 |
| Resolution (Å) | 30–2.4 | 30–2.4 | 30–2.4 | 20–1.9 |
| No. of measurements | 670,964 | 671,626 | 713,380 | 364,026 |
| No. of unique reflections | 31,464 | 31,464 | 31,464 | 23,122 |
| Completeness (%) | 93.8 (92.8) | 93.8 (92.7) | 93.8 (99.8) | 99.3 (97.5) |
| I/σ(I) | 20.4 (3.7) | 25.1 (3.2) | 25.7 (4.1) | 18.6 (4.6) |
| R₁ [sym] (%) | 6.1 (27.6) | 5.5 (32.7) | 5.6 (36.6) | 4.2 (27.3) |
| R₁ [asym] (%) | 6.2 | 4.9 | 5.0 | 5.0 |
| R₁ [sym] | 3.7 | 5.2 | 5.2 | 5.2 |
| Wilson B (Å²) | 49.1 | 50.5 | 52.5 | 29.9 |

*λ irony* was used in the refinement of the P6₁22 data. Numbers in parentheses correspond to the statistics in the highest resolution shell.

R₁ [sym] = Σ|I – (I)|/ΣI, where I is the measured intensity and (I) is the average intensity summed over all symmetry equivalent reflections.

R₁ [asym] = Σ(I(+) – I(–))/Σ[I(+) + I(–)].

R₁ [sym] = ΣF₁ [ref] – ΣF₁ [obs]/ΣF₁ [ref], where F₁ [ref] is the reference structure factor from λ irony and F₁ [obs] is the structure factor of the derivative.
Crystal Structure of E. coli MobB

| Table II | Summary of refinement and model geometry statistics |
|----------|-----------------------------------------------------|
| Resolution (Å) | 29–1.9 Å |
| Protein residues | 327 |
| Protein atoms | 2557 |
| Water molecules | 202 |
| Sulfates | 2 |
| Rwork (%) | 21.5 |
| Rfree (%) | 28.7 |
| Average isotropic thermal parameters (Å²) | |
| Subunit A overall | 44.2 |
| Subunit B overall | 43.9 |
| All main chain | 41.4 |
| All side chains | 46.8 |
| Solvent molecules | 49.6 |
| r.m.s. bond lengths (Å) | 0.012 |
| r.m.s. bond angles (°) | 1.31 |
| Overall G-factor | 0.02 |
| Ramachandran analysisb | |
| % Favourable | 90.7 |
| % Additional | 9.3 |

* r.m.s., root mean square.

G-factor and Ramachandran analysis were determined by PROCHECK (35).

Structure Solution and Refinement—The initial structure of a MobB subunit was determined using the multiwavelength anomalous dispersion method of phase determination targeting the K-edge of selenium and using the hexagonal crystal form. MobB has five methionine residues per subunit, and a homodimer constitutes the asymmetric unit. The program SOLVE (28, 29) identified 10 selenium positions that provided phases to 2.4 Å with an overall figure-of-merit of 0.48. The 10 selenium positions corresponded to the location of 5 SeMet residues in one subunit and 4 in the other subunit with one SeMet side chain displaying a dual conformation. Density modification (without the use of non-crystallographic symmetry averaging) using RESOLVE (30) raised the figure-of-merit to 0.58. An initial round of model building, using O (31), provided only 165 out of a possible 348 residues, 118 of which were associated with a single subunit. One subunit was reasonably well defined in the experimental density, but the other was of poor quality. This model was used for molecular replacement with the P2₁ data set to utilize the higher resolution data. The program AMoRe (32) provided a clear molecular replacement solution for two subunits using data within the resolution limits of 15–3 Å, which, after rigid body refinement, gave an R-factor of 47.7% and correlation coefficient of 44.1%. Prior to refinement using REFMAC (33), 5% of the data were set aside for the calculation of the Rfree (34). Refinement was carried out with a bulk solvent correction but without the use of non-crystallographic symmetry restraints. Rigid body refinement of the dimer was used to extend the resolution to 1.9 Å, and several cycles of refinement, inter-dispersed with rounds of model building, solvent, and sulfate identification, produced a model with an Rwork of 21.4% and Rfree of 28.7%. For completeness, this model was used to initiate refinement with the hexagonal data by superimposing the dimer onto the well defined subunit described above. Rigid body refinement and several rounds of graphics inspection, rebuilding, and refinement gave an Rwork of 31.1% and an Rfree of 33.8%. PROCHECK (35) was used to assess the geometry of models during the analysis, and refinement statistics are presented for the monoclinic crystal form in Table II.

RESULTS AND DISCUSSION

The Structure of MobB—Initial phases to 2.4 Å were obtained using the multiwavelength anomalous dispersion method for phase determination on the hexagonal crystal form. These crystals diffracted only to medium resolution and were poorly ordered but nevertheless enabled us to construct the first model of MobB. This model proved suitable for molecular replacement into and refinement with the high resolution native monoclinic data. Although the hexagonal crystal structure was subsequently refined, the data and resulting model are inferior to those derived from the monoclinic form, and consequently, our discussion concentrates on the 1.9-Å resolution structure.

MobB consists of 175 residues with an approximate molecular mass of 19.4 kDa. The refined model comprises residues 6–45 and 58–174 for subunit A and residues 6–175 for subunit B; this accounts for 327 out of a possible 350 residues for this homodimer. Secondary structure elements were assigned by a combination of automated methods, using PROMOTIF (36), and visual inspection. They are mapped onto the amino acid sequence in Fig. 2A.

The MobB subunit exhibits a mixed a/b-fold with an overall topology of eight β-strands and seven α-helices (Figs. 2 and 3) and is divided into two domains. The major domain is constructed with a core six-stranded twisted parallel β-sheet with strand order 8-7-6-1-5-2, two helices on one side of the sheet (α1 and α7), three shorter helical sections on the other side (α3, α4, α6) with a single turn of helix (α5) at the C-terminal edge of the sheet. The minor domain consists of a distorted α2, then antiparallel strands β3 and β4. We note that α2 is only ordered in one of the two subunits that comprise the asymmetric unit (Fig. 4).

The symmetric dimer of MobB is formed by the insertion of a minor domain from one subunit between the major and minor domains of the other. Strands β3 and β4 from one subunit are positioned between β2 and β4 of the partner and arranged such that although β2 is parallel to β3, the β4 strands are antiparallel to each other (Figs. 2B and 3). This arrangement produces a dimer, with overall dimensions of ~90 × 38 × 26 Å³, dominated by an elongated β-sheet composed of 16 strands. The sheet is flat in the central section of the dimer and twisted at either end (Fig. 3). The non-crystallographic symmetry 2-fold axis is perpendicular to the central section of the extended β-sheet.

Although GDP was present in the crystallization conditions, we note that there was no electron density corresponding to the putative ligand in either crystal form. However, sulfate ions were identified binding at the Walker A motif that lies between β1 and α1. The oxyanion binding is likely influenced by the helix macrodipole of α1 (Fig. 3) (37). Further details of the sulfate-binding site will follow.

Structural Homologues—Sequence homology among GTP-binding proteins (G-proteins) is well characterized (38), and the GDP-binding site is typically described by three highly conserved sequence motifs (39). The first, the Walker A motif (13), occurs at residues 14–21 (Fig. 2A). This phosphate-binding region adopts a well conserved structure in G-proteins, consisting of a β-strand and α-helix with a loop between them (39). The helix dipole interacts favorably with the nucleotide phosphates. The conformation of the region surrounding the Walker A motif and the position of the sulfate ion in MobB follow the general trends displayed for phosphate binding by G-proteins. The second motif, KxG, is linked to a conformational switch in G-proteins dependent on whether GDP or GTP is present. Although E. coli MobB carries the sequence DxG close to the phosphate-binding loop at residues 51–54, we note that such a motif is absent in MobB homologues. The third motif, which is predicted to determine the specificity of an enzyme for guanine (NXXD), is not present in MobB.

In addition to the conservation of three motifs, G-proteins share a common core structure of five α-helices and a six-stranded β-sheet. The β-sheet has five parallel strands and one antiparallel strand that, unusually for α-β-proteins, is placed at the edge of this sheet. Since MobB displays some of the basic structural elements of G-proteins, a search for structural homologues was considered necessary to investigate the possible function of the protein.

An architectural comparison, using DALI (40) and DEJAVU (41), indicated that the MobB subunit is structurally similar to...
a number of purine nucleotide-binding binding proteins including a nitrogenase iron protein (NIP; Protein Data Bank accession code 1FP6; Z score = 8.3; see Ref. 42), the ATPase MinD (Protein Data Bank accession code 1G3Q; Z score = 8.1; see Ref. 43), and Solfolobus solfactorius MobB. Residues colored dark gray are strictly conserved in four of the sequence homologues, and those residues colored light gray have conserved properties in at least five homologues. Here the Walker A motif is boxed for clarity.  

**Fig. 2. Sequence and structure of MobB.** A, the amino acid sequence of *E. coli* MobB with the assigned secondary structure. Residues encased in black are strictly conserved in *Haemophilus influenzae*, *Rhodobacter sphaeroides*, *Methanobacterium thermoautotrophicum*, *Bacillus subtilis*, and *Solfolobus solfactorius* MobB. Residues colored dark gray are strictly conserved in four of the sequence homologues, and those residues colored light gray have conserved properties in at least five homologues. Here the Walker A motif is boxed for clarity. B, the topology of the MobB dimer prepared with TOPDRAW (47). Subunit A is shown in red and is missing α2. Subunit B is complete, and the structural elements of this subunit that aligned with three structural homologues are shown in dark blue, with the rest shown in blue. The position of the Walker A motif of subunit B is in green.
similarities to the Ras-related GTPases. They were aligned on the most complete subunit of MobB using LSQKAB (45), and secondary structure matching was carried out using the secondary structure matching server at the European Bioinformatics Institute (www.ebi.ac.uk/msd-srv/ssm/cgi-bin/ssmserver). The root mean squared fit was calculated from the $C_\alpha$ atoms of matched residues at the best superposition of the structures. The fractions of the $C_\alpha$ atoms in the MobB subunit that overlay on NIP, MinD, and FtsY are 71, 64, and 54% with root mean squared deviations of 3.2, 2.2, and 2.0 Å, respectively. The secondary structure elements of MobB found to align with all three structural homologues were $\beta_1$, $\alpha_1$, the P-loop, $\beta_2$, $\alpha_4$, $\beta_5$, $\beta_6$, $\beta_7$, $\beta_8$, and $\alpha_7$, which constitutes almost the entire major domain of MobB. Since the searches for structural homologues revealed homology only with the major domain, a further search was carried out using only the minor domains as organized in the dimer, but no structural homologues were identified.

A superposition reveals that the phosphate-binding region ($\beta_1$, the P-loop, and $\alpha_1$) assumes an almost identical conformation in all four structures (Fig. 5). ADP is present in the crystal structures of both NIP and MinD, and the nucleotides superpose well (not shown). The sulfate in the MobB structure occupies the same position as the $\beta$-phosphate group of the bound ADP molecules. This sulfate accepts hydrogen bonds from the main chain amides of Gly16, Gly18, Lys19, and Thr20, and we note that both NIP and MinD have identical residues at equivalent positions that interact with $\alpha$- or $\beta$-phosphate oxygen atoms (Fig. 6). In both NIP and MinD, a threonine (Thr18, MinD numbering) binds the $\alpha$-phosphate oxygen using the side chain hydroxyl and main chain amide group (43), and in MobB, the equivalent residue (Thr21) interacts with a water molecule occupying the same position as an $\alpha$-phosphate oxygen in the MinD complex (Fig. 6). It is likely that MobB would bind diphosphate in similar fashion to NIP and MinD.

Although their interactions with oxyanions appear similar, the structural comparison of the four homologues reveals significant differences between MobB and the others in the nucleotide-binding pocket. MobB lacks a section of 20 residues between $\beta_8$ and $\alpha_6$ (Fig. 5). In MinD and NIP, these residues extend the $\beta_8$ and $\alpha_6$ equivalents of MobB and form a helix-loop combination that creates the nucleotide-binding pocket. In particular two residues (with MinD numbering) Pro198 and Asp200, which are positioned on these extensions, are conserved in MinD and NIP and provide hydrogen bonding interactions between the main chain and the nucleotide. MobB is also different in the $\beta_7$-$\beta_8$ section. In MinD, NIP, and FtsY, the strand corresponding to MobB $\beta_7$ is extended toward the adenine position, creating an integral part of the nucleotide-binding pocket. This loop consists of about 10 amino acids (leading into $\alpha_8$ of MinD) with a structurally conserved asparagine in all three homologues (not shown). In MinD, this Asn171 directly

![Fig. 3. Stereo view of the MobB dimer depicted in ribbon format and viewed perpendicular to the extended $\beta$-sheet. Subunit A is colored red, and subunit B is colored blue and shaded such that the color fades from the N to the C terminus. The $\alpha$-helices in subunit B are labeled along with the sulfate molecule (Corey-Pauling-Koltun (CPK) representation) positioned at the N terminus region of $\alpha_1$. The position of the Walker A motif of subunit B is in green. Figs. 2, 4, and 5–7 were prepared using MOLSCRIPT (48).](image)

![Fig. 4. Stereo view of the electron density for $\alpha_2$ of subunit B. The $\alpha$A-weighted $2F_o - F_c$ map (49) was calculated from the final model and is contoured at the 1$\sigma$ level. A stick representation of the protein is shown colored according to atom type: gray, carbon; blue, nitrogen; red, oxygen.](image)
contacts the base. In FtsY, further hydrogen bonds to the adenine are provided by an additional asparagine present in this loop (not shown) (44). From the structural overlay, it is obvious that MobB does not have the necessary structural features that are utilized by MinD, NIP, and FtsY to efficiently bind the nucleotide base. We conclude that MobB, on its own, would not be an efficient nucleotide-binding protein.

Protein-Protein Complexes—MobB has the correct sequence and structural elements for the efficient binding of the phosphate tail of a nucleotide but not of the base. In contrast, a crystal structure of MobA, the protein that is co-transcribed with MobB, reveals that MobA has little interaction with the phosphate tail of the nucleotide but strong interactions with the base (21). Also, near the putative phosphate-binding region, there is a disordered loop that is highly conserved in MobA sequences and that is termed the consensus loop (20). This loop is glycine-rich but does not contain a phosphate-binding (Walker A) motif. Moreover, the N- and C-terminal ends of the consensus loop are directed away from the phosphate tail of the bound GTP and could not contribute to its binding (21). Consequently, the possibility of MobB binding the phosphate moiety of GTP in the MobA:GTP complex was explored.

The sulfate bound to MobB occupies the equivalent position of the \( \beta \)-phosphate of ADP in the MinD complex (see above). Therefore, the ADP coordinates were taken from the aligned MinD structure and (without further manipulation) placed into the MobB structure. This ADP molecule was superimposed onto the bound GTP in the MobA complex. Since MobB is a dimer, another molecule of MobA was placed onto the second subunit of MobB in a similar fashion to generate a model for a putative MobA:MobB complex (Fig. 7).

In this model, the GTP fits neatly into a pocket formed at the protein-protein interface with no steric hindrance. There is only one area where steric clash of \( \alpha \) atoms might occur, around residues 121–125 in MobB and 154–157 in MobA (Fig. 7). These regions along with several other sections of the proteins at the putative interface are conserved in homologous proteins. In MobA, this includes residues 75–79, 128–134, 150–158, and 182–192 (20), and in MobB, this includes resi-

Fig. 5. Stereo view structural overlay of the C\( \alpha \) traces of MobB (black), MinD (green), NIP (yellow), and FtsY (cyan). The ADP molecule bound to MinD is shown (red stick model) along with the bound sulfate (ball-and-stick) in MobB. Helices \( \alpha_8 \), \( \alpha_9 \), and \( \alpha_{10} \) of MinD are labeled in green; \( \alpha_1 \), \( \alpha_6 \), \( \beta_1 \), \( \beta_7 \), and \( \beta_8 \) of MobB are labeled in black.

Fig. 6. The sulfate environment in MobB (top) and the comparable binding of the diphosphate tail of ADP in MinD (bottom). Atoms are shown as spheres colored according to type: gray, carbon; blue, nitrogen; red, oxygen; green, phosphorus; yellow, sulfur. A water molecule in the MobB structure is colored cyan. Hydrogen bonding interactions are depicted as black dashed lines.
highlight the regions of the two structures.

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