Oxyanion binding alters conformation and quaternary structure of the C-terminal domain of the transcriptional regulator ModE; implications for molybdate-dependent regulation, signalling, storage and transport.

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Abstract

The molybdate-dependent transcriptional regulator ModE of *Escherichia coli* functions as a sensor of intracellular molybdate concentration and a regulator for the transcription of several operons which control the uptake and utilisation of molybdenum. We present two high-resolution crystal structures of this domain in complex with molybdate and tungstate. The ligands bind between subunits at the dimerisation interface and analysis reveals that oxyanion selectivity is determined primarily by size. The relevance of the structures is indicated by fluorescence measurements which show that the oxyanion binding properties of the C-terminal domain of ModE are similar to those of the full length protein. Comparisons with the apoprotein structure have identified structural rearrangements which occur on binding oxyanion. This molybdate-dependent conformational switch promotes a change in shape and alterations to the surface of the protein and may provide the signal for recruitment of other proteins to construct the machinery for transcription. Sequence and structure-based comparisons lead to a classification of molybdate binding proteins.

Keywords. Ion selectivity / ModE / molybdate / Mop / oxyanion / tungstate
Introduction

Molybdenum is an essential trace element required for the catalytic activity of several enzymes in animals, plants and bacteria. In some cases the transition metal is complexed with a unique pterin forming the molybdopterin cofactor, in others it forms part of an iron-molybdenum cluster cofactor (1, 2). *Escherichia coli* acquires molybdenum in the form of MoO$_4^{2-}$ by using a high-affinity ABC-type molybdate transporter system encoded by the *modABCD* operon (3). ModA, the structure of which has been determined (4, 5), is similar to the sulfate-binding protein of *Salmonella typhimurium* (SBP, 6) and a member of the periplasmic binding protein family. ModA binds and transfers molybdate to ModB at the outer surface of the cytoplasmic membrane. ModB is an integral membrane protein, ModC a membrane associated protein; together they transport the oxyanion across the membrane using an ATP-dependent mechanism. The role of ModD is at present unknown. Exposure of *E. coli* to high levels of molybdate leads to repression of *modABCD* by a mechanism which involves the molybdate-dependent transcriptional regulator known as ModE (7-9). The *modE* gene, situated immediately upstream from the *modABCD* operon, codes for a transcriptional regulator able to control the uptake and utilisation of this transition metal. ModE binds molybdate and the complex can function as a repressor by binding at a site that overlaps the transcription start of the *modABCD* operon (10-12). In addition, the ModE-molybdate complex acts as a positive regulator of several genes including those coding for the molybdoenzymes DMSO reductase (13) and nitrate reductase A (14), and for *hyc*, the hydrogenase 3 structural operon. It also mediates expression of the *moaABCDE* operon which encodes the first enzymes required for the biosynthesis of molybdopterin (15).

*E. coli* ModE functions as a homodimer of subunits of 262 amino acids each. The protein binds MoO$_4^{2-}$ and WO$_4^{2-}$ with a high affinity ($K_d$ ~0.8 µM in each case) and stoichiometry of two oxyanions per dimer. Oxyanion binding produces a large quench (50%) of the intrinsic protein fluorescence accompanied by a blue-shift of the emission spectrum maximum which indicates that a conformational
change is induced by these ligands (11). The crystal structure of apo-ModE revealed that the protein forms distinct N-terminal and C-terminal domains (Figure 1, 16). The N-terminal domain, comprised of 121 residues, is mostly α-helical (60% α-helix, 20% β-strand) with a winged helix-turn-helix DNA binding motif (wHTH). The C-terminal molybdate-binding domains, which display a pronounced asymmetry, comprise residues 122 to 262, contains 60 % β-strands which form two β-barrels. The first barrel is constructed from a combination of strands β6-β9 with β15 and the second from strands β10-β14. Each barrel constitutes a sub-domain (Mop1A and Mop2A for example, Figure 1) of similar folds although the order of secondary structure elements with respect to the sequence varies. The sub-domain fold is related to the oligomer binding-fold (OB-fold, 17), a five-stranded Greek-key β-barrel capped by an α-helix. The sub-domains share significant sequence and structural homology with each other and also with the 7 kDa molybdenum-containing Mop proteins from \textit{Clostridium pasteurianum} (18, 19) and \textit{Sporomusa ovata} (20). These proteins are implicated in the molybdenum metabolism of a variety of micro-organisms (3, 11, 21). We have previously shown that each sub-domain corresponds to the structure of a single Mop unit and termed the C-terminal domain of ModE a DiMop domain (16). The term molbindin has been introduced (19) to identify Mop-like proteins and we make the further distinction that this refers to proteins composed of only Mop domains.

Whilst the structure of ModE has been determined, there is a paucity of data concerning how the oxyanion ligands are bound since we have been unable to determine the structure of the complete ModE-molybdate complex. Recent analysis of the Mop protein from \textit{S. ovata} provided the first structural details of oxyanion binding to a molbindin, in this case tungstate (20), results of which will be described later. In order to study the protein-oxyanion interactions relevant to the function of ModE we have cloned and characterised the C-terminal (DiMop) domain. We show that this domain retains the ability to bind oxyanions selectively with properties similar to the intact protein and have determined high-resolution crystal structures of both the molybdate and tungstate complexes. These
structures provide high-resolution information on the protein-ligand interactions and allow us to compare the apo-DiMop component of ModE with the oxyanion-loaded domain, a comparison which identifies a conformational change induced by oxyanion binding. Sequence and structural comparisons with other Mop proteins lead us to propose a classification of the proteins that utilise the Mop domain.

Materials and methods

Expression of the C-terminal DiMop domain of ModE.

A fragment of the modE gene coding for the C-terminal domain was amplified by PCR using pHW121 as the template plasmid, which carries a fragment covering the modE region in the vector pUC18 (8). The oligonucleotide primer used for the 5′ end of the C-terminal domain-encoding DNA was (5′) G CGC CAT ATG CAA ACC AGC (3′) which incorporated an NdeI recognition site (underlined) and covered the first 3 codons of the C-terminal domain. The primer used for the 3′ end of the C-terminal domain was (5′) GCG CGG ATC CCA CGC TTA GCG CAG (3′) which covered the last 6 codons of modE and contained a BamHI recognition site (underlined). The PCR-amplified fragment was cloned as a 0.45 kB NdeI-BamHI-insert in the N-terminal hexa-His-tag expression vector pET15b (Novagen) to create plasmid Plaa7. The expression of protein is under the control of the T7-Lac-promoter and the recombinant DiMop domain carries a thrombin cleavage site between the protein and the His-tag.

Plasmid Plaa7 was heat-shock transformed into E. coli BL21(DE3) and transformants were selected on Luria-Bertani (LB) agar plates containing 100 µg mL⁻¹ ampicillin. A transformant was grown at 37 °C in LB broth with ampicillin to mid-log phase at which point 0.4 mM (final concentration) isopropyl-β-D-thiogalactopyranoside was added and growth continued with vigorous aeration for a further 4 hours. Cells were harvested by centrifugation (2500 g) at 4 °C then resuspended in 50 mM Tris-HCl pH 7.6,
250 mM NaCl, 5 mM benzamidine and stored at -20 °C. Bacterial cells were broken by passage through a French press, the insoluble cell debris pelleted by centrifugation at 4 °C (18000 g) for 15 minutes and the cell extract passed through a 0.2 μm filter and applied to a 5 mL metal chelate affinity column (Hi-Trap; Pharmacia) charged using nickel chloride. The column was washed with four column volumes of 50 mM Tris-HCl pH 7.6, 250 mM NaCl, and 5 mM benzamidine. The Histagged protein was eluted with a 0 to 500 mM imidazole gradient in the same buffer and then incubated with thrombin (Pharmacia) for 12 hours at 20 °C to remove the histidine tag. The DiMop domain was separated from the thrombin, uncleaved fusion protein and N-terminal peptide by strong anion exchange chromatography using a RESOURCES™ Q (Pharmacia) column on a BioCAD 700E workstation. Pooled fractions were concentrated to 20 mg mL⁻¹ in 50 mM Tris-HCl pH 7.6 and sample purity was assessed by SDS-PAGE electrophoresis and MALDI-TOF mass spectrometry (Voyager DE STR; PerSeptive Biosystems). Protein concentration was estimated spectrophotometrically at 280 nm using the calculated molar extinction coefficient of 12660 M⁻¹cm⁻¹ (22). The yield of purified protein was approximately 40 mg L⁻¹ of bacterial culture. Following thrombin-cleavage the DiMop domain consisted of residues 124 to 262 of ModE with a Gly-Ser-His extension at the N-terminus. For reasons of consistency we have maintained the amino acid numbering of the intact ModE.

**Fluorescence measurements**

Intrinsic protein fluorescence spectra were recorded using a Perkin Elmer LS50B spectrofluorimeter maintained at 25 °C. Excitation was at 295 nm (5nm bandpass for both excitation and emission) at a scan rate of 100 nm min⁻¹ with a protein concentration of 29 μM of monomer in 50 mM Tris-HCl pH 7.6. Binding parameters were determined by changes in fluorescence at 347 nm on successive additions of sodium molybdate to a solution of protein (38 μM monomer). Corrections were made for dilution effects but not for the inner filter effect since molybdate does not absorb significantly at the wavelengths used.
Crystals were grown at 20 °C using the hanging drop method by mixing 3 µL of protein solution (16 mg mL⁻¹ protein, 50 mM Tris-HCl pH 7.6, 10 mM Na₂MoO₄ or Na₂WO₄) with 3 µL of a reservoir solution. Similar crystals were obtained with reservoirs of 400 µL of 1.0 M trisodium citrate, 100 mM HEPES pH 7.6, 10 % mM ethylene glycol or 1.4 M ammonium sulfate in 100 mM Tris-HCl pH 8.4. The crystals, which attained a size of 0.3 mm in all dimensions over a period of two days, were cryo-protected with ethylene glycol, cooled to -173 °C and data then measured using an RAXIS IV image plate with a RU-200 rotating anode (Cu Kαλ = 1.54 Å). Two data sets were measured, one from a crystal grown in the presence of WO₄²⁻ using tri-sodium citrate as the precipitant and the other from a crystal obtained in the presence of MoO₄²⁻ using ammonium sulfate as precipitant. The crystals are tetragonal with space group P4₁ with two DiMop domains in the asymmetric unit and about 42% solvent content. All diffraction data were processed, reduced and scaled using the HKL suite (23) then analysed using CCP4 software (24) with 5 % of the reflections used for calculation of the R-free; further details are given in Table 1.

The structure of the DiMop WO₄²⁻ complex was solved by molecular replacement with the program AMORE (25) using a poly-alanine search model constructed from a monomer of the DiMop domain from apo-ModE (16). An optimal position for one molecule was found by using an integration radius of 20 Å and all data between 15 and 3 Å resolution (correlation coefficient 0.57). Positioning the second molecule in the asymmetric unit gave a correlation coefficient of 0.56. The resulting electron density maps indicated significant structural changes between the search model and the oxyanion complex. The tungstate positions were identified and incorporated into the model which was then used to generate phases for use in the warpNtrace mode of ARP/wARP (26). Data to 1.9 Å were used and a new model containing 268 out of 280 residues in the model with an R-factor of 25.1 % and an R-free of 27.7 %, was obtained.
Interactive computer graphics model building was carried out using the program O (27). A Ni\(^{2+}\) ion was located in the same position of the tungstate complex as seen in one of the subunits of the apo-ModE structure and is considered an artifact of the purification procedure (16). Refinement was completed using REFMAC (28) combined with ARPP (29) to place the water molecules. The molybdate complex was solved using the structure of the tungstate-bound form with the water molecules removed and the tungstate replaced with molybdate as a starting model for calculating phases for use in the auto model building procedure in ARP/wARP. The model was refined in a procedure identical to that used for the tungstate complex and relevant statistics are presented in Table 1. The molybdate and tungstate complexes are essentially identical with a root-mean-square (r.m.s.) deviation of 0.3 Å for superposition of all C\(\alpha\) atoms. We concentrate on the molybdate-bound structure though for completeness some details of the tungstate complex are provided. Figures were produced using ALSCRIPT (30), MOLSCRIPT (31) and RASTER-3D (32).

Coordinates

Atomic coordinates and structure factors have been deposited with the Research Collaboratory for Structural Bioinformatics Protein Data Bank (accession codes 1H9R/1H9S for the tungstate and molybdate complexes, respectively).

Results and Discussion

Properties of the DiMop ModE fragment

The C-terminal DiMop fragment of ModE was expressed and purified in high yield and analytical gel filtration analysis (data not shown) revealed it to be a dimer in solution. The DiMop fragment retains two of the three tryptophan residues of ModE (Trp\(^{131}\), Trp\(^{186}\)) and the intrinsic protein fluorescence
emission spectrum is similar to that reported for intact ModE with $\lambda_{\text{max}}$ at 347 nm. On addition of 0.5 mM sodium molybdate to the DiMop fragment the fluorescence emission spectrum was reduced by over 60 % of its value in the absence of molybdate (data not shown). This molybdate-induced quench is slightly greater than that reported for intact ModE (50 %; 11) and clearly demonstrates that the DiMop fragment retains the ability to bind molybdate. Moreover, it suggests that the third tryptophan (Trp$^{49}$) of ModE, located in the DNA-binding domain, makes a less important contribution to the molybdate-induced fluorescence quench of ModE.

Titration of the DiMop fluorescence quench provided an estimate of the stoichiometry of molybdate bound per monomer fragment of 1 with a $K_d$ of approximately 0.5 $\mu$M (Figure 2). These values are in excellent agreement with those reported for molybdate binding to intact ModE; stoichiometry of 1 molybdate per monomer with a $K_d$ of 0.8 $\mu$M. The DiMop fragment effectively retains the molybdate binding-capacity and properties of intact ModE and this observation validated our approach to explore ModE molybdate interactions by crystallographic analysis of the ligand-bound DiMop fragment.

The molybdate binding site

The DiMop dimer binds two oxyanions, in sites designated I and II, at the dimerisation interface (Figure 3) using residues from both polypeptide chains. Each ligand binding site is created by the turn between $\beta_5$ and $\beta_6$ from one DiMop, and helix $\alpha_6$ and the C-terminal region of strand $\beta_{15}$ from the other (Figure 3B). The oxyanion is held in position by accepting nine hydrogens bonds with each oxygen participating in at least two such interactions. Five of the hydrogen bonds are donated from one polypeptide, three from the other and one from a water molecule (Table 2, Figure 4). The MoO$_4^{2-}$ interacts with a single basic residue, Lys$^{183}$. Other interactions from the same subunit involve hydrogen bonds donated from the main chain amides of Arg$^{128}$, Ala$^{184}$ and the side chain OH of Ser$^{126}$. 
The MoO$_4^{2-}$ is bound to the other subunit by hydrogen bonds donated from Ser$^{166}$ OH and both OH and main chain amide of Thr$^{163}$. Sequence comparisons showed that Ser$^{126}$, Arg$^{128}$, Thr$^{163}$ and Lys$^{183}$ are conserved in all Mop-like sequences (Figure 5) and it was proposed that the residues were involved in oxyanion binding (19). Arg$^{128}$ binds the oxyanion using the main chain amide. The side chain forms an intramolecular salt bridge to Glu$^{218}$ (not shown) and contributes to the stabilisation of the tertiary structure and formation of the ligand binding pocket.

The charge compensation for the oxyanion is achieved by interactions with a single basic side chain (Lys$^{183}$), and partial charges contributed from the three main chain NH groups in the binding site. A strong peak in the electron density near the molybdate was assigned as a water molecule, rather than a monovalent cation such as sodium, on the basis of a water-like hydrogen bonding pattern. This water forms hydrogen bonds with the side chain OD$_1$ of Gln$^{144}$ from one subunit and the carbonyl oxygen of Thr$^{232}$ of the partner subunit. Of note is a particularly short contact, 3.0 and 3.1 Å, between the metals in each site and the carbonyl oxygens of Ser$^{126}$ (Figure 4). The carbonyl is approximately equidistant from three of the molybdate oxygens and may help to align the oxyanion for binding. In S. ovata Mop (20) we note that the same structural feature is observed in the corresponding tungstate binding sites.

*Size determines oxyanion selectivity in ModE*

ModE functions by binding molybdate or tungstate but not other tetrahedral oxyanions such as phosphate, sulfate or vanadate (9). In this work, DiMop retained bound MoO$_4^{2-}$ in the presence of 1.4 M ammonium sulfate used in the crystallisation solutions. For selective binding of MoO$_4^{2-}$ ModE can exploit differences in charge (PO$_4^{3-}$ and VO$_4^{3-}$). The highly charged anions like VO$_4^{3-}$ are disadvantaged since ModE has only a single positively charged residue in the binding site. Size will be an important consideration in the case of PO$_4^{3-}$ and SO$_4^{2-}$. Previous analysis of oxyanion geometry in
small molecule structures have shown the average metal-oxygen distances of molybdate and tungstate are 1.75 ± 0.04 Å and 1.76 ± 0.002 Å respectively, while the average sulfur-oxygen bond length in sulfate is 1.47 ± 0.02 Å (5). Consequently MoO$_4^{2-}$ and WO$_4^{2-}$ are similar in size but much larger than SO$_4^{2-}$. The program VOIDOO (33) was used to calculate the volume of the binding pocket accessible to a rolling probe of radius 1.4 Å. The value obtained for the DiMop domain is 78 Å$^3$ which is comparable with the 72 Å$^3$ obtained for the molybdate binding site in *E.coli* ModA (4) and *Azotobacter vinelandii* ModA (5). The volume of these molybdate-binding pockets is larger than that calculated for the sulfate-binding pocket in SBP, 59 Å$^3$. The size of the oxyanion must therefore be important for selectivity.

ModA also exhibits a similar selectivity of tungstate and molybdate over other oxyanions even though it has a similar fold and binding site to SBP (4). ModA binds the oxyanion in a deep cleft formed at the junction of two globular domains. There are seven direct hydrogen bonds all donated from main chain amides and side chain hydroxyls of neutral protein ligands (4, 5) and a contribution to binding from N-terminal α-helix dipoles. Although the DiMop domain is a different fold compared to ModA both proteins use three OH groups from Ser or Thr residues in ligand binding and have residues that bind ligands via a chelate-type interaction using both main chain N and side chain OH groups.

Molybdate-specific proteins, irrespective of fold, have larger binding sites and use size in conjunction with charge compensation to determine oxyanion selectivity. The mode of dimerisation imposes size restrictions since the binding of a smaller oxyanion would require readjustment of the protein backbone and dimerisation interface to optimise the length and angles of the hydrogen bonds involved in ligand binding.
The overall structure of the ligand bound DiMop domain and comparisons with the apo-form

Like intact ModE, the ligand-bound DiMop forms a homodimer with similar secondary structure to the apoprotein (Figures 1 and 3). An obvious difference between the two structures is a decrease of the asymmetry between the partner subunits that was observed in the apoprotein structure (16): least-squares superposition of the Cα atoms of chain A onto chain B gives an r.m.s. deviation of 1.0 Å for the apoprotein, whereas for the DiMop-molybdate complex the r.m.s. deviation is lowered to 0.6 Å. Residues 138 to 144 were excluded from this superposition since they are disordered in chain B of apo-ModE.

The binding of molybdate induces a change in the quaternary structure that, as a first approximation, can be described as the rigid body movement of the four Mop domains against each other: when aligned on Mop1A (as described in the legend for Figure 6), the centre of mass of Mop1B moves about 3.0 Å between apo- and MoO₄²⁻-bound state. As this movement is almost perpendicular to the line connecting the centres of mass of Mop1A and 1B the distance between the Mop1s remains practically constant. In the same alignment the centre of mass of Mop2A moves about 1.9 Å, mostly towards Mop1B on the other chain. Overall these movements lead to the molybdate-bound protein being more compact than the corresponding part of the apo-structure with the buried surface area increasing from 2 x 750.5 Å² for the apo-dimer to 2 x 1442.5 Å² for the ligand-bound form. For consistency residues B138 to B144 were removed from the complex structure prior to the calculation. The reduction of exposed surface is only in part caused by the rigid body movements; a significant contribution is also made by a number of loops which move relative to their surroundings. The largest change (with Cα movements up to 12.6 Å) is observed for residues 138 to 144 (loop I, Figure 6). As mentioned above this loop is partially disordered in the apoprotein with no density observed for one chain. However it becomes ordered upon molybdate binding, moving to cover another flexible loop formed by residues 163 to 166 (loop II). This smaller loop is important because its movement is crucial for the formation
of the molybdate binding site, one half of which is constituted by residues 163 and 166 (see above) after the loop has moved ≈ 2 Å towards the anion. This movement closes the molybdate binding site and leads to the formation of new hydrogen bonds across the intersubunit interface, e.g. between the backbone oxygen of Ser^{126} and the hydroxyl of Ser^{166'}, as well as NH2 of Arg^{169'} or between the guanidino moiety of Arg^{128} and the Thr^{163'} hydroxyl (prime is used to denote a residue on the partner subunit). The latter two residues are also involved in molybdate binding and are conserved in virtually all known Mop domains (Figure 5). Residues 152 to 155 form a third mobile loop (III). In the apoprotein it contacts the symmetry-related loop of the Mop2 of the same chain (residues 224 to 226) and its movement upon molybdate binding follows, to a limited extent, that of the Mop2. We note that all mobile loops are located on the Mop1 domain and that Mop2 actually behaves like a rigid body.

We can now describe the probable order in which molybdate binding and the subsequent structural changes take place. We propose that molybdate initially binds to the half-site formed by residues 126, 128, 183 and 184 of one protein chain. The bound molybdate then effects the movement of loop II of the partner chain and with that consequently the rigid body movement of the partner subunit. This seems a reasonable assumption, as the binding site components of residues 126, 128, 183 and 184 are practically unchanged in the two structures and can be considered a preformed binding site, whereas loop II moves and is clearly influenced by molybdate binding. Further support for this half-site being responsible for initial ligand binding comes from the unequal distribution of protein-ligand interactions: as described above this is where three of the four oxygens of the molybdate are bound, whereas the loop II component provided by the other subunit binds only one.

The closing of the binding site by the movement of loop II then could allow the hitherto solvent-exposed and flexible loop I to rearrange itself and cover loop II, which leads to the formation of a number of new hydrogen bonds, e.g. Gln^{145'} backbone O and N with Ile^{162'} backbone N and O,
respectively, or Val^{143} backbone O with Ala^{164} backbone N, and also improves the hydrophobic packing. The now ordered loop I might then attract the loop formed by residues 210 to 216 of the other chain thereby effecting the observed movement of that Mop2 domain and leading to the formation of further hydrophobic contacts. Finally the loop III of the other chain moves to ‘follow’ the Mop2 domain movement.

The fluorescence perturbation experiments indicate that the environments of one or both tryptophans in the DiMop domain change on binding MoO$_4^{2-}$ and the crystal structures provide an explanation. When the apo and ligand-bound forms of the DiMop domains are compared there is no significant change in the environment of Trp$^{131}$ (not shown) which is located on strand $\beta 6$ and positioned in a cavity formed by the residues linking $\beta 7$ and $\beta 8$. Trp$^{186}$ however, is at the dimerisation interface on a 3$_{10}$ helix. In apo-ModE the Trp$^{186}$ of subunit A is aligned parallel with Trp$^{186}$ from the B subunit separated by about 6Å (Figure 1). The adjustment of the DiMop domains on binding MoO$_4^{2-}$ alters the side chain conformation, renders the tryptophans more buried and reduces their distance to 3.3Å (Figure 3B). In apo-ModE, Trp$^{186}$ contributes 1% of the DiMop dimerisation interface but in the ligand bound form this now increases significantly to 5% of a greater surface area. We therefore attribute the spectral changes observed on binding oxianion to alterations associated with the environment of Trp$^{186}$. It follows then that changes in the environment of Trp$^{186}$ on the addition of molybdate lead to the changes observed in the near UV CD spectrum noted for ModE (11).

**Implications for other Mop proteins**

Mop domains occur in a variety of proteins other than ModE: the molbindins consist of either one (e.g. Mop from *Haemophilus influenzae* or Mop I to III from *C. pasteurianum*) or two (e.g. ModG from *Azotobacter vinelandii*) Mop domains. The function of these small proteins is unclear, but it has been
suggested that they store molybdate/tungstate and are thus involved in maintaining homeostasis of these anions. As molybdate binding occurs at the interface between two Mop units, it is obvious that functional molbindins must be at least dimeric. The structure of a single-Mop protein from *S. ovata* (20) revealed a hexameric arrangement (Figure 7A) that provides three binding interfaces, similar to those described for DiMop, at which six WO$_4^{2-}$ are bound. The highly symmetric assembly creates two additional binding sites located on a three-fold axis and formed by residues from three neighboring Mop domains (A/C/E and B/D/F) and therefore do not occur in ModE (Figure 7).

A Mop-like sequence is also observed as a C-terminal extension in the ATP-binding subunit of the *mod*-encoded molybdate transporter (e.g. ModC from *E. coli* or ModD from *A. vinelandii*). Again the function of the Mop domain is unclear, but by analogy with similar extensions, e.g. of the maltose transporter ATP-binding protein MalK (34) we suggest that it could allow regulation of transporter activity depending on intracellular molybdate levels. Such rapid feedback regulation would complement the transcription-level control exercised by ModE.

Figure 5 shows a sequence alignment of Mop domains from a representative selection of Mop-carrying proteins. With the exception of some of the Mop2s from ModE proteins (see below) all sequences show conservation of the residues involved in molybdate binding. In a number of cases Ser$^{166}$ (numbering for the Mop1 domain of *E. coli* ModE) is replaced with an alanine, but from the structural data it seems perfectly conceivable that in those cases a water could take the place of the serine hydroxyl group.

Based on the known structures we propose that Mop-containing proteins can be divided into at least four groups depending on the quaternary arrangement of their Mop moieties. The first group
comprises the single-Mop molbindins. These Mops dimerise and the dimers then trimerise to form a hexameric assembly as shown in Figure 7A. Each pair of dimers provides a molybdate-binding interface like that observed in ModE, altogether supplying six binding sites. Two additional MoO$_4^{2-}$ binding sites are located on the three-fold axis. Thus the hexameric Mop proteins bind eight molecules of molybdate, all of them at subunit interfaces. DiMop molbindins with two Mop domains on a single polypeptide constitute the second structural group. Even though no structural data is currently available, it can be expected that DiMop proteins will trimerise to form an assembly similar to the Mop hexamer, the main difference being that in this case the participating dimers are covalently linked (Figure 7B). Likewise the DiMop molbindins should also possess eight molybdate binding sites.

_E. coli_ ModE is a member of the third group. Here only two DiMop chains dimerise to form a assembly which can be superimposed almost perfectly onto two ‘dimers’ from the _S. ovata_ Mop structure (Figure 7C). As the ‘missing’ dimer participated in the formation of not only the two ‘threefold’ sites, but also two of the binding interfaces, it is clear that ModE-like proteins can only bind two moles of molybdate per mole of (dimeric) protein. It is furthermore clear that two of the four Mop domains (one from each dimer, dark green and purple in Figure 7C) are no longer involved in oxyanion binding. Accordingly it is not surprising that in some ModE-like proteins (e.g. the ones from _E. coli_ or _H. influenzae_, Figure 5) the Mop2 domain has undergone amino acid changes which render molybdate binding impossible. Consider the Mop2 domain of _E. coli_ ModE: the residue corresponding to Mop1 Ser$^{126}$ is Asn$^{198}$, which, in the observed conformation, would for sterical reasons be unable to bind molybdate. Arg$^{128}$ is substituted by Asp$^{200}$, its side chain facing towards the hypothetical binding site preventing the approach of an oxyanion. Asn$^{252}$ from the Mop2 domain is the equivalent residue to Lys$^{183}$ of Mop1 – again a residue important for molybdate binding is lost. The other two binding site residues, Thr$^{163}$ and Ser$^{166}$, are replaced by Pro$^{234}$ and Glu$^{237}$, respectively, which abolishes all hydrogen bonding and introduces another negative charge incompatible with oxyanion binding.
We note that Mop proteins with a proposed storage function maximise the number of molybdates bound per Mop unit (= 1.33), whereas the oligomerisation state of other Mop proteins with a function in signalling/feedback only provide two binding sites thus ensuring that no molybdate is ‘wasted’ (e.g. *E. coli* ModE binds 0.5 molybdates/Mop).

The fourth group of Mop proteins are the ATP-binding components of the molybdate-transporting ABC-transporter system, for example *E. coli* ModC and *A. vinlandii* ModD. On the basis of sequence alignments (Figure 5), these proteins carry a Mop domain which should be able to bind molybdate. In order to do so it must at least dimerise with a Mop-domain containing protein and it is possible that this involves another subunit of ModC itself. Further work will be required to confirm this.

**Conclusions**

Our analysis shows that the molybdate binding properties of the C-terminal DiMop fragment of *E. coli* ModE are the same as for the intact protein; it has provided details of oxyanion binding together with the conformational and quaternary changes that result. Structural data have revealed that *E. coli* ModE discriminates between oxyanions based on size and charge. This molecular recognition is vital for the biological role of ModE in sensing and then controlling the internal molybdate concentration in *E. coli* by regulation of the transcription of the *modABCD* operon. The DiMop domain undergoes a ligand-induced conformational change that produces an alteration to the surface of the DiMop dimer and by implication to the surface of ModE. We previously suggested a role for the C-terminal domain of ModE in the recruitment of partner proteins to form the complexes necessary for the regulation of transcription (16). The conformational alteration that the C-terminal domain undergoes when oxyanion binds may represent a molecular switch that regulates the recruitment of such partner proteins necessary for the positive regulation of transcription. The possibility exists that this
conformational switch in the C-terminal domain may extend to the N-terminal domain and influence the interactions with DNA, though further work will be required to investigate this hypothesis.

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Figure legends

Figure 1. Ribbon diagram to depict the architecture of intact apo-ModE (16). Trp\textsuperscript{186} is shown as a ball-and-stick representation and labelled A186 and B186 to identify the subunit. The N-terminal DNA binding domains are red and orange for subunits A and B respectively and the winged-helix-turn-helix motifs are labelled. The molybdate-binding domains are divided into Mop sub-domains labelled 1A, 2A, 1B and 2B according to subunit.

Figure 2. Titration of the DiMop fragment with molybdate. The continuous curved line is the calculated curve for a stoichiometry of one molybdate bound per monomer with a $K_d$ of 0.5 µM.

Figure 3. The dimeric assembly and architecture of the DiMop domain of ModE complexed with molybdate (shown as van der Waals spheres with molybdenum cyan and oxygen red) occupying binding sites I and II. The same colour scheme as in Figure 1 is used. (A) View with Mop1B and Mop2B in a similar orientation to the corresponding subunit shown in Figure 1. (B) View orthogonal to (A) with selected elements of secondary structure labelled.

Figure 4. Stereoview of oxyanion binding site I. Nitrogen and oxygen atoms of the protein are shown as blue and red spheres respectively, a water molecule is shown as a light blue sphere. Residues are coloured blue if part of Mop1A, green where they are part of Mop1B. Dashed black lines represent hydrogen bonds and the close contact between the carbonyl oxygen of Ser\textsuperscript{126} and molybdenum is depicted as a yellow dashed line. The single letter code is used for amino acids and prime to further identify those residues from Mop1B.
Figure 5. Sequence alignment of 13 Mop domains. The residues of ModE directly involved in oxyanion binding are marked with triangles, shaded to indicate the use of a side chain functional group and open where a main chain amide is used for binding. Residues enclosed in black are conserved in at least 11 of the sequences, those enclosed in grey correspond to at least 8 out of 13 being conserved. The order of the sequences shown is as follows: The ModE Mop1 from E. coli, H. influenzae, and the two Mop domains from A. vinelandii ModA (a ModE homologue), the single Mops from C. pasteurianum, H. influenzae and S. ovata, A. vinelandii ModG Mop1 and 2, E. coli ModC and A. vinelandii ModD. The final two sequences are the Mop2 domains of E. coli and H. influenzae ModE respectively.

Figure 6. Alignment of the ModE molybdate-binding domain in its apo-form (blue) and oxyanion-bound form (green). Two ‘intermediate’ states that were generated by cartesian morphing (using the programme LSQMAN (35) are shown to illustrate the transition. The two proteins were aligned on the Cα atom of the rigid core of Mop1A and B, comprising residues 124-137, 145-151, 156-162 and 167-184. The colour gradient from blue/green to red reflects increasing Cα distances based on the separate alignments of the rigid core for every Mop domain. Even though Mop2s essentially are rigid bodies for comparability they were aligned on the Cα atoms of residues 196-209, 217-223, 227-233, 238-253. Unless stated otherwise all alignments and center-of-mass calculations use these sets of residues. The molybdate binding sites are labelled as in Figure 3.

Figure 7. Schematic of the modular arrangements for four classes of Mop proteins. (A) The hexameric organisation of the single-Mops as exemplified by S. ovata Mop. Each Mop domain is indicated by a large sphere and the molybdate ions by smaller cyan coloured spheres. The oxyanions are positioned at the Mop-Mop interfacial regions. Only those sites at the dimer interface which bind 6 oxyanions are shown; the other sites are buried at the trimer interface behind subunit A in one case and
D in the other. (B) The likely trimeric arrangement of polypeptides in *E. coli* ModG. The only difference with respect to *S. ovata* Mop is that the Mop domains are covalently linked as indicated by the cylinder joining the Mop domains. (C) The dimeric DiMop arrangement in ModE and (D) a postulated organisation for the Mop domain of ModC.
Table 1. Data collection, processing, refinement and model geometry statistics for both complexes.

|                                | Molybdate | Tungstate |
|--------------------------------|-----------|-----------|
| Unit cell lengths $a = b, c$ (Å) | 72.34     | 72.23     |
| Resolution range (Å)            | 30.0-1.82 | 30.0-1.90 |
| No. of measurements             | 272924    | 182781    |
| No. unique reflections          | 22803     | 19932     |
| Coverage overall (%)            | 98.1(84.3)| 97.7(78.9)|
| $I/\sigma(I)$ all data          | 22.79(2.9)| 27.8(9.5)|
| $R_{sym}$ overall (%)           | 7.5(38.1)| 4.9(14.7)|
| Protein residues                | 273       | 272       |
| Waters                          | 212       | 309       |
| Ni $^{2+}$                      | 0         | 1         |
| Oxyanions                       | 2         | 2         |
| R-work/R-free (%)               | 18.8/23.4 | 17.9/22.9 |
| Average isotropic thermal parameters ($\AA^2$) | | |
| Subunit A overall               | 33.7      | 26.3      |
| Subunit B overall               | 35.5      | 27.3      |
| Main chain                      | 33.2      | 26.0      |
| Side chains                     | 36.2      | 29.3      |
| Waters                          | 46.7      | 37.6      |
| Ni$^{2+}$                       |           | 44.1      |
| Oxyanion                        | 24.9      | 19.2      |
| r.m.s. bond lengths (Å)         | 0.010     | 0.012     |
| r.m.s. bond angle associated distances (Å) | 0.024 | 0.034 |
| Ramachandran plot favourable/additional (%) | 93.1/6.9 | 92.1/7.9 |
Table 2. Hydrogen bond lengths (Å) formed between the oxyanions and the protein.

|                  | Molybdate |          | Tungstate |          |
|------------------|-----------|----------|-----------|----------|
|                  | A         | B        | A         | B        |
| Ser126 OH        | 2.65      | 2.74     | 2.61      | 2.66     |
| Arg128 N         | 2.90      | 2.87     | 2.83      | 2.80     |
| Thr163 N         | 2.85      | 2.74     | 2.88      | 2.67     |
| Thr163 OH        | 2.76      | 2.73     | 2.83      | 2.74     |
| Ser166 OH        | 2.91      | 3.00     | 2.90      | 2.96     |
| Lys183 NZ        | 2.75      | 2.75     | 2.63      | 2.73     |
| Lys183 NZ        | 3.28      | 3.49     | 3.34      | 3.46     |
| Ala184 N         | 3.05      | 2.97     | 2.63      | 2.73     |
| Water            | 3.03      | 2.91     | 2.87      | 2.88     |
Fraction of sites saturated by [Ligand\textsubscript{total}] (µM)
Oxyanion binding alters conformation and quaternary structure of the C-terminal domain of the transcriptional regulator ModE; implications for molybdate-dependent regulation, signalling, storage and transport
David G. Gourley, Alexander W. Schuttelkopf, Lisa A. Anderson, Nicholas C. Price, David H. Boxer and William N. Hunter

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