Properties of the Periplasmic ModA Molybdate-binding Protein of Escherichia coli*

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The modABCD operon, located at 17 min on the Escherichia coli chromosome, encodes the protein components of a high affinity molybdate uptake system. Sequence analysis of the modA gene (GenBank L34009) predicts that it encodes a periplasmic binding protein based on the presence of a leader-like sequence at its N terminus. To examine the properties of the ModA protein, the modA structural gene was overexpressed, and its product was purified. The ModA protein was localized to the periplasmic space of the cell, and it was released following a gentle osmotic shock. The N-terminal sequence of ModA confirmed that a leader region of 24 amino acids was removed upon export from the cell. The apparent size of ModA is 31.6 kDa as determined by gel sieve chromatography, whereas it is 22.5 kDa when examined by SDS-polyacrylamide gel electrophoresis. A ligand-dependent protein mobility shift assay was devised using a native polyacrylamide gel electrophoresis protocol to examine binding of molybdate and other ligands. ModA, which is required for the assembly and function of several proteins, is subsequently reduced and then incorporated into the periplasm. ModC is proposed to contain an ATP hydrolase activity and to bind molybdate with high specificity and affinity. The ModB protein at the outer surface of the cytoplasmic membrane transports molybdate across the cytoplasmic membrane to the cell cytoplasm. ModC in conjunction with the ModD protein then transports molybdate into the cell cytoplasm. ModD is proposed to contain an ATP hydrolase activity that provides energy for the transport process (Rech et al., 1995); the ATP dependence is predicted by the sequence similarity of the modC gene product to other bacterial ABC transporter genes including hisP and malK (Ames, 1986). Mutations in the modC gene severely impair molybdate accumulation and lead to the inability of the cell to grow under conditions of low molybdate (approximately below 1 μM). This phenotype can be overcome by supplementing E. coli cells with exogenous molybdate at 100 μM (Scott and Amy, 1989).

Molybdenum is an essential trace metal for most bacteria as well as for all plants and animals. High affinity uptake of molybdate in the bacterium Escherichia coli proceeds by a specific transport system encoded by the modABCD operon (Rech et al., 1995). Following the uptake of molybdate into the cell, it is subsequently reduced and then incorporated into the molybdenum cofactor, molybdopterin guanine dinucleotide, which is required for the assembly and function of several enzymes including nitrate reductase, formate dehydrogenase, dimethyl-sulfoxide reductase, trimethylamine-N-oxide reductase, and biotin-sulfoxide reductase (Rajagopalan and Johnson, 1992). These enzymes, except biotin-sulfoxide reductase, are synthesized primarily during the anaerobic growth of E. coli and other enteric bacteria. They participate in anaerobic respiration or fermentation reactions to aid in cellular energy generation. The molybdate (modABCD) transport operon of E. coli was recently sequenced and characterized (Maupin-Furlow et al., 1995; Rech et al., 1995; Johann and Hinton, 1987). It encodes a bacterial ABC type transport system based on a comparison with other solute uptake components including the maltose, histidine, and leucine-isoleucine transporter proteins (Gilson et al., 1982; Higgins et al., 1982; Ames, 1986; Shuman, 1987). The modA gene product was predicted to encode a 28.6-kDa protein located in the cell periplasm (Rech et al., 1995). By analogy to other bacterial periplasmic binding proteins, the ModA protein binds molybdate and transfers it to the ModB protein at the outer surface of the cytoplasmic membrane. ModB in conjunction with the ModC protein then transports molybdate across the cytoplasmic membrane to the cell cytoplasm. ModC is proposed to contain an ATP hydrolase activity that provides energy for the transport process (Rech et al., 1995); the ATP dependence is predicted by the sequence similarity of the modC gene product to other bacterial ABC transporter genes including hisP and malK (Ames, 1986). Mutations in the modC gene severely impair molybdate accumulation and lead to the inability of the cell to grow under conditions of low molybdate (approximately below 1 μM). This phenotype can be overcome by supplementing E. coli cells with exogenous molybdate at 100 μM (Scott and Amy, 1989).

Molybdenum transporters similar to the E. coli modABCD have been recently reported in other bacterial species including Azotobacter vinelandii, Rhodobacter capsulatus, and Haemophilus influenzae Rd based on cloning and DNA sequence studies (Luque et al., 1993; Wang et al., 1993; Fleischmann et al., 1995). In the nitrogen-fixing bacteria, the molybdate transport process also provides molybdenum for synthesis of the molybdenum containing cofactor of nitrogenase as well as for the structurally distinct molybdopterin cofactor that is made by E. coli and most other bacteria.

At present, little is known about the operation of the Mod-ABC transport systems in bacteria. In this study we report the isolation and characterization of the modA gene product from E. coli. It is shown to be located in the cell periplasmic space and to bind molybdate with high specificity and affinity. The pre-ModA protein is processed upon secretion to give a mature periplasmic protein. The binding specificity of the ModA protein is shown to extend to the molybdate analog, tungstate, but not to other inorganic anions. Finally, the requirement for ModA protein in growth of E. coli cells in the presence of low concentrations of molybdate is documented.
**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Bacteriophages, and Plasmids**—The genotypes and origins of the *E. coli* K-12 strains, bacteriophage, and plasmids used in this study are given in Table I.

**Cell Growth**—For plasmid, phage, and strain manipulations, cells were grown in Luria broth or on solid media. When required, ampicillin was added to the medium at a concentration of 100 μg/ml. Aerobic growth and anaerobic growth were performed as described previously (Rech et al., 1995). When desired, sodium molybdate was added at a final concentration of 100 μM (Rech et al., 1995). Anaerobic cell growth experiments (see Fig. 7) were performed by growing the indicated strain in 10 ml of anaerobic minimal medium that contained 25 mM sodium nitrate and 4% glycerol as described previously (Rech et al., 1995). Where indicated, sodium molybdate was added to a final concentration of 10 μM. The cells used for inoculation were grown under the same conditions overnight.

Overproduction and Purification of the ModA Protein—For ModA protein production, strain MC4100 containing plasmid pModAT7 was grown to stationary phase in a LB medium supplemented with ampicillin (100 μg/ml). Following cell harvest, the periplasmic fraction of the cells was isolated using an osmotic shock protocol as follows. Cells were chilled to 4 °C and harvested by centrifugation at 8,000 rpm for 8 min in a Sorvall T centrifuge (DuPont, Inc.). The spent cell growth medium was discarded, and the cell pellet was resuspended in distilled water; a 20 mM buffer (0.5 M sucrose, 0.1 M Tris-Cl, pH 8.2, and 1 mM EDTA) at a ratio of 5 ml/1 g of cells (wet weight). The cells were placed on ice for 5 min and then centrifuged as described above. The supernatant was discarded, and the cell pellet was resuspended in distilled water; a 20 mM MgCl₂ solution was then added to give a final concentration of 1 mM MgCl₂. The cell suspension was centrifuged at 10,000 rpm for 10 min in a Sorvall T centrifuge (DuPont, Inc.). The spent cell growth medium was discarded, and the cell pellet was resuspended and placed on ice for subsequent ModA protein purification. The periplasmic proteins were concentrated by the addition of solid ammonium sulfate to a final concentration of 60% (w/v). The solution was then stirred gently for 1 h, and the precipitated material was removed by centrifugation for 20 min at 4 °C (10,000 rpm). The supernatant fraction was brought to a final ammonium sulfate concentration of 90% and then stirred for an additional hour. The precipitated proteins were harvested by centrifugation as described above, resuspended in 50 mM potassium acetate buffer at pH 5, and dialyzed overnight against the same buffer.

The purified protein was clarified by centrifugation for 15 min at 13,000 rpm. The supernatant fraction was then loaded onto a Mono S HiTrap column (Pharmacia Biotech Inc.), which was equilibrated with 50 mM potassium acetate buffer, pH 5, at room temperature. The ModA protein was eluted from the column using a linear KCl gradient. The fractions containing the ModA protein were pooled and dialyzed against potassium acetate buffer. The purified ModA protein was greater than 99% pure as judged by SDS-PAGE. It was stored at −70 °C for subsequent use. Protein concentration was measured according to Bradford (1976), with bovine serum albumin as the standard.

**Gel Electrophoresis**—Gel electrophoresis and Western blotting were performed as described previously (Laemmli, 1970). Native protein gel electrophoresis was done according to the instructions provided with a Pharmacia Phast protein system (Pharmacia). The isoelectric point of the ModA protein was determined experimentally by use of a Pharmacia Phast gel system and gels according to the manufacturer’s instructions. The pl was also calculated from the amino acid sequence information by using the PeptideSort routine of the University of Wisconsin Genetics Software package (Devereux et al., 1984).

**Amino Acid Analysis**—The amino acid composition of the purified ModA protein was determined using an Applied Biosystems amino acid analyzer. The amino acid sequence of ModA was obtained using an Applied Biosystems protein sequencer.

**DNA Sequence Analysis**—The DNA sequence analysis differed from the experimentally determined sequence (see “Results”). The DNA sequence analysis differed from the experimentally determined sequence (see “Results”). The DNA sequence analysis differed from the experimentally determined sequence (see “Results”).

**RESULTS**

**Overexpression and Purification of the ModA Protein**—The modA gene of *E. coli* was recently isolated, and its DNA sequence was determined (see Rech et al., 1995); sequence accession L34009). In order to purify and characterize the modA gene product, the modA gene was cloned into the plasmid expression vector, pGem7-, and the resulting plasmids that contained modA gene inserted in each orientation were identified and stocked (see “Experimental Procedures”). Following the introduction of each modA expression plasmid into *E. coli* strain MC4100, cells were grown to mid-exponential phase ($A_{600} = 0.9$), and isopropyl-1-thio-β-D-galactopyranoside was added to induce ModA protein synthesis. At 1, 2, and 3 h postinduction, no accumulation of ModA protein was observed by SDS-PAGE analysis of total *E. coli* proteins (data not shown). However, when strain MC4100 containing the modA expression plasmid pModAT7 was grown to stationary phase

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1 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
The indicated E. coli cell or protein fractions were separated by SDS-PAGE as indicated under "Experimental Procedures." Lane 1, whole cell proteins of wild-type strain; lane 2, cell shock fraction; lane 3, whole cell protein of a strain containing the overexpression plasmid; lane 4, cells following osmotic shock; lane 5, cell shock protein; lane 6, purified ModA protein. MW, molecular weight protein standards.

(i.e. 18 h postinoculation), a significant amount of the ModA protein was accumulated as evidenced by the appearance of a 22.5-kDa protein (Fig. 1, lane 3) compared with cells that lacked the plasmid (lane 1). No other growth conditions or induction protocols tested were found to improve the yield of the ModA protein.

The cellular location of the ModA protein was determined by fractionating the cells into the cytoplasmic, membrane, and periplasmic components (Fig. 1). ModA was accumulated primarily in the periplasmic space, as revealed by its release from the cell following an osmotic shock (Fig. 1, lane 5). The ModA protein was estimated to routinely comprise over 75% of the total periplasmic proteins. Very little ModA protein was found in either the particulate or soluble cell fractions.

The ModA protein was purified to homogeneity (see "Experimental Procedures") and used for subsequent characterization studies. The mature ModA protein exhibits an apparent subunit size of 22.5 kDa (Fig. 1). This differs from the 27.4-kDa size predicted from the DNA sequence (Rech et al., 1995; Maupin-Furlow et al., 1995).

Processing Site of the pre-ModA Protein—The purified ModA protein was amino acid sequenced at its N-terminal end. The pre-ModA protein deduced by DNA sequence analysis is aligned with the experimentally determined amino acid sequence of the purified periplasmic ModA protein. The numbering is relative to the predicted pre-ModA protein from E. coli. Single letter abbreviations of the amino acid code are used. Rcmd, R. capsulatus ModA protein; Avmod, A. vinelandii ModA protein.

Effect of pH on ModA-Molybdate Binding—To examine the pH dependence of the mobility shift, ModA was run in a pH gradient native gel following preincubation with and without molybdate present (Fig. 4). A noticeable mobility shift was seen for ModA across the entire pH range tested from pH 3 to 9. It appears that ModA protein is able to effectively bind molybdate at any of these pH values; the mobility of ModA increases toward the anode at increasing pH. The relative displacement between the molybdenum bound and unbound forms of ModA increased from pH 3 to 4.5, and then remained relatively constant until a pH value of 6.5 to 8 was achieved.

UV-Visible Absorption Spectrum of ModA—The UV-visible absorption spectrum of the ModA protein was examined in the
absence and presence of sodium molybdate (Fig. 5). As anticipated from the amino acid content of the ModA protein, an absorption maximum at 280 nanometers was seen for the protein when no ligand was present. The addition sodium molybdate to a final concentration of 10 mM caused a slight but noticeable change in the ModA spectrum in the high UV wavelength range; a shoulder appeared on the ModA absorption peak at about 290 nm, while the maximum absorbance peak at 280 nm was enhanced relative to ModA when no ligand was present.

This ligand-dependent spectral change in ModA upon binding of molybdate was further examined by taking the difference spectra at higher resolution (Fig. 6). As evidenced by the spectral properties of the ModA protein in the absence as well as in the presence of increasing amounts of sodium molybdate, it was apparent that molybdate binding alters the environment of the tryptophan and/or tyrosine residues in the ModA protein. Upon addition of molybdate, two absorption maxima at 281 and 287 nm were observed (Fig. 6A). Analysis of the bound versus free ModA protein, based on the absorption maxima at 281 and 287 nm, revealed an apparent dissociation constant for sodium molybdate of 3 mM. When analogous studies were performed using sodium tungstate, a molybdate anion analog (Fig. 6B), the absorption maxima of the ModA protein was similar to that revealed for sodium molybdate except that the peaks were shifted slightly in the UV range (i.e. blue-shifted). Titration of ModA protein with sodium tungstate revealed an apparent dissociation constant of 7 mM at wavelengths of 280 and 286 nm.

Proteolytic Susceptibility of ModA—Limited proteolysis of the ModA protein was performed in the presence and absence of sodium molybdate to determine if it undergoes a conformational change upon binding of ligand. When ModA was incubated for 40 min with chymotrypsin at 1 μg/ml, it was partially digested as evidenced by a reduction in the intensity of the 22.5-kDa species (data not shown). No long-lived intermediate cleavage products were detected. However, when sodium molybdate was also present during the digestion, significantly less degradation of ModA was observed. These studies support the proposal that ModA can exist in either of two protein conformations depending on whether molybdate is bound or not. In control experiments to test if molybdate affected the activity of chymotrypsin, ovalbumin was digested as effectively when molybdate was present versus when absent.

Identification of an E. coli modA Phenotype—To determine if the wild-type modA gene is required for molybdate-dependent expression of E. coli fumarate reductase (frdABCD) genes, we screened a number of previously isolated mod mutants (e.g. chlorate resistance) for defects in the modA gene using the procedure of Kalman and Gunsalus (1988, 1990). Based on the ability of a modA plasmid to restore the molybdate-dependent nitrate repression of frdA-lacZ gene expression, one modA mu-
tant was identified. It was designated E. coli strain LK82RG77. The mod phenotype could also be suppressed when the modA mutant was grown in the presence of 100 \( \mu M \) sodium molybdate. Additionally, a wild-type copy of the modA gene was found to be essential for molybdate-dependent regulation of the anaerobic respiratory pathway genes, narGHJl and frdABCD (data not shown). These genes encode the respiratory nitrate reductase and fumarate reductase enzymes required for anaerobic respiration to nitrate and fumarate, respectively (Kalman and Gunsalus, 1990).

Requirement of the ModA Binding Protein for Cell Growth under Molybdate-Limiting Conditions—The phenotype of the modA mutant identified above suggested that a defect in the modA gene might prevent anaerobic cell growth of the mutant on a glycerol-nitrate medium under molybdenum limiting conditions. To test this, the modA strain was grown in the minimal medium without sodium molybdate added (Fig. 7). The mutant was unable to grow, presumably due to an inability to transport low levels of molybdate, whereas it could grow when transformed with the modA plasmid. These results demonstrate that the modA encoded molybdate-binding protein is an essential component of the high affinity molybdate transport system encoded by the E. coli modABCD operon. Since one of the complementing plasmids only contained the modA gene, the original modA defect was apparently not due to a polar mutation. When the modA or wild-type cells were grown in medium supplemented with 10 \( \mu M \) molybdate, they grew as well as the wild-type strain in medium lacking molybdate, conditions where molybdate apparently enters the cell via a different route.

**DISCUSSION**

In this study, we report the overexpression, purification, and characterization of the ModA periplasmic molybdate-binding protein of E. coli. The purification procedure we used was similar to the isolations of previous bacterial binding proteins (Ames, 1994) and allowed us to rapidly purify ModA to homogeneity. The protein was released from the periplasmic space using osmotic shock, concentrated, and dialyzed into buffer at pH 5. The low pH treatment led to the precipitation of many contaminating proteins and lipopolysaccharides, but, as shown in previous reports (Ames, 1994), it did not harm the periplasmic binding protein. The resistance of ModA to low pH allowed us to load the protein preparation directly onto a cationic exchange column as a final purification step. The overproduction of ModA occurred from a multicopy plasmid containing the native modA promoter. Interestingly, ModA only accumulated to high levels when cells were allowed to grow overnight to stationary phase.

The homogenous preparations of ModA were used to determine the biochemical characteristics of the protein for comparison to other binding proteins. The presence of a signal sequence was confirmed by N-terminally sequencing the purified ModA protein. Comparison to the nucleotide sequence shows that the first 24 amino acids have been removed upon transport to the periplasmic space (Fig. 2). This leader sequence has several characteristics that are common to prokaryotic signal sequences (Oliver, 1987; Izard and Kendall, 1994). The amineterminal end has one positively charged amino acid, arginine at position 3, which is followed by a stretch of predominantly neutral amino acids that form the hydrophobic core. The peptide ends in the consensus processing site AXB as described previously (Oliver, 1987) in which A and B are alanine.

We examined the ModA protein-ligand interactions using isoelectric focusing, pH titration, a ligand-dependent gel shift assay, as well as UV-visible spectroscopy methods. It has been reported previously that the equilibrium for formation of the enzyme-ligand complex can shift the apparent pI of a protein. Rudnick et al. (1990) showed that the pI of N-myristoyltransferase was shifted by 3 pH units when the ligand was bound. This shift was not based on the calculated change of the pI due to the additional charges provided by the ligand. Therefore the observed shift in pI was thought to indicate a change in the protein conformation caused by formation of a reaction intermediate. In the case of the ModA protein, we observed a similar shift in the pI in the presence of molybdate as seen in the pH titration curve in Fig. 4. When incubated with molybdate, the pI of the protein decreased by about 1.4 pH units, which we interpret as a change in protein conformation upon ligand binding. This may be due in part to the two negative charges of molybdate that may balance positive charges on the protein upon binding of the anion. Further studies are needed to resolve this possibility.

Both molybdate and tungstate ions had the ability to influence the mobility of ModA on native PAGE gels (Fig. 3). No other inorganic anions tested had the same effect even when present at 10\(^3\)-fold higher levels than used for molybdate binding (–10 \( mM \)). The specificity of the molybdate interactions with ModA supports the previously proposed role of the periplasmic protein in molybdate uptake (Rech et al., 1995). These results are also consistent with the in vivo observation that molybdate as well as tungstate can be transported by the molybdate uptake operon (Miller et al., 1987). It is noteworthy that sulfate did not bind to the ModA protein, since it has been suggested previously that when present at high concentrations (>100 \( \mu M \)), molybdate is taken up via the sulfate transporter (Lee et al., 1990). Our observations suggest that sulfate is not likely to enter the cell via the ModABC transport system.

The ModA mobility shift seen using the Native Phast gels (Pharmacia) also allowed us to determine the apparent dissociation constants for molybdate and tungstate binding to ModA. These values are in the range reported for \( K_d \) values of other binding proteins for their anions such as citrate (2 \( \mu M \)) and phosphate (0.8 \( \mu M \)) (Tam and Saier, 1993). Therefore the ligand-induced mobility shift assay appears to be a rapid and reproducible method that should be applicable to examine the
specificity and affinity of other periplasmic binding proteins. The molybdate and tungstatelidgand-dependent ModA mobility shift data and the observation that the ModA pl changes upon ligand binding indicate that the protein undergoes a conformational change when it binds ligand. Similar conclusions have been made for the leucine, isoleucine, valine binding protein of E. coli using x-ray scattering and computer modeling approaches (Olah et al., 1993). We further investigated the ligand-dependent changes in ModA protein conformation by using UV spectroscopy and limited proteolysis methods. Addition of molybdate resulted in an increase in the absorption maximum observed at 281 nm as well as the appearance of the absorbance peak at a higher wavelength (i.e. red-shift to 287 nm) (Fig. 5). These observations indicate that one or more tryptophans or tyrosines experience a change to a more hydrophobic environment upon binding of ModA to molybdate (Copeland, 1995).

It has been shown recently that a tryptophan residue is also involved in the interaction of sulfate with the sulfate periplasmic binding protein (Pflugrath and Quiocho, 1988). The absorbance difference spectra of ModA show two absorption maxima at about 281 and 287 nm, which increase with the addition of up to 10 μM of ligand. The Kₐ values for molybdate (3 μM) and tungstate (7 μM), calculated based on the absorbance changes, show that ModA has a 2-fold higher affinity for molybdate. The pattern of the difference spectra confirm that tryptophan and/or tyrosine residues are in close vicinity of the conformational change occurring in ModA upon molybdate binding. Additionally, the absence of a characteristic third peak at 292 nm indicates that the largest contributor to the ModA spectrum appears to be tyrosine (Copeland, 1995). Examination of the deduced amino acid sequence of ModA (Fig. 2) reveals the presence of a pair of tryptophans and one tyrosine at positions 106, 124, and 133, respectively, relative to the ModA N terminus. This region is thus likely to be involved in molybdate binding. However, direct evidence for the position of the binding site needs to be established from the examination of the crystal and/or NMR structures of the ModA protein.

Limited proteolysis of the ModA protein using either trypsin or chymotrypsin did not yield two polypeptides corresponding to the two domains typical of several other periplasmic binding proteins including the leucine, isoleucine, valine and sulfate binding proteins (Adams and Oxender, 1989). Even though no stable polypeptide intermediates were observed, the binding of molybdate to ModA slowed the proteolytic attack. This suggests that the protein-ligand complex has reduced solubility accessible sites for the protease.

In support of the biochemical data for ModA in its role as the periplasmic molybdate binding protein, we were able to show that this protein is required for molybdate uptake by E. coli in vivo. A modA mutant could not respire with nitrate unless complemented with a modA+ plasmid (Fig. 7). The modA mutant also had a similar phenotype to a modC mutant for chloride resistance (data not shown and Rech et al. (1995)). The present studies demonstrate that a modA mutant containing a defect in the molybdate binding protein is unable to take up the trace amounts of molybdate present in the medium even though the ModBCD proteins are apparently present (Fig. 7).

In summary, using biochemical methods as well as in vivo studies, we have been able to demonstrate that modA, the first gene of the modABCD operon, encodes the periplasmic molybdate binding protein (Table II). This protein has a characteristically low homology to other binding proteins except for the presence of a leader peptide (Fig. 2). The specificity of protein-ligand interaction, and the requirement of ModA for molybdate transport in vivo confirm its role as a periplasmic binding protein. Analysis of the ModA crystal structure data should reveal what tertiary features are shared between ModA and the other periplasmic binding proteins, and it should aid in elucidating the amino acid interactions of ModA with the molybdate and tungstate ligands.

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Table II

| Subunit Size | Properties of the ModA molybdate binding protein |
|--------------|-------------------------------------------------|
| From DNA sequence | Subunit size (Kₐ) |
| Experimental from SDS PAGE | 24.9 kDa |
| Native Size | 22.5 kDa |
| Superoxide gel minus MoO₄ | 31.6 kDa |
| Superoxide gel plus MoO₄ | 31.6 kDa |
| Calculated | |
| Determined (minus MoO₄) | 6.82 |
| Plus MoO₄ | 7.00 |
| Kₐ | 5.60 |
| Molybdate | 3 μM |
| Tungstate | >7 μM |
| Permanganate | 2 μM |

* Predicted size without leader region.