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Molybdate binding by ModA, the periplasmic component of the *Escherichia coli mod* molybdate transport system

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Abstract

ModA, the periplasmic-binding protein of the *Escherichia coli mod* transport system was overexpressed and purified. Binding of molybdate and tungstate to ModA was found to modify the UV absorption and fluorescence emission spectra of the protein. Titration of these changes showed that ModA binds molybdate and tungstate in a 1:1 molar ratio. ModA showed an intrinsic fluorescence emission spectrum attributable to its three tryptophanyl residues. Molybdate binding caused a conformational change in the protein characterized by: (i) a shift of tryptophanyl groups to a more hydrophobic environment; (ii) a quenching (at pH 5.0) or enhancement (at pH 7.8) of fluorescence; and (iii) a higher availability of tryptophanyl groups to the polar quencher acrylamide. The tight binding of molybdate did not allow an accurate estimation of the binding constants by these indirect methods. An isotopic binding method with $^{99}MoO_4^{2-}$ was used for accurate determination of K_D (20 nM) and stoichiometry (1:1 molar ratio). ModA bound tungstate with approximately the same affinity, but did not bind sulfate or phosphate. These K_Ds are 150- to 250-fold lower than those previously reported, and compatible with the high molybdate transport affinity of the *mod* system. The affinity of ModA for molybdate was also determined in vivo and found to be similar to that determined in vitro. © 1998 Elsevier Science B.V.

Keywords: ModA; Molybdate binding; Tungstate binding; Molybdenum transport; *Escherichia coli*

1. Introduction

Molybdenum is an essential component of a large number of enzymes (for a recent review, see Ref. [1]), where it is present in the form of two types of cofactor: the iron–molybdenum cofactor, in nitrogenase [2,3], and the molybdopterin cofactors in all the other known molybdoenzymes [4]. Molybdenum is a relatively abundant element in the earth's crust [5], in two main chemical forms, MoS_2, and MoO_4^{2-}. Only Mo(VI) in the form of molybdate is readily soluble and stable in aqueous solutions, and it is thus the only relevant source of molybdenum for biological systems [5]. It has been known for a long time that bacteria are able to scavenge traces of molybdate from the medium, concentrating it in the cell for molybdoenzyme synthesis, and in some cases actively storing it [6]. This capacity is illustrated by the difficulties researchers have encountered in eliminating traces of molybdenum in culture media [7–9].

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Recently, the genes encoding a molybdate transport system (*mod*) have been isolated and sequenced from *Escherichia coli* [10–13] and similarly from two diazotrophs, *Rhodobacter capsulatus* [14] and *Azotobacter vinelandii* [15,16]. In addition, genome sequencing projects with unrelated bacteria are uncovering the widespread occurrence of the *mod* system [17,18]. In *E. coli*, the *mod* genes were isolated by complementation of a subset of chlorate-resistant mutants ($ChlD^-$) characterized by a very high requirement of molybdate in the medium for molybdoenzyme biosynthesis [11,19,20]. Four genes *modABCD*, organized in an operon, share strong structural similarity and sequence homology with genes encoding binding-protein dependent solute (or ABC-type) transport systems [10–13]. The first gene, *modA*, encodes a periplasmic binding protein, which has been recently purified and characterized [21]. ModB and ModC have sequence homology with integral membrane and cytoplasmic ATP hydrolysis components of ABC transport systems, respectively. ModD does not show sequence similarity to any gene product in current databases and its role in transport is unclear. Mutants in the *mod* operon are unable to synthesize molybdoenzymes at molybdate concentrations (0.1 μM) in the medium sufficient for the wild type, presumably because they lack the high-affinity *mod* transport system. Two more genes, *modEF*, are also involved in molybdate transport. The *modE* gene encodes a regulatory protein that effects molybdenum-dependent repression at the *modA* promoter [22–24]. The exact role of *modF* is not yet clear [22].

Despite these genetic advances, relatively few studies aimed at the biochemical characterization of the transport systems have been published, many of them at an earlier period [6,7,25–28]. As a result, an adequate description of molybdate uptake at the physiological and biochemical level is still lacking. Our laboratory has recently undertaken a comprehensive description of the process of $^{99}MoO_4^{2-}$ uptake by *E. coli* cells [10]. Our results suggest that the *mod* operon encodes a very high affinity ($K_m \approx 15$ nM) molybdate transport system. The low K_m values agree well with previous results obtained with the enteric diazotroph *K. pneumoniae* ([7]; $K_m \approx 20$ nM, J. Imperial, unpublished results), and adequately explain the high molybdenum scavenging capacity observed with most bacteria.

As a first step in the characterization of the molybdate uptake by *E. coli*, we undertook the isolation and characterization of ModA, the periplasmic binding component of the *mod* system. A report on the purification and characterization of ModA has appeared recently [21]. Through the use of indirect assays based on changes in gel mobility and UV-visible spectra of ModA upon addition of molybdate, the authors estimated a K_D for molybdate of 3–5 μM. This is one of the highest substrate K_D values reported for any periplasmic binding protein [29–31] and contrasts with the low K_m values for molybdate uptake. In this report, we reexamine the substrate binding properties of ModA, both by a direct isotopical method and by indirect spectrophotometric and spectrofluorometric methods, and discuss their relevance for the mechanism of molybdate transport.

2. Materials and methods

2.1. Overexpression of ModA

Chromosomal DNA segments containing *mod* genes were subcloned from clone λ10F3(180) from the Kohara ordered library (a gift from Dr. Kohara, Japan) into plasmid pBlueScript KS(+) (Stratagene, La Jolla, CA) and identified by their ability to restore nitrate reductase activities of several *mod* mutants to wild-type levels when the cells were grown without any additional molybdate in the media, and by DNA sequencing. Plasmid pMH2c contained ca. 4.7 kb *Eco*RI–*Eco*RV fragment spanning *modEABC*. In order to isolate *modA*, pMH2c was digested with *Bal*I that cut at the positions 258 bp upstream of the start codon of *modA* and 660 bp downstream of the stop codon of *modA*, resulting in ca. 1.7 kb fragment that was further ligated to pBlueScript KS(+) linearized with *Eco*RV. The orientation of *modA* relative to the T7 promoter was determined by restriction enzyme digestion and plasmid pMHmodA2, with T7 promoter located upstream of *modA*, was used for ModA overexpression. Bacteriophage T7 RNA polymerase-dependent overexpression was achieved in strain BL21(DE3) containing plasmid pMHmodA2, essentially as described by Studier et al. [32]. Briefly, cultures were grown in 2 × YT medium [33] containing 400 μg ml^{-1} ampicillin under aeration. When

cultures reached an A_{600} of ≈ 0.6, IPTG was added to 0.4 mM, and incubation continued until optimal induction was achieved (4 h; data not shown).

2.2. Purification of periplasmic ModA protein

ModA was purified from induced cultures by an osmotic shock procedure based on the work of Ames [34] for HisJ and other binding proteins. All procedures were carried out at 4°C except where indicated. Cultures were chilled, brought to 50 mM Tris–HCl, pH 7.8, and centrifuged (10 min, $8000 \times g$). Cell pellets were resuspended (1/20 original culture volume) in 50 mM Tris–HCl, pH 7.8, 40% sucrose, and EDTA added to 2 mM final. After 5 min incubation at room temperature, cells were centrifuged (10 min, $12{,}000 \times g$). Cell pellets were carefully drained, loosened with a spatula, and shocked by adding iced water (1/20 original culture volume). After complete resuspension, shocked cells were centrifuged (15 min, $20{,}000 \times g$). An almost quantitative release of periplasmic ModA was achieved by this procedure (data not shown). ModA was purified from the osmotic shock supernatant by cation-exchange chromatography with a 5-ml Macroprep-S Hiprep column (Bio-Rad, Hercules, CA). The osmotic shock supernatant (10 ml fractions) was brought to 25 mM sodium acetate, pH 5.0, and loaded on the column equilibrated with the same buffer. The column, run at 2 ml min^{-1}, was washed with the same buffer for 24 min, and eluted with a linear 50–400 mM NaCl gradient in the same buffer (60 min, 1 min fractions collected). ModA eluted as a sharp A_{280} peak with a maximum at ca. 110 mM NaCl and was essentially pure. A yield of ca. 20 mg pure ModA protein was routinely obtained per liter of induced culture. In some preparations, an ammonium sulfate precipitation step, as described by Rech et al. [21], was carried out before the cation-exchange chromatography step. Briefly, the osmotic shock supernatant was brought to 60% ammonium sulfate and gently stirred for 1 h. The precipitated protein was eliminated by centrifugation (15 min, $20{,}000 \times g$). The supernatant was brought to 90% ammonium sulfate and processed as above. The protein pellet was resuspended in 25 mM sodium acetate, pH 5.0 and dialyzed against the same buffer. The precipitate that formed was eliminated by centrifugation and discarded. The clear supernatant containing ModA was subjected to cation-exchange chromatography as above. Inclusion of the ammonium sulfate precipitation step did not significantly improve purification.

2.3. Assays of molybdate binding by ModA

2.3.1. Direct assay with $^{99}MoO_4^{2-}$

The procedure used is based on the method of Pardee et al. [35] for studying $^{35}SO_4^{2-}$ binding by the sulfate-binding protein. No commercial sources for $^{99}MoO_4^{2-}$ were available in the US when this study was undertaken. Hence, carrier-free $^{99}MoO_4^{2-}$ was prepared in our laboratory from spent ^{99m}Tc generators exactly as described by El-Kolaly et al. [36]. Binding assays were carried out in 1-ml volumes with 10 mM Tris–HCl pH 7.8, 500,000 dpm carrier-free $^{99}MoO_4^{2-}$, varying amounts of non-radioactive MoO_4^{2-}, and ModA protein. After 5 min incubation at room temperature, 0.25 g Dowex AG-1X8 (100–200 mesh, Cl^- form; Bio-Rad) resin was added as a slurry (1 vol. water/vol. resin) to remove unbound Mo from solution. The mixture was allowed to settle for 5 min and 100-μl portions were counted in vials containing Ecolume (ICN Biochemicals, Costa Mesa, CA) in a Packard scintillation counter. Control experiments showed that the Dowex resin did not strip any Mo off the ModA protein within the time frame of the assays (data not shown). In view of the short half-life of ^{99}Mo (66.4 h) and of its decay to radioactive ^{99m}Tc and ^{99}Tc, all counts were corrected for decay and $^{99}MoO_4^{2-}$ stocks were not used after two half-lives. WO_4^{2-} was substituted for non-radioactive MoO_4^{2-} in the assays for competition studies. Binding parameters were estimated from the general rectangular hyperbolic binding equation:

$$r = pC_s/(K_D + C_s) \quad (1)$$

where r and C_s represent the binding function (bound ligand/total acceptor) and the free ligand concentration, respectively, and p and K_D are the number of sites and dissociation constants, respectively [37]. Optimal non-linear regression fitting of the data to the equation was performed with the SigmaPlot program (Jandel Scientific, Corte Madera, CA).

2.3.2. Fluorometric and spectrophotometric assays

Changes in the intrinsic UV fluorescence and UV absorption spectra of ModA upon molybdate binding

were recorded at room temperature with a Perkin-Elmer 650-LS10 spectrofluorometer ($\lambda_{ex} = 290$ nm, λ_{em} in the 300–400 nm range using 5-nm bandwidths) and with a Shimadzu UV160U spectrophotometer, respectively, in 25 mM Tris–HCl, pH 7.8 and, for fluorescence spectra, also in 25 mM sodium acetate, pH 5.0. Spectra were corrected for dilution effects and, in the case of fluorescence spectra, for inner-filter effects due to molybdate.

Titration of intrinsic fluorescence of ModA and the ModA · molybdate complex by acrylamide was performed in 25 mM Tris–HCl, pH 7.8 at $\lambda_{ex} = 290$ nm and $\lambda_{em} = 339$ nm, by serial additions from a 4.2-M acrylamide stock. Data were plotted as normal and modified Stern–Volmer plots, and the effective collisional quenching constant (K_{SV}) was calculated as previously described [38].

2.4. Binding of molybdate by ModA in *vivo*

In order to measure molybdate binding by ModA in whole cells, a strain lacking the molybdate transport system but synthesizing large amounts of ModA was constructed. *E. coli* strain RK5202 (*chlD202*::Mu *c*ts [39]) lacks an active *mod* molybdate transport system because of a Mu *c*ts insertion in *modC* [12]. Thus, synthesis of ModA should be completely derepressed at low molybdate concentrations in the medium [40]. To ensure synthesis of even larger amounts of ModA, plasmid pMHmodA2 (see above) was introduced into strain RK5202. Although the T7 promoter is inactive in this strain, enough expression was observed from its native promoter, resulting in the accumulation of high levels of mature ModA protein in the periplasmic space (data not shown). The strain was grown at 30°C in modified M63 medium [41] which was buffered with 0.1 M Tris–HCl, pH 7.6, and potassium phosphate was added to 0.5 mM, in order to minimize molybdenum contamination. Late exponential phase cultures were washed, resuspended in fresh medium, and used for binding assays. Binding assays were carried out in 0.5-ml volumes with ca. 0.5 A_{600} units of cells, $^{99}MoO_4^{2-}$ (250,000 dpm), and varying amounts of cold molybdate. After 30 s incubation, cells were separated by centrifugation (2 min, $12{,}000 \times g$), and portions of the supernatant counted as above. In preliminary experiments, cells were centrifuged through a $\rho =$ 1.021 (two parts $\rho = 1.05$ plus one part $\rho = 0.963$, both from Aldrich Chemical) silicone oil layer [42] to ensure good separation of cell pellet and aqueous supernatant, but this was later found unnecessary. The effect of non-specific binding of molybdate to cell surface components was minimized by the high ModA expression levels. However, an estimate for the non-specific component was determined from cells where the *mod* system was completely repressed (wild-type strain BL21(DE3) grown as above but in the presence of 1 μM molybdate to ensure maximum repression of the *mod* system, washed and resuspended in medium without molybdenum, and assayed) and subtracted. Binding parameters were estimated as above.

2.5. Protein determination

Protein was routinely assayed with the dye-binding assay [43] (Bio-Rad) with bovine serum albumin as a standard. For purified ModA fractions, protein was estimated from the A_{280}, using a theoretical extinction coefficient of 1.045 for a 1 mg ml^{-1} solution, as estimated from the sequence of the mature protein by the method of Gill and Von Hippel [44]. A compositional bias correction factor was determined by total amino acid analysis of purified ModA performed at the Protein Structure Laboratory, University of California-Davis. Both the dye-binding and A_{280} assays overestimated ModA protein contents by factors of 1.39 and 1.25, respectively.

3. Results

3.1. Changes in UV absorption spectrum of ModA upon molybdate and tungstate binding

Fig. 1A shows the absorption spectrum of ModA in the 250–310 nm range. The spectrum shows a maximum at 281 nm and a shoulder at 290 nm. In the presence of excess molybdate, there was a slight increase in the 281 nm peak and a more pronounced increase in absorbance near the shoulder, which were clear in the difference spectrum (Fig. 1B). Similar changes were observed in the presence of tungstate (Fig. 1B), but not in the presence of phosphate or

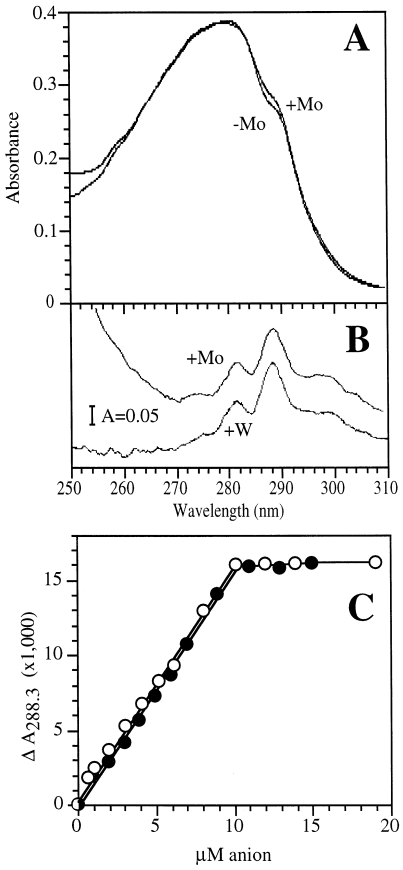

Fig. 1. Effect of molybdate and tungstate binding on the ModA UV absorption spectrum. Spectra were recorded with 9.6 μM ModA in 25 mM Tris–HCl, pH 7.8. (A) Raw spectra in the presence (+Mo) or absence (−Mo) of molybdate (14.1 μM). (B) Difference spectra of ModA plus molybdate (14.1 μM; +Mo) and ModA plus tungstate (15.0 μM; +W) vs. ModA with no metal addition. The vertical bar indicates an absorbance of 0.05. (C) Titration of ModA absorption at 288.3 nm by molybdate (○-○) or tungstate (●-●).

sulfate (data not shown). Titration of ModA with molybdate or tungstate resulted in a linear increase of the differential spectrum peak at 288.3 nm (Fig. 1C) which stopped abruptly when the anion reached a concentration approximately equal to that of protein. The plots for molybdate and tungstate were very similar, with tungstate promoting a slightly lower absorption increase and requiring a slightly higher concentration for saturation. These changes in the absorption spectrum probably represent conformational changes in the protein upon anion binding, and suggest that ModA binds molybdate and tungstate very tightly, with very similar affinities, and in a 1:1 molar ratio.

3.2. Changes in the intrinsic fluorescence spectrum of ModA upon molybdate binding

The ModA intrinsic fluorescence spectrum at pH 7.8 showed an excitation maximum at 283 nm and an emission centred around 339 nm. The long emission wavelength indicates that the primary fluorophore is tryptophan, and that tryptophanyl residues in the protein are largely exposed to the solvent. Addition of saturating levels of molybdate resulted in a slight blue-shift in the emission spectrum to a maximum at 337 nm, and in an overall increase in the relative fluorescence of 13% at the peak (Fig. 2A). Equivalent results were obtained in 25 mM HEPES pH 7.8

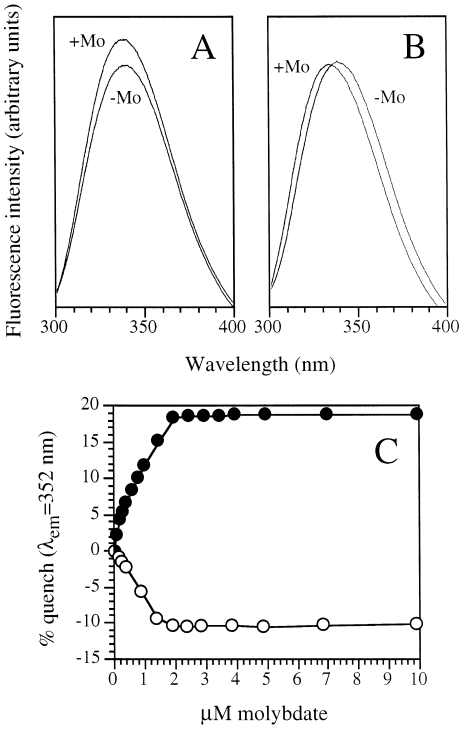

Fig. 2. Effect of molybdate binding on the intrinsic fluorescence emission spectra of ModA. (A) Spectra were recorded with 2.0 μM ModA in the presence (+Mo) or absence (−Mo) of molybdate (9.9 μM) in 25 mM Tris–HCl, pH 7.8. (B) Spectra were recorded as in (A) but in 25 mM sodium acetate, pH 5.0. (C) Titration of ModA fluorescence by molybdate at $\lambda_{em} = 352$ nm in 25 mM sodium acetate, pH 5.0 (●-●) and in 25 mM Tris–HCl, pH 7.8 (○-○).

(data not shown). At pH 5.0, the ModA fluorescence spectrum was very similar, with an emission centred around 340 nm. However, addition of excess molybdate resulted in a more pronounced blue-shift in the emission spectrum to a maximum at 335 nm and in a slight decrease in the overall fluorescence (Fig. 2B). The change in the emission peak suggests that a conformational change upon substrate binding exposes tryptophan residues to a more hydrophobic environment. Differences in the way the ModA fluorescence spectrum responds to molybdate at different pH are probably a reflection of large changes in the overall charge of the protein upon molybdate binding (pI without substrate = 7.0; pI with molybdate = 5.62; Ref. [21]). Changes in ModA fluorescence were used to follow molybdate binding to the protein (Fig. 2C). Assays at both pH 5.0 and 7.8 resulted in an almost linear response of fluorescence to molybdate addition, which stopped abruptly after a stoichiometric amount of metal (2.0 μM) had been added, indicating that each ModA molecule binds one molybdate very tightly. This tight binding precludes accurate determination of the K_D for the ModA · molybdate complex. In fact, the data fit poorly to an equation describing the titration curve for a second order binding process [45] (data not shown). The data obtained by lowering the protein concentration in the assay were not reliable due to a lower signal to noise ratio. However, an upper limit for the K_D of 0.1 μM can be estimated from the data in Fig. 2C by assuming that a maximum of 5% of the added molybdate remains unbound at concentrations below the equivalence point.

Acrylamide quenching of the intrinsic tryptophan fluorescence of ModA in the absence or presence of saturating molybdate levels, was used to probe conformational changes in ModA upon molybdate binding. Molybdate binding to ModA resulted in higher quenching by acrylamide (Fig. 3), suggesting that fluorophores become more accessible to acrylamide. This is shown by the higher effective collisional quenching constant ($K_{SV} = 10.4\ M^{-1}$ vs. $K_{SV} = 9.0\ M^{-1}$) for acrylamide, as calculated from modified Stern–Volmer plots (Fig. 3). Standard Stern–Volmer acrylamide quenching plots curved upward (data not shown). Since three tryptophanyl residues are present in the mature ModA protein [21], this result indicates either that the three residues are equally fluorescent or that the spectrum is dominated by a single fluorophore [38].

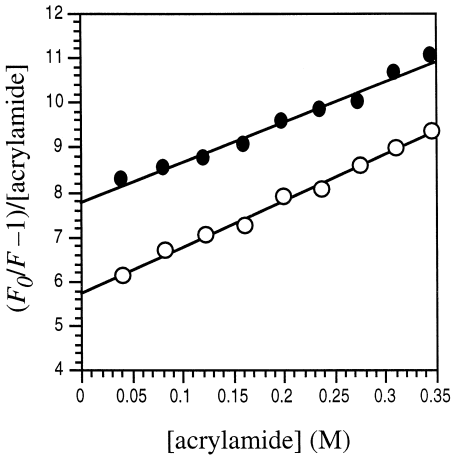

Fig. 3. Modified Stern–Volmer plots for acrylamide quenching of ModA intrinsic fluorescence. Quenching data were obtained at the emission peak ($\lambda_{em} = 338$ nm) with 2.0 μM ModA in 25 mM Tris–HCl, pH 7.8 and in the presence (○-○) or absence (●-●) of molybdate (9.9 μM). F_0 and F are the fluorescence intensities in the absence and presence of quencher.

3.3. Direct assay of molybdate binding by ModA with $^{99}MoO_4^{2-}$

Attempts to isotopically measure molybdate binding to ModA by means of filter-binding assays were unsatisfactory even using low-binding polyvinyl difluoride filters, due to high non-specific adsorption of the isotope to the membranes and lack of reproducibility (data not shown).

A method, derived from that of Pardee et al. [35] for the sulfate-binding protein, proved useful for molybdate binding studies. Fig. 4 shows a typical molybdate-binding curve for ModA. The binding constants were estimated as 0.97 ± 0.09 for the number of binding sites in ModA and 20 ± 8 nM for the dissociation constant. Competition studies were used to characterize tungstate binding (Fig. 4). Essentially equivalent results were obtained, showing that ModA does not discriminate between molybdate and tungstate. Phosphate and sulfate did not have any effect on molybdate binding by ModA (data not shown).

$^{99}MoO_4^{2-}$ was also used to determine the effect of pH and temperature on molybdate binding by ModA.

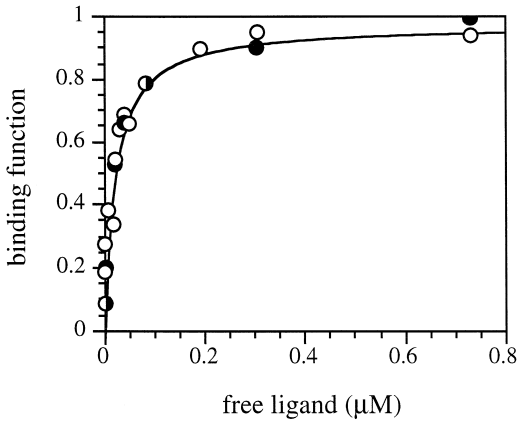

Fig. 4. Isotopic determination of molybdate binding (○) and of tungstate inhibition of molybdate binding (●) by ModA. Assays were carried out with 0.2 μM ModA and $^{99}MoO_4^{2-}$ as described in Section 2 with varying amounts of non-radioactive molybdate (○) or tungstate (●). Data were plotted as binding function (bound ligand/total ModA) vs. free ligand. The curve represents optimal fit of the molybdate-binding data to Eq. (1), as described in Section 2.

Binding was relatively unaffected by pH in the physiological range between 5.0 and 8.5 (data not shown). Molybdate binding by ModA was also relatively unaffected by heating (80°C, 30 min).

3.4. Molybdate binding by ModA in vivo

E. coli cells accumulating large amounts of ModA in the periplasmic space, but unable to transport molybdate through the *mod* transport system, were used to determine the molybdate binding affinity of ModA in vivo. The results are shown in Fig. 5. An apparent $K_D = 67$ nM was estimated, a value not too dissimilar to that obtained in vitro. These results are relevant in view of the widely different ModA protein concentration in the periplasmic space and in the assays in solution, and indicate that results obtained with the purified protein are physiologically relevant.

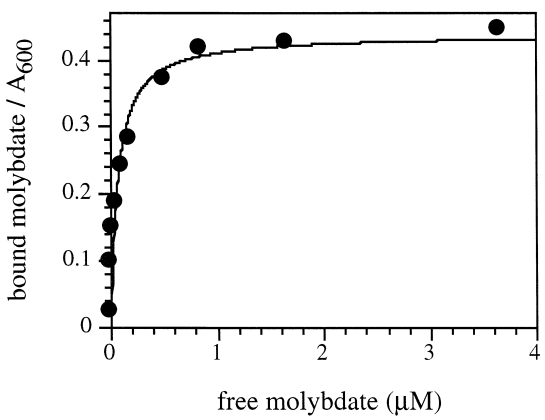

Fig. 5. Molybdate binding by ModA in vivo. Experimental points are indicated with full circles. The curve represents the best fit of the data to Eq. (1), as described in Section 2.

4. Discussion

The overexpressed *E. coli* ModA protein was efficiently exported into the periplasmic space from where it could be isolated in large amounts by a simple osmotic shock procedure, and purified to homogeneity by a single cation-exchange chromatography step. As it is the case with many periplasmic binding components of ABC-type transport systems [34,35], ModA was very stable to low pH and high temperature, which aided in its purification. One concern with this family of proteins is that their tight substrate binding properties can result in purified preparations containing both free and ligand-bound molecules [34,46]. This might have been the case with ModA, in view of the difficulties in eliminating traces of molybdate from culture media [7–9]. However, as Rech et al. [21] have shown, the apparent pI of ModA shifts from 7.0 to 5.6 upon substrate binding. The recently solved crystal structure of ModA shows that this is not due to charge compensation of the bound anion by interaction with positively charged amino acid residues [47]. At any rate, the observed pI shift ensures that substrate-bound ModA molecules would be selectively lost during the cation-exchange chromatographic step at pH 5.0.

We were especially intrigued by the report of Rech et al. [21] that ModA binds molybdate with a K_D of 3 μM and tungstate with a K_D of 7 μM. These are among the highest substrate K_Ds reported for periplasmic binding proteins [29–31], at the level of periplasmic components of transport systems for carbon and energy substrates, such as maltose and citrate. This result is clearly at odds both with the well known molybdate-scavenging capacity of bacteria and with the measured molybdate transport affinities of enterobacteria (in the submicromolar range [7,10]).

As it has been repeatedly observed, and mathematically elaborated by Boos and Lucht [31], Bohl et al. [48], Merino et al. [49] and Bohl and Boos [50], the overall substrate affinity of the complete transport system under physiological conditions, reflects that of the isolated periplasmic component, which shows a K_D that is often higher, but rarely lower, than the K_m for transport [29–31]. We reexamined substrate binding by purified ModA by following changes in the ModA UV absorption and fluorescence emission spectra and also by a direct isotopic procedure with $^{99}MoO_4^{2-}$.

Minor, but significant changes in the UV absorption spectrum of ModA upon molybdate or tungstate binding were observed. These changes are similar to those previously reported [21], and were used by these authors to estimate apparent K_Ds for molybdate and tungstate. A plot of our titration data revealed that ModA binds both molybdate and tungstate with high affinity and in a 1:1 molar ratio, but the tight binding did not allow, in our hands, an accurate estimation of the K_Ds at the protein concentrations used, which are similar to those used by Rech et al. [21]. Interpretation of these authors' results is further complicated by the fact that their titrations were carried out at pH 5.0. At this pH, and depending on the anion (molybdate or tungstate) concentration, significant condensation into polymers can take place. This is especially true for tungstate [51,52] and could play a role in the lower apparent affinity of ModA towards tungstate observed by Rech et al. [21].

ModA exhibited an intrinsic fluorescence emission spectrum characteristic of tryptophanyl residues. Molybdate binding by ModA determined changes in the spectrum. These changes were strongly affected by pH. At pH 5.0, fluorescence quenching was observed, whereas at pH 7.8 molybdate binding resulted in an enhancement of fluorescence. At both pH values, a blue-shift in the emission peak was observed, suggesting that tryptophanyl residues are exposed to a more hydrophobic environment upon molybdate binding. Three W residues, at positions 52, 96, and 129 of the mature protein, are present in ModA [12,13,21]. Acrylamide quenching was used to determine exposure of these residues to the solvent [38]. The upward-bending Stern–Volmer plot suggests that all three tryptophanyl residues are spectroscopically equivalent or that the spectrum is dominated by a single tryptophan [38,53]. The fluorophores were highly accessible to the polar quencher ($K_{SV} = 9.0$ M^{-1}), and this accessibility increased ($K_{SV} = 10.4$ M^{-1}) upon molybdate binding. The above observations suggest that fluorescence of the tryptophanyl residues is modified by the charge of adjacent residues, which in turn is modified by molybdate binding. These changes are probably related with the strong effect of molybdate binding on the apparent pI of the protein, which decreases by 1.4 units in the ligand-bound form [21]. As it was the case with UV absorption spectra, titration of the fluorescence emission spectra with molybdate resulted in a linear response up to a molybdate concentration equal to that of protein. This shows that molybdate is very tightly bound by ModA in a 1:1 molar ratio, but makes accurate estimation of the binding affinity difficult. An upper limit of 0.1 μM for the K_D was estimated based on the assumption that a maximum of 5% of the substrate remains unbound below the equivalence point.

A method for direct measurement of molybdate binding by ModA with $^{99}MoO_4^{2-}$ was optimized. This method relies on the use of an anion-exchange resin for removal of unbound molybdate from solution and is directly adapted from that of Pardee et al. for the sulfate-binding protein [35]. A K_D of 20 nM and a 1:1 molar ratio were determined for molybdate. A similar affinity was observed for tungstate in molybdate-binding inhibition experiments. These very high affinities are characteristic of periplasmic components of high-affinity ABC-type transporters [29,30] and are compatible both with the molybdate- (and tungstate-) scavenging ability of bacteria and with the observed K_m for molybdate transport (see above). Lopez-Corcuera et al. [27] had previously studied molybdate binding by a crude osmotic shock preparation of an *E. coli* ChlD$^-$ mutant, and determined an apparent K_D of 9 nM for molybdate. Our results suggest that they were indeed observing molybdate binding by ModA in their preparations.

ModA specifically bound molybdate or tungstate. No binding of sulfate or phosphate was observed. This is important for efficient molybdate transport, since both sulfate and phosphate are present in the medium and are required by the cell at levels several orders of magnitude higher. *E. coli* has specific periplasmic binding proteins for these anions which

are part of specific transport systems. These two anions have similar geometry, and the specificity of their respective proteins is based on the discrimination by the protein of the proton in the phosphate anion, which is absent in the sulfate anion [54]. In the case of ModA, a larger molecular size of the cognate ligand might explain the lack of binding of sulfate or phosphate. This has been confirmed by the recently solved crystal structure of ModA with bound ligand [47]. However, molybdate and tungstate are very similar in geometry and charge, and it had been hypothesized that transport systems might not be able to discriminate both anions unless they undergo redox reactions [5,51]. Our above results with ModA and with the molybdate uptake system [10] show that, at least for *E. coli*, the *mod* transport system does not discriminate between molybdate and tungstate, which are equally taken up by the cell. Consistent with this, the crystal structures of ModA with bound molybdate or tungstate have been shown to be essentially identical, and rule out any redox reaction of the anions upon binding [47]. Discrimination between both anions must then occur along the cofactor biosynthetic pathway, probably at some step involving reduction of the metal [55,56].

Molybdate binding by ModA was also studied in vivo in cells that do not transport molybdate inside the cell, so that the amount of molybdate bound by the cell reflects binding by ModA. The normal concentration of binding protein in the periplasmic space has been estimated as 30–40 mg ml^{-1} for several systems [31]. When in vitro binding experiments have been attempted at these high protein concentrations, extremely low substrate-binding affinities have been observed [31]. Although the explanation for these observations is not clear, they raise questions as to whether these low affinities are found in vivo. Our results, in a situation where even higher ModA levels in the periplasmic space are ensured by protein overexpression, show that this is in fact not the case, and that the dissociation constants obtained in vitro reflect the physiological characteristics of the system in vivo.

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