An Analysis of the Binding of Repressor Protein ModE to modABCD (Molybdate Transport) Operator/Promoter DNA of Escherichia coli

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Expression of the modABCD operon in Escherichia coli, which codes for a molybdate-specific transporter, is repressed by ModE in vivo in a molybdate-dependent fashion. In vitro DNase I-footprinting experiments identified three distinct regions of protection by ModE-molybdate on the modA operator/promoter DNA, GTTATT (−15 to −8; region 1), GCCTACAT (−4 to +4; region 2), and GTTCACAT (+8 to +14; region 3). Within the three regions of the protected DNA, a pentamer sequence, TAYAT (Y = C or T), can be identified. DNA-electrophoretic mobility experiments showed that the protected regions 1 and 2 are essential for binding of ModE-molybdate to DNA, whereas the protected region 3 increases the affinity of the DNA to the repressor. The stoichiometry of this interaction was found to be two ModE-molybdate per modA operator DNA. ModE-molybdate at 5 nM completely protected the modABCD operator/promoter DNA from DNase I-catalyzed hydrolysis, whereas ModE alone failed to protect the DNA even at 100 nM. The apparent Kₐ for the interaction between the modA operator DNA and ModE-molybdate was 0.3 nM, and the Kₐ increased to 8 nM in the absence of molybdate. Among the various oxyanions tested, only tungstate replaced molybdate in the repression of modA by ModE, but the affinity of ModE-tungstate for modABCD operator DNA was 6 times lower than with ModE-molybdate. A mutant ModE(T125I) protein, which repressed modA-lac even in the absence of molybdate, protected the same region of modA operator DNA in the absence of molybdate. The apparent Kₐ for the interaction between modA operator DNA and ModE(T125I) was 3 nM in the presence of molybdate and 4 nM without molybdate. The binding of molybdate to ModE resulted in a decrease in fluorescence emission, indicating a conformational change of the protein upon molybdate binding. The fluorescence emission spectra of mutant ModE proteins, ModE(T125I) and ModE(Q216*), were unaffected by molybdate. The molybdate-independent mutant ModE proteins apparently mimic in its conformation the native ModE-molybdate complex, which binds to a DNA sequence motif of TATAT-7bp-TAYAT.

Molybdenum is necessary for the activity of several enzymes found in animals, plants, and bacteria, such as sulfite oxidase, xanthine dehydrogenase, nitrate reductase, formate dehydrogenase, and nitrogenase (1–3). From structural studies, it has been determined that molybdenum exists in molybdoenzymes in the form of a pterin-containing molybdenum cofactor, with the exception of dinitrogenase, which has an iron-molybdenum cofactor (1, 3, 4).

For Escherichia coli, the production of its complement of molybdoenzymes requires the efficient uptake of molybdate via the molybdate-specific transporter encoded by the modABCD operon (5–8). Analysis of the proteins encoded by the modABCD operon suggests that ModA functions as a molybdate-specific periplasmic binding protein (9), ModB as an integral membrane channel-forming protein, and ModC as an ATP-binding energizer protein. Mutations in genes coding for the molybdate-specific transporter in E. coli result in a loss of active molybdoenzymes, such as nitrate reductase and formate dehydrogenase (8). However, when sufficient levels of molybdate are added to the culture medium of these mutant strains, activity of the molybdoenzymes is restored, suggesting that in molybdate-supplemented media, molybdate enters the cell through alternate transport systems (10). It was determined that expression of the modABCD operon is regulated by the intracellular concentration of molybdate. High levels of intracellular molybdate resulted in reduction of transcription of the modABCD operon (10–12), which implies that E. coli has a molybdate-dependent repressor that regulates expression of the modABCD operon.

Isolation of E. coli mutants that did not exhibit the classical repression of the modABCD operon upon molybdate-supplementation of the growth medium enabled us to identify a putative molybdate-dependent repressor (ModE) of the modABCD operon (13). Similar genetic analysis also identified modE product as a potential repressor of the modABCD operon (7, 14). Purified ModE protein prevented transcription of modAB' template DNA in an in vitro coupled transcription-translation experiment (13). Molybdate is an essential component of this repression, and ModE-molybdate is the active form of the repressor. Molybdate-independent mutant forms of the repressor have been described (13, 14). Some of these mutant forms carried point mutations within the amino acid sequence TSARNQXXG (positions 125–133), which is conserved in ModE-homologs from several organisms and in Clostridium pasteurianum molybdopterin-binding proteins (8, 13). Other molybdate-independent mutants were missing different lengths of the C terminus of the ModE protein (13, 14). However, the kinetics of interaction of the mutant proteins with the modA operator DNA are yet to be established.

Confirmation of the binding of ModE to E. coli modA operator/promoter DNA was provided by electrophoretic mobility.
shift and by DNase I-footprinting of this DNA in the presence of ModE (15–17). McNicholas et al. (17), using the modABCD coding strand, determined that ModE protected a 28-bp stretch of modA DNA spanning from −18 to +10. It was proposed that two inverted repeat sequences located within the ModE-protected region serve as possible ModE binding sites. This inverted repeat sequence is similar to a consensus sequence, CGTTATATAN_{4-6}TATAACG, identified for molybdenum-regulated genes based on sequence similarity (18). However, McNicholas et al. (17) reported that ModE protected the modA operator DNA even in the absence of added molybdate, which is in contrast to the in vivo observations of strict molybdate dependence. A K_{D} value of about 25 nM was reported for the interaction between ModE-molybdate and the operator DNA (15, 17). This value of 25 nM is significantly higher than the subnanomolar K_{D} values reported for binding of other repressors to their cognate operator DNA (19–21). We reevaluated the kinetics of interaction between ModE and modA operator DNA, as well as the role of molybdate in this interaction, and the results are presented in this communication. Additional information on the interaction of various mutant forms of ModE with modA operator DNA is also presented.

**Experimental Procedures**

**Reagents**—Biochemicals were purchased from Sigma. Inorganic and organic chemicals were obtained from Fisher Scientific Co. and were analytical or molecular biological grade. Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs, Inc., or Promega. Sequenase 2.0 was obtained from United States Biological Corp. All oligonucleotides used in this study were synthesized by National Biosciences, Inc., or Genosys.

**Bacterial Strains**—E. coli K-12 strain SE2069 (ΔlacU169, rpsL, F\textit{modA}−lacZ102) and its isogenic \textit{modE} mutant, strain SE1811 (ΔlacU169, rpsL, =lacZ102, F\textit{modA}−lacZ102), were described previously (13). Strain BL21(DE3) carries ADE3, which contains phage T7 gene 1 (RNA polymerase) under the control of lac at the attB site. The plasmids used in this study are listed in Table I.

**Media and Growth Conditions**—L broth (LB), which served as rich medium, was supplemented with glucose (0.3%) or sodium molybdate (1 mM). Antibiotics, as needed, were included in media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml. Genetic and molecular biological experiments were performed essentially as described previously (6, 10, 13).

**DNA Sequencing Experiments**—All DNA sequences generated in the course of this study were obtained using the Sanger dye-末端 method and appropriate primers and plasmids isolated by alkaline lysis procedure (21). Restriction endonuclease-modified DNA was ligated to plasmid pUC19. Plasmid pAM6, was obtained by Bal-31 exonuclease digestion of plasmid pUC19. All mutations were confirmed by sequencing the DNA. Throughout this paper, the numbering of the bases.

**Construction of ModE Mutant Proteins**—\textit{ModE(T125I)} and \textit{ModE−}

![Image](https://www.jrc2jm.com/interaction-between-mod-e-and-modabcd-operator-dna.png)

1 The abbreviation used is: bp, base pair(s).
cleotides covering the modA operator region between 18 and +25, were synthesized (National Biosciences, Inc.) and biotin was incorporated into the 5'-end of one of the oligonucleotides that upon annealing would be located in the 5'-end of the DNA. All cuvettes were prepared by applying the same amount of modA operator DNA for generation of the immobilized ligand. The quantity of DNA present in the cuvette was further verified by monitoring the response of the instrument to a standard amount of ModE-molybdate.

The biotin cuvette in the optical sensor was rinsed with phosphate-buffered saline containing 0.1% Tween 20 (PBST). Streptavidin (100 μl; 1 mg/ml) in 10% PBST was added to the cuvette (200 μl reaction volume) and incubated for 6.5 min with mixing. After removing the unbound streptavidin, DNA was added and incubated until no further response was noted. Excess DNA was removed from the cuvette by 3 washes with PBST. ModE protein with or without molybdate (1 mM) was added to the cuvette, and the rate of increase in refractive index (response units in arc s) was recorded. After the maximum response was attained, free ModE in the cuvette was removed by a wash with PBST, and the rate of dissociation of ModE from the ModE-DNA complex was monitored. The experiment was repeated with various ModE concentrations, as well as with various mutant forms of ModE and mutated modA operator DNA.

In Vitro DNase I-Footprinting Experiments—DNase I protection experiments were performed as described previously (31). For these experiments, a 448-bp-modA-HindIII fragment from plasmid pAM4, which carries the modA operator/promoter DNA spanning from −247 to +25, was used after purification using a 10–30% sucrose gradient (32).

DNA Mobility Shift Experiments—DNA mobility shift experiments were performed as described by Fried and Crothers (33), with modification (31, 34). These experiments used a binding reaction buffer (10 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 50 mM NaCl, 1 mM diithiothreitol, and 5% glycerol). DNA (0.1 pmol; −18 to +25 region of the modA operator/promoter DNA or mutant modA operator derivative), and protein in a final volume of 10 μl. Sodium molybdate or other oxanions at a final concentration of 1 or 10 mM were also included in the reaction mixtures, gels, and electrophoresis running buffer, as needed.

Determination of the Molecular Weight of the ModE-DNA Complex—In order to determine the stoichiometry of the ModE-DNA association, binding reactions containing 0.1 pmol of a 43-bp DNA (−18 to +25 region) and 25 nM of ModE were prepared as described for the DNA mobility shift experiments. The binding reaction samples were then subjected to electrophoresis through a 5.0, 6.0, 7.0, or 8.0% polyacrylamide-Tris-borate-EDTA-nondenaturing gel as described for DNA mobility shift experiments (31). The distance of migration of ModE-molybdate DNA complex was compared with values obtained with protein standards. This information was used to produce a Ferguson plot (35), and the apparent molecular weight of the ModE-DNA complex was determined by extrapolation from the Ferguson plot. For determination of the Rf values of the protein standards, 15 μg of lactalbumin (14,200), 20 μg of carbonic anhydrase (29,000), 20 μg of chicken egg albumin (45,000), 15 μg of bovine serum albumin (monomer, 66,000; dimer, 132,000) and 6 μg of urease (dimer, 240,000; tetramer, 480,000) were subjected to electrophoresis along with the ModE-DNA complex.

Fluorescence Spectroscopy Measurements—Emission spectra were collected using an Amino Bowman Series 2 spectrofluorometer (Spectronic) at a bandpass of 4 nm. The detector voltage was set at 610 V. ModE proteins were diluted in Tris buffer (pH 8.0) with 0.5 mM dithiothreitol for measurement using a 1-cm path length. Three separate concentrations of ModE and mutant ModE proteins (1, 2.4, and 4.8 μM) were analyzed for intrinsic fluorescence with and without added molybdate.

RESULTS AND DISCUSSION

Identification of ModE Binding Sites in modA Operator/ Promoter DNA Using DNase I-Footprint Analysis—ModE has previously been shown to repress transcription of the modABCD operon only in a molybdate-dependent fashion (7, 13, 14). However, the purified ModE was reported to bind to modA operator DNA, even in the absence of added molybdate, and it protected the DNA from hydrolysis by DNase I (17). This raised the possibility that a fraction of ModE used in these experiments is contaminated with molybdate and the analytical sensitivity of molybdate determination methods is not high enough to detect its presence. In order to evaluate this possibility, ModE purified as described previously (13) was compared with ModE purified using the nickel affinity method (*ModE). The nickel affinity purification method was chosen because it involves only a limited number of steps, thereby decreasing the exposure of ModE protein to large volumes of buffer, which could be a possible source of molybdate contamination. The E. coli host used for expression of modE in our experiments, strain BL21 (DE3), was found to carry an unidentified mod operon mutation and thus is incapable of transporting molybdate when grown in rich medium not supplemented with molybdate.2 ModE binding to DNA was used as the assay for molybdate contamination of ModE, and to increase the sensitivity, DNase I footprinting was used as the analytical tool.

The results presented in Fig. 1 (top panel) show that ModE alone partially protected modA DNA from DNase I hydrolysis at a concentration of 50 nM (lanes 2–4). A hypersensitive A at position +5 can also be seen at this concentration of ModE. Addition of molybdate to the binding reaction reduced the amount of ModE-molybdate required for complete protection to less than 5 nM (Fig. 1, lane 6). In the presence of *ModE protein purified using the nickel affinity procedure, protection of modA operator/promoter DNA from DNase I hydrolysis was dependent on molybdate (Fig. 1, lanes 11–18). Even at 100 nM *ModE, the modA operator DNA was not protected from DNase I (Fig. 1, lane 14). These results suggest that greater than 95% of the *ModE in this preparation is free of molybdate and that the active form of ModE binding to modA operator DNA is ModE-molybdate.

Analysis of the ModE-molybdate protected region of the modA operator/promoter DNA after DNase I cleavage of the coding strand (Fig. 1, lanes 6–9 and 15–18) revealed that there are three areas of protection detectable at a ModE-molybdate concentration as low as 5 nM. The first protected region, GT-TATATT, spans from −15 to −8 and overlaps the modA −10 sequence (Fig. 1, bottom panel). The second ModE-protected region, GCCTACAT spans from −4 to +4 of the modA operator/promoter DNA, whereas the third protected region, GTTACAT, is located at bases +8 to +14. Each of these three DNase I-cleavage protected regions contains either a TATAT (protected region 1) or a TACAT (protected regions 2 and 3) sequence, which suggests that it is these sequences that are recognized by ModE as the target binding site. The independent protected sites are separated by three bases, and the pentameric sequences in regions 1 and 3 are preceded by the same two bases, GT. Aside from the three protected regions, the base A at position +5, located next to the 3' end of the second protected region, is hypersensitive to DNase I cleavage. An inverted repeat of bases (−16 to +8) also exists within the ModE-protected region of the modA operator/promoter DNA. Mccollins et al. (17), based on DNase I footprinting, reported that ModE protected the entire region of DNA spanning from −18 to +10. The hypersensitive A at position +5 was not detectable in the reported ModE footprint (17). Although the hypersensitive site is recognizable in the ModE-modA DNase I footprint reported by Anderson et al. (15), the unprotected bases between the regions 1 and 2 were not discernable. The fact that the DNase I footprint presented in Fig. 1 resolves the modA operator/promoter region into discrete ModE-binding regions with a distinct DNase I hypersensitive site delineating protected regions 2 and 3 suggests that ModE binds and protects the three indicated regions in vivo also. The *ModE also protected the same bases with the hypersensitive adenine in the modA operator DNA, but the amount of protein required for complete protection was slightly higher (10 nM versus 5 nM for ModE-molybdate). Similar DNase I footprinting of the noncoding
failed to bind ModE-molybdate (data not presented). The modA operator DNA from plasmid pAM17 that lacks the third pentamer sequence did bind ModE-molybdate, and the DNA-protein complex migrated at a lower rate than the DNA alone during electrophoresis (data not shown). The apparent $K_d$ for the interaction between the DNA lacking the region three and ModE-molybdate in an optical sensor experiment was about three times higher (1 nM) than the native DNA with all three sites (0.3 nM). Based on these results, the DNA in the first and second protected regions is essential for binding ModE-molybdate, whereas the third pentamer enhances ModE-molybdate binding.

The GTTA sequence found in the ModE-protected regions 1 and 3 repeats itself nine times within the presented 81-bp region of the modA operator/promoter DNA (Fig. 1, upper panel). The function of this tetramer sequence in modA DNA is not known. However, the presence of this tetramer sequence in the ModE-molybdate-protected region in both the hyc and narXL promoter DNA (31) suggests that ModE-molybdate does recognize the sequence GTTA.

**Determination of the Stoichiometry of the Association of ModE with modABCD Operator DNA**—The DNA mobility shift experiments with native and mutant forms of modA operator DNA indicated that a stable ModE-molybdate-DNA complex may involve contact with only two of the three ModE-protected regions. If this is the case, and given the size of the ModE protein (28,711 Da) and the target binding sites (TATAT and TACAT), it is likely that ModE would bind to each of the two ModE-protected regions as a monomer. To determine the stoichiometry of the ModE-DNA association, ModE was bound to a 43-bp modA operator/promoter DNA that contains all three ModE-protected regions in a standard DNA mobility shift binding reaction. The calculated apparent molecular weight of ModE-molybdate-DNA complex, based on its migration through a set of different percentage polyacrylamide gels and the resulting Ferguson plot (35) of these data, was 81,247. This molecular weight compares favorably with an apparent molecular weight of 83,026 expected for the association of a ModE dimer (56, 424) with the 43-bp DNA (26, 602) (Fig. 2). Thus, the ModE-DNA complex consists of a ModE dimer associated with the 43-bp-long DNA. For this stoichiometry determination, the axial ratio and electrophoretic mobility of the protein-DNA complex was considered to be similar to the characteristics of the protein itself, because the DNA used in this experiment was only 43 bp. Sedimentation equilibrium analysis of ModE also revealed that the protein exists as a homodimer in solution (15). The ModE-molybdate apparently binds to the modA operator/promoter DNA with 2-fold symmetry as a dimer using the palindromic consensus operator with the proteins centered around the pentamer sequence TATAT or TACAT.

**Molybdate-independent Mutant ModE**—Grunden et al. (13)
previously described several ModE mutants that are either inactive (A76V) or molybdate-independent (T125I and Q216*) in modA repression. These mutant proteins were purified using His tag, nickel affinity chromatography. In agreement with the in vivo results, the *ModE(A76V) did not protect modA operon/promoter DNA from DNase I hydrolysis (Fig. 3, lanes 9–11). Inclusion of molybdate in the reaction mixture did not increase the affinity of *ModE(A76V) for the DNA (data not presented). Although the *ModE(T125I) mutant protein protected the same region of DNA protected by native ModE, the amount of protein required for complete protection in the absence of molybdate was close to 100 nM (Fig. 3, lanes 6–8). At this concentration, the level of protection by the *ModE(T125I) protein was about 2 times better than the *ModE protein. The higher affinity of *ModE(T125I) in the absence of molybdate is in agreement with the observed inability of ModE-molybdate-DNA complex.

**Kinetics of Interaction between ModE and modA Operator DNA**—The apparent $K_d$ for binding of MoeE-molybdate to modA operator DNA was previously reported to be about 25 nM (15, 17). This $K_d$ value is significantly higher than the <5 nM of ModE-molybdate required for complete protection of modA operator/promoter DNA from DNase I hydrolysis (Fig. 1, lane 6). To resolve this difference, the interaction between ModE and modA operator/promoter DNA was determined using an evanescent wave biosensor. Upon addition of 13.5 nM *ModE-molybdate, a rapid response signifying binding was observed, and the maximum binding was achieved within 80 s (Fig. 4A). In the absence of added molybdate, ModE bound to the same DNA at a lower rate, and the total response signifying the amount of protein bound was also lower. However, both forms of protein showed a concentration response to association with DNA in the cuvette (Fig. 4, B and C). Upon removal of excess ModE, the ModE without molybdate dissociated from DNA at a higher rate than the sample with molybdate. ModE-molybdate did not bind to a mutant modA operator DNA (TACAT between −1 and +4 changed to CTTGG; plasmid pAM18) (Fig. 4A), demonstrating the specificity of ModE-Mo for native modA operator DNA. The small increase in response observed with the mutant DNA and ModE-molybdate was seen with the native DNA also and is apparently due to a change in refractive index of the buffer associated with ModE addition. However, nonspecific adsorption of the protein to the DNA cannot be ruled out.

The apparent $K_d$ for this interaction between *ModE and modA operator/promoter DNA (−18 to +25) was calculated to be 0.3 nM. An apparent $K_d$ of 0.4 nM was obtained when native ModE was used in these experiments. This apparent $K_d$ value is similar to the subnanomolar apparent $K_d$ values reported for other repressor/operator DNA interactions (19–21). In the absence of molybdate, this apparent $K_d$ value for the interaction between ModE and modA operator/promoter DNA increased to 8 nM.

When *ModE(T125I) mutant protein was used in these experiments, no significant difference was observed either in the rate of association or dissociation of the protein with the DNA both in the presence and absence of molybdate (Fig. 5). The apparent $K_d$ for the interaction between *ModE(T125I) and modA operator DNA was 3 nM in the presence of molybdate and 4 nM in the absence of molybdate. These values are about 10 times higher than the values obtained with native ModE-molybdate but about 1⁄2 of the $K_d$ value obtained with ModE alone. The *ModE(A76V) protein did not bind to the DNA at a protein concentration as high as 54 nM, either with or without molybdate (Fig. 5A). This is in agreement with the observed inability of this mutant protein to repress modA-lac in vivo or to protect the DNA from DNase I hydrolysis (Fig. 3) (19). These results also show that the interaction between ModE-Mo and modA operator DNA is specific. The kinetics of *ModE(Q216*) interaction with DNA was found to be complex, and the apparent $K_d$ value for this interaction was not determined.

It is interesting to note that the calculated apparent $K_d$ for *ModE(T125I) in the absence of molybdate is about 3 nM, whereas that of native ModE is 8 nM. However, in vivo, ModE(T125I) repressed modA-lac expression in the absence of molybdate, whereas native ModE failed to repress it in the absence of molybdate. The rate of dissociation of the two pro-
from the cuvette to monitor the dissociation of ModE from the DNA. B, for the interaction of a mutant protein has been previously described than does the native ModE. This phenomenon of a lower rate of dissociation of the protein-DNA complex at an appreciably lower rate compared with the native ModE characteristics can possibly be reconciled by the observation that the ModE(T125I) dissociates with different concentrations of *ModE-molybdate and native modA operator DNA. Each dot in the response curve represents a single data point. C, pseudo-first order rate constant (k_a) values versus [ModE]. Total change in refractive index due to binding obtained at different [ModE] was analyzed with the IAsys FASTfit software assuming a single exponential association. The linear plot of [ModE].

**FIG. 4.** Optical sensor response curves for ModE binding to modABCD operator/promoter DNA. A, *ModE protein (15.5 nM) in the presence of native modA operator DNA (top two lines) or mutant modA operator DNA from plasmid pAM18 (bottom line). Molybdate concentration was 1 mM. Upward arrow indicates addition of ModE protein, and the downward arrow denotes the time at which the excess ModE was removed from the cuvette to monitor the dissociation of ModE from the DNA. B, response curves with different concentrations of *ModE-molybdate and native modA operator DNA. Each dot in the response curve represents a single data point. C, pseudo-first order rate constant (k_a) values versus [ModE]. Total change in refractive index due to binding obtained at different [ModE] was analyzed with the IAsys FASTfit software assuming a single exponential association. The linear plot of k_a value at various protein concentrations has the association and dissociation rate constants k_a and k_d as the slope and y intercept, respectively (29). The equilibrium constant K_d was obtained as the ratio of k_d/k_a.

**FIG. 5.** Optical sensor response curves for mutant ModE binding to modABCD operator/promoter DNA. A, *ModE(T125I) (top two lines) or *ModE(A76V) (bottom line) at 13.5 nM with native modA operator DNA. Molybdate, when present, was at 1 mM. B, response curves with different concentrations of *ModE(T125I) and native modA operator DNA. C, k_a values for *ModE(T125I) versus *ModE(T125I) concentration. See Fig. 3 for details.

Proteins from the DNA was found to be significantly different (Figs. 4 and 5). Thus, the apparent discrepancy between the in vivo activity of ModE(T125I) and its in vitro kinetic parameters compared with the native ModE characteristics can possibly be reconciled by the observation that the ModE(T125I) dissociates from the protein-DNA complex at an appreciably lower rate than does the native ModE. This phenomenon of a lower rate of dissociation of a mutant protein has been previously described for the interaction of trp superrepressor with its operator DNA (36).

**Fluorescence Characteristics of ModE Mutant Proteins—** Anderson et al. (15) reported that the intrinsic fluorescence of ModE protein decreased upon binding molybdate and this could be a result of conformational change of the protein. Because the ModE mutant proteins are molybdate-independent, it is possible that the mutant proteins structurally mimic the ModE-molybdate complex. If this is indeed the case, the intrinsic fluorescence of the mutant proteins should not be altered by addition of molybdate. When the *ModE protein was excited by irradiation at 290 nm, the emission spectrum had a peak at 347 nm and a shoulder at 370 nm (Fig. 6). The peak of emission shifted down to 343 nm in the presence of molybdate, and the relative fluorescence was also reduced by about 60%. This reduction in fluorescence was dependent on the molybdate concentration. Relative fluorescence of ModE decreased linearly between the molybdate concentrations of 0.2 and 2.0 μM, and maximum response was observed at about 10 μM. The amount of molybdate required for a reduction of 1/2 of the total extent of fluorescence change was about 0.75 μM, and this value is similar to the 0.8 μM reported by Anderson et al. (15) for an equivalent change. ModE has three tryptophans (positions 49, 131, and 186) (Fig. 7), and the tryptophan at 131 is located within a sequence motif (125TSARNQWF133) that is conserved in several ModE homologs from other organisms ((T/S)SARNQXXG) and also in a molybdopterin-binding protein from C. pasteurianum (8). It is possible that molybdate binding to ModE buried the tryptophan at 131, and its fluorescence is not readily observable. Because a mutation that changed the Thr (to Ile at 125) or Gly (to Asp at 133) in E. coli ModE converted the mutant proteins to molybdate-independent forms (13), it is possible that the conformation of the mutant protein mimics ModE-molybdate. In agreement with the possible conformational change, the relative fluorescence of ModE(T125I) was not altered by molybdate (Fig. 6). The peak of emission was 339 nm, which is closer to the peak of emission of ModE-molybdate (343 nm) than that of ModE (347 nm). The intrinsic fluorescence of the C-terminal deletion protein ModE(Q216*) was also not affected by the addition of molybdate (Fig. 6). These results suggest that the molybdate-independent ModE mutant proteins resemble ModE-molybdate in its conformation. This “locked-on” conformation may have allowed the protein to bind to DNA, even in...
the absence of added molybdate. However, this consequence is apparently not the same as that of ModE-molybdate because than the native ModE-molybdate (Figs. 4 and 5).

Other ModE Mutant Proteins—The C-terminal 47 amino acids (217–262), which contain all three cysteines in the protein (positions 217, 230, and 262) (Fig. 7) are apparently essential for normal function of ModE, because ModE(Q216*), which lacks the amino acids from 216 to the C terminus, is molybdate-independent for repression of modABCD operon. The conformation of ModE(Q216*), based on intrinsic fluorescence emission characteristics, is also significantly different from that of native ModE. It is possible that one or more of these cysteines play a role in the conformation change of ModE in response to molybdate. In order to evaluate this possibility, two different sets of ModE mutants were constructed. In the first set, two additional deletion derivatives lacking one or two cysteines were constructed (ModE-220hyb and ModE-N252*). In ModE(220hyb), the cysteines at positions 230 and 262 were removed, and in ModE(N252*), only the C-terminal cysteine was removed. Both of these ModE mutants were found to be molybdate-independent for repression of modABCD operon (Table II). In an alternate experiment, the three cysteines were changed individually by mutagenesis, and the length of the protein was maintained at the full length of 262 amino acids. The mutant proteins repressed modA-lac expression only in modE-lac strains, and the H. influenzae ModE protein can functionally replace the E. coli ModE in vivo (16). Deleting these amino acids eliminated the biological activity of the ModE protein (Table II), suggesting that the N-terminal part of the protein is critical for repression of modABCD expression.

Changing the amino acids in the SARNQ region (amino acids 126–130; Fig. 7), especially the basic amino acids RN, to PG would be expected to cause a structural change, and such a mutant protein was found to be inactive (Table II). McNicholas et al. (14) replaced some of the amino acids in the SARNQ region individually and found that the repression ratio was altered ($\beta$-galactosidase activity from mod-lac; $-\text{Mo}^+/\text{Mo}$). Taken together, these results indicate that the amino acids in the TSARNQXXG region, as well as the C-terminal 11 amino acids, are critical for molybdate-dependent conformational

TABLE II

| Amino acid | Mutation | $\beta$-Galactosidase activity |
|------------|----------|-------------------------------|
| Wild type  | None     | 650 < 10                      |
| 220hyb     | $\Delta$66-end | -10 < 10                  |
| N252*      | $74^{\text{AAT}}$ to TAA | -10 < 10                 |
| C217L      | $69^{\text{TGGCA}}$ to CTCGAG | 660 < 10              |
| C230E      | $69^{\text{GTGC}}$ to CGAG | 620 < 10              |
| C262E      | $78^{\text{GTCG}}$ to CGAG | 710 < 10             |
| $\Delta$5ILLTL9 | $\Delta$13–27 | 670 < 620             |
| RN(128,129)/PG | $383^{\text{GTAAC}}$ to CCGGG | 580 540               |

also conserved in Hemophilus influenzae ModE protein (37), and the H. influenzae ModE protein can functionally replace the E. coli ModE in vivo (16). Deleting these amino acids eliminated the biological activity of the ModE protein (Table II), suggesting that the N-terminal part of the protein is critical for repression of modABCD expression.

Fig. 6. Fluorescence emission spectra of ModE and mutant ModE proteins. Excitation wavelength was 290 nm. Protein concentration was 1 $\mu$M. A, fluorescence emission spectra of *ModE at various molybdate concentrations. B and C, mutant ModE proteins. Solid line, without added molybdate; dashed line, with 1 mM molybdate.
change of the ModE protein.

**Oxyanion Specificity of ModE.**—The possibility that other oxyanion analogs of molybdate could functionally substitute for molybdate in promoting the association of ModE with modA operator/promoter DNA was investigated under both in vivo and in vitro conditions. It was found that molybdate and tungstate can support ModE-mediated repression in vivo, because addition of 1 mM sodium molybdate or sodium tungstate to strain SE2069 culture medium resulted in extremely low modA-‘lacZ expression (30 and 40 units of β-galactosidase activity, respectively, compared with 1300 units of activity produced by strain SE2069 grown without molybdate or tungstate). Neither sodium sulfate nor sodium vanadate could substitute for molybdate in vivo, as evidenced by the resultant high or nonrepressed β-galactosidase activity levels. These results show that tungstate is the only other oxyanion tested that is effective in vivo. The apparent Kd also is borne out by direct ModE-binding experiments with the sic fluorescence characteristics of ModE-molybdate complex.

Therefore, the successful activation of ModE relies solely on the binding of an appropriately sized and shaped moiety to affect DNA.

**Interaction between ModE and modABCD Operator DNA**

The possibility that other repressors (19–21) may be modulated in this regard is similar to that of the native ModE-molybdate complex with its cognate operator DNA. Three regions in the modA operator DNA can be identified as ModE-binding areas (−15 to +15), and only the segment between −15 and +10, which also includes the sequence motif TATAT-7bp-TACAT, appears to be critical for binding of ModE-molybdate as a dimer.

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