MalT, the transcriptional activator of the maltose regulon from *Escherichia coli*, is the prototype of a new family of transcription factors. Its activity is controlled by multiple regulatory signals. ATP and maltotriose (the inducer) are two effectors of the activator that positively control its multimerization, a critical step in promoter binding. In addition, MalK, the ABC component of the maltodextrin transport system, and the two enzymes MalY and Aes down-regulate MalT activity in *vivo*. By using a biochemical approach, we demonstrate here that (i) Aes controls MalT activity through direct protein-protein interaction, (ii) Aes competes with maltotriose for MalT binding, (iii) ATP and ADP differentially affect the competition between Aes and the inducer, and (iv) part, if not all, of the Aes binding site is located in DT1, the N-terminal domain of the activator, which also contains the ATP binding site. All of these characteristics point toward an identical mode of action for MalT and Aes. However, we have identified an amino acid substitution in MalT that suppresses MalT inhibition by Aes without interfering with its inhibition by MalY, suggesting that the binding sites of the two inhibitory proteins do not coincide. The differential effects of ATP and ADP on the competition between the inducer and Aes (or MalY) suggest that the ATPase activity displayed by MalT plays a role in the negative control of its activity.

MalT, the transcriptional activator of the maltose regulon of *Escherichia coli*, is the archetype of a new family of bacterial transcriptional activators of approximately 100 kDa (1, 2). It displays distinctive features compared with the other types of regulatory proteins. Transcription activation by MalT involves cooperative binding of the protein to an array of sites located upstream from the −35 region of the target promoters and requires the presence of two positive effectors, maltotriose (the inducer) and ATP (reviewed in Ref. 3). Recent studies revealed that the unliganded form of the protein is monomeric and that the function of both of these effectors is to induce MalT self-association, thought to play a critical role in promoter binding (4). MalT is endowed with a weak ATPase activity whose role is thus far elusive; activation of open complex formation does not require ATP hydrolysis (5).

The activity of MalT is also negatively controlled by three different proteins, MalK, MalY, and Aes. MalK is the ABC component of the maltodextrin transport system (3). Its role as a phenotypic repressor of the maltose system was recognized with the finding that a malK null mutation renders expression of the regulon constitutive, whereas overexpression of MalK abolishes its induction (6–9). Genetic data suggest that MalK acts as a repressor of MalT when the maltodextrin transport system is resting, thereby preventing induction of the maltose regulon by endogenous maltotriose in the absence of maltodextrins in the growth medium (10). In contrast, little is known regarding the raison d’être of the negative controls exerted by MalY and Aes on MalT. MalY (2 × 43.5 kDa) is a cytoplasmic protein that exhibits BC-S lyase activity (11, 12). It is coexpressed with MalX, an enzyme II of the phosphotransferase system whose natural substrate is unknown. Induction of the *malY* gene by a null mutation in *malI*, the gene encoding the repressor of the *malXY* operon, abolishes constitutive expression of the maltose regulon in a *malK* strain and considerably delays induction of the maltose regulon by external maltodextrins in a wild-type strain (13). Aes is a cytoplasmic protein displaying esterase activity whose overexpression severely depresses expression of the maltose regulon in a *malK* strain (14, 15). The basal level of expression of the chromosomal *aes* gene is very low (14), and the physiological conditions causing induction of the *aes* gene as well as the physiological role of the enzyme are not obvious.

The mechanism by which MalT activity is negatively controlled is best understood in the case of MalY. *In vitro* studies have revealed that MalY interferes with transcription activation by MalT by directly interacting with the activator. MalY and maltotriose compete for binding to MalT (16). The available data are consistent with a model in which MalT is in equilibrium between two forms: an inactive, monomeric form stabilized by MalY and an active, monomeric form stabilized by maltotriose and prone to multimerize. In the inactive form, the maltotriose binding site would be either masked or altered so as to show a lower affinity for maltotriose, whereas in the active form, the MalY binding site would be either masked or distorted. As revealed by the x-ray structure of MalY, amino acid substitutions in MalY that impair MalT inhibition delineate a patch on the surface of the protein that most likely represents the MalT contact site (17). Based on *in vivo* observations, it was proposed that MalK and Aes also inhibit MalT by antagonizing maltotriose binding (3): overexpression of MalT relieves repression by MalK and Aes (10, 14), and MalT constitutive mutants that have a higher affinity for maltotriose than wild-type MalT are less sensitive to MalK and Aes in *vivo* (18, 33). Panagiotidis et al. (10) have also provided direct evidence for a physical interaction between MalT and MalK.

Structural studies have begun to provide insights into the mechanism by which some of these positive and negative regulatory signals are integrated at the level of the MalT protein.
The activator (103 kDa) is made of four structural domains: domain 1 (DT1; residues 1–241), domain 2 (DT2; residues 250–436), domain 3 (DT3; residues 437–806), and domain 4 (DT4; residues 807–901) (20, 21). DT1 binds ATP and MalY (21, 23). DT3, whose x-ray structure is known (22), binds maltotriose with a low affinity; high affinity binding is observed only with DT2-DT3 (21). DT4, a member of the LuxR-type DNA-binding domain family, contains the DNA binding fold; it is also thought to contact RNA polymerase (23). As revealed by limited proteolysis, maltotriose binding triggers a conformational change in MalT that involves domains 1–3 and most likely reflects part of the postulated transition from the inactive form to the active form of MalT (21). Several lines of evidence suggest that the three N-terminal domains of the protein represent a new signal integration module (21).

To obtain a better understanding of how the activity of MalT is negatively modulated, we have analyzed in vitro the mechanism whereby the Aes protein inhibits MalT. We show that Aes alone interferes with transcription activation by MalT in a purified transcription system and that Aes acts via the same mechanism as MalY, although their cognate recognition sites on MalT are likely to be distinct.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**Strain pop7164, an MC1401 derivative harboring ΔmalT720, a deletion of the entire malT gene, is described by Schreiber et al. (16). Plasmid pOM2nlmalT7 T38R was constructed as follows. The mutation leading to the T38R amino acid substitution (codon change ACC→CGC) was introduced in plasmid pOM2nlmalT7 (24) by using the QuickChange™ site-directed mutagenesis kit (Stratagene). After verification of its sequence, the mutated PstI-AatII fragment (509 bp), which contains the malT7 T38R mutations, was gel-purified and ligated to the gel-purified AatII-PstI fragment (6616 bp) of plasmid pOM2 (25). Plasmid pOM163 was constructed by inserting a blunt-ended Strep tag-encoding linker (sequence of the coding strand, 5′-GCTTCGAAGGCACCCGATTTGAAAAA) in the EcoH site of the pOM150 plasmid (21) and cloning the NeoI-HindIII fragment of the plasmid obtained between the NeoI and HindIII sites of pET28h (+) (Novagen). pOM163 T38R was obtained by amplifying the DT1 T38R-encoding fragment of pOM2nlmalT7 by PCR using oligonucleotides MalT-nt (40 m M HCl, pH 8.0), 33 mM tri-potassium citrate, 10 mM magnesium acetate, 1 mM dithiothreitol, 220 μM acetylated bovine serum albumin (Sigma), 5 mM malTp7 DNA fragment, the indicated concentrations of maltotriose and adenine nucleotide, 0.1 μM pyridoxal-5'-phosphate (in the assays with MalY), and Aes or MalY when indicated. RNA polymerase solution (2 μL) (0.54 μM in 40 mM HEPES-KOH (pH 8.0), 33 mM tri-potassium citrate, 10 mM magnesium acetate, 0.12 mM EDTA, 1.1 mM dithiothreitol, 220 μM acetylated bovine serum albumin (Sigma), 5 mM malTp7 DNA fragment, the indicated concentrations of maltotriose and adenine nucleotide, 0.1 μM pyridoxal-5'-phosphate (in the assays with MalY), and Aes or MalY when indicated. The reaction products were separated from free [α-32P]CTP by chromatography on Whatman 3MM paper as described by McClure (30). The chromatograms were dried and scanned on a PhosphorImager, and the amount of ApApC synthesized was quantified. The malTp7 DNA used as template is a 320-bp fragment containing the malPp gene, is described by McClure et al. (16). Plasmid pOM2—The Binding Assay—The 30-μL reaction mixture contained 22 mM HEPES-KOH (pH 8.0), 10 mM Tris-HCl (pH 7.7), 22 mM tri-potassium citrate, 6 mM magnesium acetate, 0.07 mM EDTA, 0.1 mM ATP, 1 μM [α-32P]malT (850 μCi/mmol), 0.2 mg/ml acetylated bovine serum albumin, and MalT and Aes as indicated. After 5 or 30 min of incubation at 20 °C, the tube was chilled on ice for 2 min, and the proteins were precipitated by adding 100 μL of a solution containing 3.0 M ammonium acetate and 50% (v/v) ethanol and centrifuging at 14,000 × g for 20 min at −80 °C. The precipitated proteins were washed with 70% (v/v) ethanol containing 0.1 M NaCl and 0.1 M EDTA, air-dried, and then resuspended in 50 μL of sample buffer containing 50 mM Tris-HCl (pH 8.0), 33 mM tri-potassium citrate, 10 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, 100 μM ATP or ADP (or 500 μM AMP-PNP) and, when indicated, 1 mM maltotriose. Twenty μL of sample were then injected onto a Superose 12 column (3.2 × 300 mm) or onto a Superdex 200 column (3.2 × 300 mm) (Amersham Biosciences) installed on a SMART system (Amersham Biosciences) and equilibrated with the sample buffer. Filtration was performed at 6 °C at a flow rate of 40 μL/min. When needed, 50-μL fractions were collected. The columns were calibrated with globular proteins: bovine thyroglobulin (669 kDa), sweet potato β-amylase (200 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and horse heart cytochrome c (12.5 kDa).

**Affinity Chromatography—**Soluble extracts containing DT1S or DT1S T38R were prepared from 125 mL of LB medium (31) containing 20 μg/ml ampicillin and 50 μg/ml chloramphenicol incubated with (pOM163) or BL21(DE3) AesT220 (pOM163 T38R) at A900 = 0.1 and grown to A900 = 1.5 at 37 °C. Isopropyl-thio-β-D-galactoside (1 mM) was then added, and growth was continued at 24 °C for 4 h. Cells were collected, washed, and resuspended in Tris-HCl buffer (50 mM; pH 7.7).

1 The abbreviation used is: AMP-PNP, adenylyl-imidodiphosphate.
containing 0.5 mM KCl, 10% sucrose, and 10 mM MgCl₂. The cells were disrupted in a French press (16,000 p.s.i.) and centrifuged (30 min at 180,000 × g). The supernatant was flash-frozen and kept at -20 °C.

Affinity chromatography was performed at 6 °C in Micro Biospin® Bio-Rad columns packed with 50 µl of Strep-Tactin® Sepharose® resin (IBA). Solutions were passed through the columns by spinning at 11 × g for 1 min in a refrigerated benchtop centrifuge. The columns were equilibrated with cell disruption buffer containing 0.1 mM ATP and loaded with 2 × 50 µl of DT1S soluble extract or 4 × 50 µl of DT1ST38R soluble extract supplemented with 1 mM ATP (after each load, the protein was allowed to bind for 5 min). The columns were washed with 6 × 100 µl (DT1S) or 8 × 100 µl (DT1ST38R) of washing buffer (50 mM Tris-HCl (pH 7.7), 0.1 mM KCl, 10% sucrose, 1 mM MgCl₂, and 0.1 mM ATP). Aes (50 µl at 1 mg/ml in washing buffer) or carbonic anhydrase (50 µl at 2 mg/ml in washing buffer) was allowed to flow through the column, and unbound proteins were washed out with 4 × 50 µl of the same buffer containing 2.5 mM desthiobiotin (Sigma-Aldrich). Nine 50-µl fractions were collected, starting with the input of Aes or carbonic anhydrase, and analyzed by 12% SDS-PAGE (37:5:1, acrylamide:bisacrylamide).

RESULTS

Aes Inhibits Transcription Activation by MalT in Vitro—MalT inhibition by Aes was assessed in vitro by measuring the ability of MalT to activate open complex formation at malPp, a MalT-dependent promoter, in the presence of Aes. MalT was preincubated with malPp in the presence of ATP, maltotriose, and various concentrations of Aes before adding RNA polymerase and allowing open complex formation. The amount of open complexes formed was then quantified by measuring the rate of abortive product synthesis. Given that maltotriose might compete with Aes (9), the Aes effect was investigated in the presence of various concentrations of inducer. Note that for each combination of ligand concentrations and MalT variant used in this work, we determined the response curve, i.e. the amount of open complexes formed, as a function of MalT concentration. The MalT concentration used to assess the Aes effect was that eliciting about half of the maximum response under the chosen conditions (the inset of Fig. 1A shows the response curve obtained in the presence of 50 µM ATP and 0.1 mM maltotriose). This ensures that MalT inhibition by Aes, if any, would be detected.

As shown in Fig. 1A, 9 µM Aes strongly depressed open complex formation at malPp in the presence of 0.1 mM maltotriose, whereas no significant inhibitory effect was observed in the presence of 1 mM maltotriose. The observation that MalT inhibition by Aes was relieved in the presence of a high concentration of inducer suggests that the inhibitory effect observed in vitro is functionally relevant.

The malT26 mutation, which generates the R242P substitution in the DT1-DT2 linker, confers constitutive expression of the maltose regulon by favoring the transition from the inactive state to the active state of MalT (18, 21), and it is known to abolish MalT sensitivity to Aes in vivo (33). To confirm that the inhibition caused by Aes in vitro had a functional significance, we tested whether the MalT26 variant displays a reduced sensitivity to Aes in vitro. As shown in Fig. 1B, MalT26 was scarcely inhibited by Aes in the presence of 0.1 mM maltotriose, i.e. a maltotriose concentration allowing repression of wild-type MalT by Aes (Fig. 1A). This therefore demonstrates that the inhibitory effect observed in vitro reflects the phenomenon of MalT repression by Aes observed in vivo. Furthermore, MalT26 was inhibited by Aes when assayed in the absence of maltotriose (Fig. 1B). All these data therefore indicate that MalT26 is still intrinsically sensitive to Aes and that one effect of malT26 is to decrease the concentration of maltotriose required to relieve MalT repression by Aes. Together with the fact that MalT26 displays a higher affinity for maltotriose (18), this observation further supports the hypothesis of a competition between maltotriose and Aes.

MalT and Aes Form a Complex—To test the hypothesis that MalT inhibition by Aes involves a direct protein-protein interaction, we tried to isolate a MalT-Aes complex by gel filtration. Samples containing MalT and/or Aes were preincubated for 20 min at 20 °C and passed through a Superose 12 column at 6 °C with ATP present throughout the experiment (Fig. 2A). In the absence of Aes, MalT eluted as a 96-kDa globular protein (elution volume, 1.30 ml), consistent with the fact that MalT is mainly monomeric under these conditions (4). Aes alone eluted as a 57-kDa globular protein (elution volume, 1.38 ml). When MalT and Aes, present in a 1:1 (protomer:protomer) ratio, were chromatographed together, a single peak was observed that eluted earlier than MalT, with an apparent molecular mass of 153 kDa (elution volume, 1.23 ml). SDS-PAGE analysis confirmed that both proteins were present in the peak fractions (Fig. 2B). Therefore, Aes and MalT do form a complex.

To find out whether maltotriose antagonizes Aes binding to MalT, we performed gel filtration experiments in the presence
of both ATP and 1 mM maltotriose, the concentration of inducer relieving inhibition of wild-type MalT by Aes in our transcription system (Fig. 2C). When chromatographed alone, Aes eluted at the same position as described above, whereas MalT eluted as a $\sim$300-kDa protein, consistent with the fact that MalT is multimeric in the presence of ATP and maltotriose (4). Filtration of a sample containing both MalT and Aes gave two peaks at the positions at which MalT and Aes, respectively, elute when chromatographed alone. These data clearly demonstrate that maltotriose inhibits interaction between MalT and Aes.

In the above-mentioned experiments, complex formation was observed with a sample containing 9 $\mu$M MalT and 9.5 $\mu$M Aes, and no additional peak was observed, thereby suggesting that the MalT:Aes ratio in the inhibition complex is 1:1 (protomer: protomer). This interpretation was confirmed by showing that filtration of samples containing MalT and Aes in a 2:1 or 1:2 ratio (18 $\mu$M MalT + 9.5 $\mu$M Aes or 9 $\mu$M MalT + 19 $\mu$M Aes) gave an additional peak at the position at which the protein present in excess is expected to elute (data not shown).

Finally, chromatography of samples containing increasing concentrations of both proteins present in a 1:1 (protomer: protomer) ratio showed that the apparent molecular mass of the complex keeps increasing with the concentration of the partners. For instance, chromatography of a sample containing 4.5 $\mu$M of each protein through a Superdex 200 column (whose separation range extends up to 600 kDa) gave a unique peak at 1.45 ml (apparent molecular mass, 125 kDa), whereas filtration of a sample containing 36 $\mu$M of each protein gave a peak at 1.34 ml (apparent molecular mass, 210 kDa) (data not shown). This suggests that the MalT-Aes complex is actually in rapid equilibrium with dissociated MalT and Aes forms, with the fraction of the complexed proteins increasing with the concentration of the proteins, and the position of the peak reflecting the weighted average Stokes radius of the species in equilibrium.

Aes Inhibits Maltotriose Binding by MalT—Having observed that maltotriose antagonizes Aes binding by MalT, we tested whether, inversely, Aes inhibits maltotriose binding. Maltotriose binding by MalT was measured at 1 $\mu$M substrate by ammonium sulfate precipitation. As shown in Fig. 3, Aes reduced the amount of maltotriose bound by wild-type MalT by about two-thirds, whereas it did not affect maltotriose binding by the
MalTc26 variant. The same values were obtained for 5-min and 30-min incubation times, thereby excluding the possibility that the difference observed between the two MalT variants was due to kinetic effects. Together with the ability of maltotriose to prevent Aes binding by MalT, these data conclusively demonstrate that, like MalY, Aes competes with the inducer for MalT binding.

The MalT/ADP Form Is Insensitive to Competition by the Inducer—Both AMP-PNP and ADP can replace ATP as a positive effector of MalT (5). Previous work had revealed that competition between the inducer and MalY is affected by the nucleotide bound to MalT (16). In the presence of ADP, MalT inhibition by MalY was not relieved by maltotriose, even when present in a large excess (100 mM), whereas in the presence of AMP-PNP, MalY action was counteracted by 0.1 mM inducer (16). Thus, we investigated whether the nucleotide bound to MalT also affects the competition between Aes and the inducer. This possibility was examined by determining the concentration of maltotriose needed to relieve MalT inhibition by a fixed amount of Aes in the presence of AMP-PNP or ADP in our transcription system. As before, the concentration of MalT was adjusted to elicit half of the maximum response under the chosen concentrations of ligands. This was necessary because of the higher ability of MalT/AMP-PNP and the reduced ability of MalT/ADP to self-associate in the presence of maltotriose, compared with MalT/ATP (4). In the presence of AMP-PNP, MalT inhibition by Aes was relieved by 0.1 mM maltotriose (Fig. 4A), whereas in the presence of ADP, Aes action was not counteracted by maltotriose, even when present at 100 mM (Fig. 4B). In contrast, 1 mM maltotriose was needed to obtain resistance against Aes in the presence of ATP (Fig. 1A). Thus, the nucleotide bound to MalT similarly affects competition between the inducer and MalY or Aes. The intermediate results obtained with ATP are best explained by the presence of both ATP- and ADP-bound forms of MalT due to ATP hydrolysis during the assay.

The T38R Substitution in MalT Specifically Prevents Aes Binding—The above-mentioned results point toward identical mechanisms for MalT inhibition by MalY and Aes. However, as
shown by Schlegel et al., (33) the T38R substitution, isolated\(^2\) by Notley-McRobb and Ferenci (32) and located in MalT ddomain 1 (DT1), renders the protein insensitive to Aes in vivo, whereas it slightly increases its sensitivity to MalY, thereby suggesting that the substitution specifically alters the Aes binding site. To test this hypothesis, we purified the T38R MalT variant and analyzed its sensitivity to both inhibitory proteins in vitro. In the presence of AMP-PNP and a saturating concentration of maltotriose, the purified T38R variant was as active as the wild-type protein, provided that the nucleotide concentration was increased to 0.5 mM (data not shown). The T38R substitution, which is located just upstream of the putative P-loop structure (Walker A motif), most likely hampers ATP binding. Because a high concentration of ATP interferes with abortive initiation at malPp, whereas a high concentration of AMP-PNP does not, all of the assays involving the T38R variant were performed in the presence of 0.5 mM AMP-PNP.

When assayed in the presence of AMP-PNP without maltotriose, the T38R variant displays the same level of residual activity as the wild-type protein\(^3\) (data not shown). Adding a suboptimal concentration of maltotriose (10 \(\mu\)M) similarly increased the activity of both proteins (Fig. 5A). Therefore, these results indicate that the T38R substitution does not alter the balance between the inactive and the active forms of MalT, nor does it increase MalT affinity for maltotriose. As shown in Fig. 5A, at 10 \(\mu\)M maltotriose and 0.5 mM AMP-PNP, the T38R variant is fully resistant to Aes, whereas the activity of the wild-type protein is severely reduced (by 72%). In contrast, the mutated protein remains sensitive to repression by MalY (Fig. 5B). Comparison of the responses to increasing concentrations of MalY indicates that the T38R substitution actually slightly increases MalT susceptibility to inhibition by MalY, as observed in vivo (33). Furthermore, gel filtration experiments performed in the presence of 0.5 mM AMP-PNP showed that Aes forms a complex with wild-type MalT, but not with MalT T38R (data not shown; Fig. 6A), and that MalY forms a complex with MalT T38R (Fig. 6B). These results suggest that the T38R substitution specifically alters the Aes binding site and that the Aes binding site (or part of it) might be located on DT1.

To test this possibility, we examined whether immobilized DT1 would bind Aes. The Aes protein was passed through a Strep-Tactin\textsuperscript{®} Sepharose\textsuperscript{®} column preloaded with C-terminally Strep-tagged DT1 (DT1S). After washing, the proteins adsorbed to the column were eluted with desthiobiotin. SDS-PAGE analysis of the eluted fractions revealed that a significant amount of Aes bound to the column and co-eluted with DT1S (Fig. 7A). Controls showed that carbonic anhydrase is not retained by immobilized DT1S (data not shown) and that Aes does not bind a Strep-Tactin\textsuperscript{®} column preloaded with the DT1S-T38R variant (Fig. 7B), which demonstrates that Aes adsorption to the DT1S column involves a specific interaction between both proteins. We conclude that DT1 contains part of the Aes binding site.

**DISCUSSION**

The in vitro analysis reported here establishes that negative control of MalT activity by Aes is direct. In addition, this work demonstrates that Aes is a negative effector of MalT that competes with maltotriose, as previously hypothesized on the basis of in vivo data (3). These conclusions are supported by the following observations: (i) Aes inhibits MalT in a purified transcription system, (ii) Aes and MalT form a complex stable enough to be isolated by gel filtration, (iii) Aes inhibits maltotriose-binding by MalT, and (iv) reciprocally, maltotriose inhibits Aes binding to MalT. The reduced sensitivity of the MalT\textsuperscript{c26} variant to Aes action makes us confident that the repression phenomenon observed in vitro mimicks the in vivo situation. Furthermore, we have shown that part, if not all, of the Aes binding site is located on DT1, the N-terminal domain of MalT. As demonstrated by affinity chromatography, the purified DT1 domain specifically interacts with Aes. Finally, we have shown that the amino acid substitution T38R specifically impairs the interaction between Aes and DT1 or full-length MalT. The substituted residue is located just upstream from the Walker A motif thought to form a P-loop interacting with the \(\beta\) and \(\gamma\) phosphates of the bound ATP (19). The T38R substitution does not alter the specific activity of MalT (in the presence of a saturating concentration of nucleoside triphosphate), nor does

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\(^2\) The T38R substitution was isolated as a mutation increasing the basal expression of the LamB porin, a protein encoded by the maltose regulon, in a glucose-limited chemostat (32).

\(^3\) In the presence of AMP-PNP, MalT activity is partially independent of maltotriose (E. Richet, unpublished results).

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**Fig. 6.** Gel filtration: effect of MalT substitution T38R on MalT interaction with Aes and MalY. A, samples containing MalT T38R (9 \(\mu\)M) + Aes (9.5 \(\mu\)M), MalT T38R (9 \(\mu\)M) alone, or Aes (9.5 \(\mu\)M) alone were incubated and filtered through a Superose 12 column in the presence of 0.5 mM AMP-PNP. B, samples containing MalT T38R (9 \(\mu\)M) + MalY (4.5 \(\mu\)M dimer), MalT T38R (9 \(\mu\)M) alone, or MalY (4.5 \(\mu\)M dimer) alone were incubated and filtered through a Superose 12 column in the presence of 0.5 mM AMP-PNP.
it suppress its ability to interact with MalY. Therefore, it is unlikely that this substitution affects Aes binding by grossly altering DT1 folding. This inference is further supported by the observation that the *Vibrio cholerae* MalT ortholog has an arginine residue at the corresponding position. Hence, substitution T38R presumably precludes Aes binding either directly via a steric effect or indirectly via a local perturbation of the tertiary structure of DT1.

Although the gel filtration experiments clearly show that MalT and Aes are present in a 1:1 (protomer:protomer) ratio in the complex, the quaternary structure of the complex remains unknown. The MalT-Aes complex is in rapid equilibrium with free MalT and Aes on the time scale of the chromatography, and the apparent molecular mass of the complex increases with increasing concentrations of the partners without reaching a limit value within the protein concentration range used. MalT is known to be predominantly monomeric in the presence of ATP alone (4). Aes was reported to behave as a monomer upon gel filtration (15), but in our hands, when chromatographed alone, Aes (the His-tagged polypeptide, 37.7 kDa) elutes at the position of a 57-kDa globular protein, irrespective of the concentration of the protein in the injected sample. Thus, Aes could be either an asymmetric monomer or a dimer whose elution is delayed because of a weak interaction with the column matrix. As a result, the Aes-MalT complex might contain one protomer of each protein (predicted molecular mass, 141 kDa) or one Aes dimer and two MalT monomers (predicted molecular mass, 281 kDa).

![Affinity chromatography](image)

**Fig. 7. Affinity chromatography.** Aes was applied on Strep-Tactin® Sepharose® microcolumns preloaded with DT1S or DT1S T38R. Lane 1, Aes input; lane 1, flow-through; lanes 2–5, wash; lanes 6–9, elution with desthiobiotin. A, DT1S column; B, DT1S T38R column.

![Reaction scheme](image)

**Fig. 8. A model for the control of MalT activity.** Both the ATP-bound and the ADP-bound forms of MalT are in equilibrium between inactive and active monomeric forms designated a and i, respectively, in the case of ATP/MalT and a’ and i’, respectively, in the case of ADP/MalT. X corresponds to Aes or MalY. The event that would cause ATP hydrolysis and the MalT form that undergoes ATP hydrolysis are both unknown. The species thought to be involved in transcription activation and the negative control of the protein activity are boxed.

Interestingly, Aes seems to down-regulate MalT activity via the same mechanism as MalY. In both cases, the inhibitory protein is a negative effector of MalT that competes with the inducer for MalT binding and whose binding site is located on the N-terminal domain of the activator. Hence, the model according to which MalY would stabilize the inactive form of MalT applies equally to Aes, but it is presently unclear whether the molecular details of the inhibitory reactions are the same. The fact that the competition between the inhibitory protein and the inducer is similarly influenced by the nucleotide bound to MalT strongly suggests that MalY and Aes interact with the same conformational MalT species. It is also worth noting that the kinetic parameters of the inhibitory reaction are probably similar. MalT inhibition by MalY and Aes is observed for similar concentrations of inhibitory proteins and is counteracted by the same concentrations of maltotriose (16). In addition, in both cases, the complex is in rapid equilibrium with the free forms of the proteins. However, the binding sites of the inhibitory proteins on MalT do not coincide. Because the two proteins are unrelated at the amino acid sequence level, it indeed seems unlikely that they interact with the same surface determinant of MalT. Moreover, the T38R substitution abolishes Aes binding without impairing MalY binding.

MalK, another negative effector of MalT (10), is also thought to prevent maltotriose binding like MalY and Aes (3), but thus far, we do not know where its binding site is located on MalT. *In vivo* characterization of the *malT T38R* mutant revealed that besides rendering MalT fully resistant to repression by Aes, the mutation also significantly decreases MalT sensitivity to repression by MalK (33). This last property may explain why the substitution has been selected in a glucose-limited chemostat (32) (by reducing MalT sensitivity to repression by MalK in the absence of exogenous maltodextrins, the T38R substitution would lead to an increased basal expression of the LamB porin). Given that the T38R substitution does not change MalT affinity for maltotriose *in vitro* as shown here, the reduced sensitivity to MalK conferred by the substitution is not caused by an increased affinity for maltotriose but might result from an impaired interaction between MalT and MalK. Whereas the absence of MalK titration by DT1 *in vivo* excludes the possibility that the N-terminal domain of MalT harbors a major determinant of the MalK binding site (33), it remains possible that DT1 contributes to MalK binding and that substitution T38R alters the DT1 element of the MalK binding site.

One intriguing feature of MalT is the complex interplay between ATP or ADP binding and the binding of the inducer and the negative effectors, MalY and Aes. *In vitro*, both ATP and ADP promote maltotriose binding and MalT self-association (Fig. 8). However, given that MalT/ADP has a higher affinity for maltotriose and is more prone to self-association than MalT/ATP (4, 16), MalT/ATP/maltotriose is expected to be the species responsible for transcriptional activation *in vivo*. 

**How Aes Modulates MalT Activity**

- ATP-MalTₐ-X
- ATP-MalTₐ
- ATP-MalTₐ-maltotriose
- ATP-MalTₐₚ-maltotriose
- ATP-MalTₐ-maltotriose
- ATP-MalTₐₚ-maltotriose
- ATP-MalTₐₚ-maltotriose

**Fig. 8. A model for the control of MalT activity.** Both the ATP-bound and the ADP-bound forms of MalT are in equilibrium between inactive and active monomeric forms designated a and i, respectively, in the case of ATP/MalT and a’ and i’, respectively, in the case of ADP/MalT. X corresponds to Aes or MalY. The event that would cause ATP hydrolysis and the MalT form that undergoes ATP hydrolysis are both unknown. The species thought to be involved in transcription activation and the negative control of the protein activity are boxed.
Both nucleotides allow MalT inhibition by MalY and Aes in vitro, but they clearly differentially affect the equilibrium between inactive complexes (MalT-Aes or MalT-MalY) and active MalT-maltotriose complexes, with ATP favoring the interaction with the inhibitory protein (Fig. 8). Indeed, as observed in vitro, the ADP-bound form of MalT remains sensitive to MalY (16) or Aes (this work) even in the presence of an excess of maltotriose (up to 100 mM), whereas inhibition by Aes or MalY of the AMP-PNP bound form of MalT is relieved by 0.1 mM maltotriose. Hence, these results suggest that MalT/ATP binds the inducer or the inhibitory protein, depending on their relative concentrations, whereas MalT/ADP does not occlude binding of Aes and MalY even at very high maltotriose concentrations. What is the functional meaning of the differential control exerted by ATP and ADP on the competition between the inducer and the inhibitory proteins? Given that the ATPase activity of MalT does not play any role in the activation of open inducer and the inhibitory protein, depending on their relative concentrations? With the low affinity of MalY for ADP, it is tempting to propose that it actually binds the ADP-bound form of MalT, whereas MalT/ADP does not occlude binding of Aes and MalY even at very high maltotriose concentrations. Hence, these results suggest that MalT/ADP form is the actual target of MalY and Aes in vivo.

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