Conserved Motifs Involved in ATP Hydrolysis by MalT, a Signal Transduction ATPase with Numerous Domains from *Escherichia coli*^{∇†}

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The signal transduction ATPases with numerous domains (STAND) are sophisticated signaling proteins that are related to AAA⁺ proteins and control various biological processes, including apoptosis, gene expression, and innate immunity. They function as tightly regulated switches, with the off and on positions corresponding to an ADP-bound, monomeric form and an ATP-bound, multimeric form, respectively. Protein activation is triggered by inducer binding to the sensor domain. ATP hydrolysis by the nucleotide-binding oligomerization domain (NOD) ensures the generation of the ADP-bound form. Here, we use MalT, an *Escherichia coli* transcription activator, as a model system to identify STAND conserved motifs involved in ATP hydrolysis besides the catalytic acidic residue. Alanine substitution of the conserved polar residue (H131) that is located two residues downstream from the catalytic residue (D129) blocks ATP hydrolysis and traps MalT in an active, ATP-bound, multimeric form. This polar residue is also conserved in AAA⁺. Based on AAA⁺ X-ray structures, we proposed that it is responsible for the proper positioning of the catalytic and the sensor I residues for the hydrolytic attack. Alanine substitution of the putative STAND sensor I (R160) abolished MalT activity. Substitutions of R171 impaired both ATP hydrolysis and multimerization, which is consistent with an arginine finger function and provides further evidence that ATP hydrolysis is primarily catalyzed by MalT multimers.

Signal transduction ATPases with numerous domains (STAND) represent a recently recognized class of widespread, sophisticated signaling proteins (1, 8, 21), which often control fundamental processes. The STAND class notably includes proapoptotic factors (*Homo sapiens* APAF1, *Caenorhabditis elegans* CED4) (2), proteins controlling innate immunity responses in plants and mammals (14, 45), and the *Mycobacterium tuberculosis* PknK serine/threonine kinase involved in host infection (22), as well as bacterial transcription factors (23, 24).

STAND proteins are signaling hubs that sense negative and positive regulatory signals and relay positive signaling by activating other proteins. These multiple roles are reflected in their complex modular design (8) (see Fig. 1A). Their hallmark is a NOD (nucleotide-binding oligomerization domain) module which comprises a nucleotide binding domain (NBD), a helical domain (HD), and a winged-helix domain (WHD). The C terminus of NOD is typically followed by a sensor domain which binds the inducer. STAND proteins additionally exhibit

one or several effector domains located at either protein end, which are responsible for downstream signaling.

The mechanism of signal transduction seems to be conserved throughout the STAND class (8). The emerging picture is that of a regulated molecular switch, with the off position corresponding to a long-lived, monomeric, ADP-bound form stabilized by negative effectors and referred to as the resting form and the on position corresponding to an ATP-bound, oligomeric form, competent for downstream signaling (see Fig. 1B). In the resting form, the NOD module is in a closed conformation, with the ADP sandwiched between the WHD and the NBD/HD face harboring the nucleotide binding site. This closed conformation is thought to be stabilized by the unliganded form of the sensor domain. In the active form, the NOD module is in an open conformation with the WHD away from the NBD/HD-bound ATP. The ATP-bound, open NOD mediates protein self-association into closed or open multimers, via head-to-tail interactions with the ATP located at the interface (29, 51, 54). Conversion of the resting form into the active form is triggered by inducer binding. It is predicted to involve a series of conformational changes, including the relief of the auto-inhibitory effect exerted by the apo sensor domain, opening of the NOD module, nucleotide exchange, and protein self-association (Fig. 1B) (8).

ATP hydrolysis is not involved in downstream signaling, but it plays a fundamental role in signal integration by generating the ADP-bound form (23). It is catalyzed by the NBD/HD, yet little is known about the residues involved. Although the NBD and HD are structurally related to the nucleotide binding domain and the helical domain that make up the conserved core

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FIG. 1. The MalT protein. (A) Domain structure. NBD, nucleotide-binding domain; HD, helical domain; WHD, winged-helix domain. The MalT arm is as of yet structurally ill defined. See Steegborn et al. (44) for the X-ray structure of the MalT sensor domain. SUPR, superhelical repeats. The effector domain is a LuxR-type DNA-binding domain. aa, amino acids. (B) MalT cycling between the resting, ADP-bound form and ATP-bound homopolymers, the active form. The colors correspond to those in panel A. Resting MalT is characterized by a NOD module in a closed conformation, as observed for ADP-bound Δ WD40 APAF1 (36). This closed configuration is thought to be stabilized by the apo form of the sensor domain (8). Resting MalT is further stabilized by three proteins acting independently: MalY, Aes, and MalK. MalT activation would involve maltotriose-dependent relief of the sensor domain inhibitory effect, NOD opening, nucleotide exchange, and MalT assembling into polydisperse homopolymers, via head-to-tail interactions with the ATP located at the protomer interface. Note that this model relies on the assumption that the ATP-bound NOD cannot close. The oligomerization process is probably coupled to promoter binding *in vivo*, with the size of the oligomers governed by the number of MalT binding sites present in the target promoters (20). (C) Sequence alignment of the MalT clade STAND proteins, highlighting conservation of the Walker B region, the sensor I motif, the putative arginine finger, and predicted β -strand secondary structures. The partially conserved arginine that characterizes the MalT clade (R173 in MalT) was not considered, because its substitution by a histidine confers a gain-of-function phenotype (O. Danot and E. Richet, unpublished results).

TABLE 1. Strains

Strain	Genotype	Reference or source
MC4100	<i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	6
pop1951	MC4100 (F ⁺)	9
pop7164	MC4100 <i>ΔmalT220</i> ^a	40
pop7170	MC4100 <i>ΔmalB107^b trp:::(Kan^r-malEpΔ92-lac)_{op}^c aroB glpD</i>	35
pop7175	MC4100 <i>ΔmalB107 trp:::(Kan^r-malEpΔ92-lac)_{op}</i>	23
pop7185	MC4100 <i>ΔmalB107 malI::Tn10 trp:::(Kan^r-malEpΔ92-lac)_{op} aroB glpD</i>	35
pop7186	MC4100 <i>ΔmalB107 malI::Tn10 trp:::(Kan^r-malEpΔ92-lac)_{op}</i>	23
pop7190	MC4100 <i>ΔmalB107 trp:::(Kan^r-malEpΔ92-lac)_{op} ΔmalT220</i>	35
pop7191	MC4100 <i>trp:::(Kan^r-malEpΔ92-lac)_{op} ΔmalT220</i>	35
pop7192	MC4100 <i>ΔmalB107 malI::Tn10 trp:::(Kan^r-malEpΔ92-lac)_{op} ΔmalT220</i>	35
pop7254	MC4100 <i>malEpKpΔ100:::(P_{tac}-P_{KAB-TTGG})^d trp:::(Kan^r-malEpΔ92-lac)_{op} aroB glpD</i>	23
pop7255	MC4100 <i>malEpKpΔ100:::(P_{tac}-P_{KAB-TTGG}) trp:::(Kan^r-malEpΔ92-lac)_{op}</i>	23
pop8012	BL21 <i>ΔmalT220</i> (DE3)	O. Danot (Institut Pasteur)

^a *ΔmalT220* is a deletion of the entire *malT* gene (40).

^b *ΔmalB107* deletes most, if not all, of the *malK* gene (17).

^c The subscript of indicates that the strain carries a *lacZYA* operon fusion.

^d *malEpKpΔ100:::(P_{tac}-P_{KAB-TTGG})* corresponds to a 260-bp deletion of the *malEp-malKp* intergenic region that extends from position -6 with respect to *malEp* to position -7 with respect to *malKp*, with the divergent *malEp* and *malKp* promoters replaced by *P_{tac}* and *P_{KAB-TTGG}*, respectively. *P_{KAB-TTGG}* is a weak, constitutively active, semi-synthetic promoter variant with the TTGG sequence from position -17 to -14 (5).

of the ATPases associated with diverse cellular activities (AAA⁺) family, a heterogeneous class of proteins that couple switch function or substrate remodeling to ATP binding/hydrolysis via oligomeric assemblies (12), they have inherited only a few of the conserved AAA⁺ motifs involved in ATP hydrolysis (8). The best-conserved motif shared with the AAA⁺ is the Asp/Glu that is located immediately after the Walker B motif (see Fig. 1C) (21), which has been shown to correspond to the catalytic residue in STAND (23, 46).

Here, we use MalT, the transcriptional activator of the *Escherichia coli* maltose regulon and one of the best-studied STAND (Fig. 1A), as a model system to identify other key residues that are involved in ATP hydrolysis. As a typical STAND, MalT cycles between an ADP-bound resting form and ATP-bound oligomers, the active form. The latter bind cooperatively the multiple MalT binding sites decorating the target promoters (20) and recruit RNA polymerase via an interaction involving a DNA-binding domain surface determinant (10). MalT activation is tightly controlled by three negative effectors, the proteins MalK, MalY, and Aes, which stabilize the resting form (23), and maltotriose, which triggers conversion of the resting form into the active form by binding the sensor domain (7). MalK, the ABC component of the maltodextrin transporter, couples induction of the maltose regulon to the presence of maltodextrins in the growth medium (3). The functions of MalY (a β-C-S lyase) and Aes (an acyl esterase) are unknown (3). ATP hydrolysis seems to be catalyzed by the multimeric forms, thereby ensuring signal extinction and MalT recycling under an ADP-bound form (23).

To identify STAND motifs that are involved in ATP hydrolysis, we changed well-conserved polar or charged residues in MalT and determined the effects of these substitutions on MalT properties *in vivo* and/or *in vitro*. We showed that a histidine that is located two residues downstream from the catalytic Asp (21) and that is highly conserved in the MalT clade plays an essential role in the hydrolytic attack. We provide evidence that the arginine of the STAND S/T R motif, which is proposed to be equivalent to the AAA⁺ sensor I, is

essential for MalT activity and that MalT R171 might correspond to the canonical AAA⁺ arginine finger. Finally, we provide additional evidence that MalT multimers are the catalytically competent forms, which clearly distinguishes MalT from APAF1 (33), another STAND, and indicates that the timing of ATP hydrolysis may vary with the protein.

MATERIALS AND METHODS

Material. ATP was purchased from Roche. Maltotriose and ADP (high-performance liquid chromatography [HPLC] grade) were purchased from Sigma.

Strains and plasmids. All strains (Table 1) are derivatives of *E. coli* K-12. The mutations altering residues H131, S159, R160, and R171 were introduced in the M13malT5 phage DNA (23) by site-directed mutagenesis as described by Kunkel et al. (19) together with silent mutations that introduced a restriction site next to the mutated codon (for the codon changes and the silent mutations, see Table S1 in the supplemental material). The *malT* mutations were transferred into the chromosome of strain pop1951 by a two-step transfer technique with the M13 derivatives containing the *malT* mutations as shuttles (9). *malT* derivatives of pop7175, pop7186, and pop7255 were obtained by P1 transduction of pop7170, pop7185, and pop7254, respectively, to Aro⁺ Gly⁺ using P1 phage lysates of the corresponding pop1951 mutants. pOM206 is a pET24a(+) derivative encoding N-terminally His-tagged MalT (23). Mutated pOM206 derivatives were obtained by cloning the AatII-AflIII fragment from the M13malT5 variants between the corresponding sites of pOM206. The pOM206 derivatives were cloned and maintained in pop7164, which does not encode T7 RNA polymerase.

β-Galactosidase assays. Cells were grown at 30°C in M9 medium (25) supplemented with 0.4% glucose or glycerol, 0.01% tryptophan, 1 mg/ml thiamine, and 0.5% L broth as described previously (35). β-Galactosidase was assayed as described by Miller (26), using chloroform and 0.0037% SDS to permeabilize the cells. Each culture was assayed in duplicate. Enzyme activities (expressed in Miller units) represent the means ± standard deviations (SD) of the values obtained from at least two independent cultures and are corrected for the background levels (9 and 2.9 units for cells grown in the presence of glycerol and glucose, respectively) as measured with isogenic *ΔmalT* strains.

Immunoblots. Total extracts from cells grown in the presence of glycerol as described above were analyzed on a 9% SDS-polyacrylamide gel (acrylamide-bisacrylamide, 75:1). Western blotting was performed with rabbit polyclonal anti-MalT or anti-sigma 70 (from R. Ebricht, Rutgers University) and horseradish peroxidase-conjugated secondary antibodies to rabbit immunoglobulin G, followed by ECL Plus (GE Healthcare) detection on a PhosphorImager. The MalT levels were obtained by normalizing the MalT band volume to the σ⁷⁰ volume and subtracting the value obtained for the isogenic *ΔmalT* strain. The immunoblot analyses were performed under linear dose-response conditions.

Purification of His-tagged MalT proteins. MalT proteins were purified from pop8012 (pOM206 *malTx*), as described previously (23). Three chromatography steps were required to get rid of contaminating ATPases: an Ni-NTA agarose (Qiagen) affinity chromatography procedure, a hydrophobic chromatography procedure, and a Sephacryl S-300 (GE Healthcare) gel filtration. For wild-type MalT, hydrophobic chromatography was performed with butyl-Sepharose (GE Healthcare), as described except that the protein was eluted with a 0.4 M KSCN buffer. For MalT-H131A, -R171A, and -R171E, propyl-agarose (Sigma) was used instead of butyl-Sepharose, with column adsorption and washing performed at 0.95 M and 0.9 M $(\text{NH}_4)_2\text{SO}_4$, respectively, and the proteins were eluted with a 0.25 M $(\text{NH}_4)_2\text{SO}_4$ buffer (for MalT-H131A and -R171A) or a 0.4 M KI buffer (for MalT-R171E). The proteins eluted from the butyl-Sepharose or propyl-agarose columns were concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and/or concentrated with the Centrprep Millipore YM-30 and filtered through a Sephacryl S-300 column, as described previously (23). The absence of contaminating ATPases was verified by monitoring the maltotriose-dependent and -independent ATPase activities across the MalT peak eluted from the molecular sieve. Free ATP was removed by ammonium sulfate precipitation (39) and filtration through a G-25 Sephadex column equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.7), 10% sucrose, 0.2 M KI, 10 mM magnesium acetate, and 0.1 mM EDTA.

ATPase assays. MalT (2 μM) was incubated at 30°C in a 20 μl reaction mixture containing 40 mM HEPES-KOH (pH 8.0), 5 mM Tris-HCl (pH 7.7), 27 mM tri-potassium citrate, 20 mM KI, 11 mM magnesium acetate, 1 mM dithiothreitol (DTT), 100 $\mu\text{g/ml}$ acetylated bovine serum albumin (BSA) (Sigma), and 25 μM [γ - ^{32}P]ATP (0.15 Ci/mmol). Aliquots (1 μl) were spotted at different time points onto polyethyleneimine cellulose plates (Merck) and developed in 1 M formic acid/0.5 M LiCl. The chromatograms were scanned in a PhosphorImager.

Gel filtration. The 25- μl protein sample contained 50 mM Tris-HCl (pH 7.7), 104 mM KI, 16 mM tri-potassium citrate, 10 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, proteins, and, when indicated, 1 mM maltotriose and/or 0.1 mM nucleotide. After a 15-min incubation at 20°C, 20 μl of sample were injected onto a Superdex 200 column (3.2 by 300 mm) installed on a SMART system (GE Healthcare) and equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.7), 33 mM tri-potassium citrate, 10 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, and, when indicated, 1 mM maltotriose and/or 0.1 mM nucleotide. Filtration was performed at 6°C at a flow rate of 40 $\mu\text{l/min}$. Standard globular proteins bovine thyroglobulin (669 kDa), β -amylase (200 kDa), and BSA (66 kDa) eluted at 1.00 ml, 1.25 ml, and 1.48 ml, respectively.

Quantification of protein-bound adenine nucleotides. The ATP and ATP plus ADP contents were determined with a bioluminescence assay kit (Sigma). A sample of 4 μl MalT was added to 82 μl of 1.2 mM EDTA. The mixture was heated for 10 min at 92°C. After the addition of 12 μl of a solution containing 0.56 M Tris-HCl (pH 7.7), 8.3 mM maltotriose and/or 0.1 mM nucleotide, and 1.5 mM phosphoenolpyruvate, the mixture was incubated for 50 min at room temperature in the absence or in the presence of pyruvate kinase (4 units) (Roche). ATP was then assayed directly with luciferase as described by the manufacturer.

RESULTS

Substitution H131A freezes MalT at a prehydrolytic step. Most of the STAND proteins whose catalytic acidic residue is located right after the Walker B motif also possess a conserved polar residue (His, Gln, or Asp) that is located 2 amino acids further downstream (21). In particular, the MalT clade is typified by a highly conserved histidine at this position (Fig. 1C). Considering the possibility that this conserved residue plays a key role in the hydrolytic attack, we replaced the corresponding histidine in MalT (H131) by an alanine and characterized the modified protein *in vivo* and *in vitro*.

The effect of the H131A substitution on MalT activity was first assessed *in vivo* in the absence of MalT repressors (MalK, MalY, and Aes). For this purpose, we used a ΔmalK strain (pop7175) grown in minimal medium supplemented with glycerol. Under these conditions, the strain is phenotypically MalY[−] and Aes[−] as well (see hereinafter and Richet et al. [35]) and the maltose regulon is induced due to the presence of endogenous maltotriose (3, 11). The ΔmalK strain used har-

bors the *lac* operon in the chromosome under the control of *malEp* Δ 92, a MalT-dependent promoter (34), which allows assessment of MalT activity through measurements of β -galactosidase activity. Because the combined action of the maltodextrin-catabolizing enzymes MalP, MalQ, and MalZ, whose expression is controlled by MalT, marginally alters the level of endogenous maltotriose (11), any mutation effect on MalT activity will not cause any significant, concomitant changes in the level of endogenous inducer that might amplify or partially mask variations in MalT activity level.

Comparison of β -galactosidase levels in *malT*⁺ and *malT-H131A* derivatives of the ΔmalK reporter strain revealed that MalT-H131A was 4-fold more active than wild-type MalT in the absence of repressors (Fig. 2A). MalT-H131A activity was also enhanced to a similar extent under conditions in which the cellular level of MalT was lower. MalT-H131A was indeed 5-fold more active than wild-type MalT when the cells were grown in the presence of glucose instead of glycerol, i.e., under conditions in which the activity of the *malT* promoter is decreased by a factor of 3 because of catabolic repression (30) (Fig. 2B). This therefore excludes the possibility that MalT-H131A hyperactivity, as measured on cells grown in the presence of glycerol, is underestimated due to saturation of the *malEp* Δ 92 promoter by an excess of active protein. Last, immunoblots show that the cellular concentration of MalT-H131A is 40% higher than that of wild-type MalT (Fig. 2C and D), thereby indicating that MalT-H131A hyperactivity is only marginally due to an increase in protein concentration.

We then asked whether substitution H131A alters MalT sensitivity to the MalK repressor by comparing the levels of β -galactosidase in the ΔmalK reporter strain used above and an isogenic *malK*⁺ strain (pop7255). To avoid the possibility that changes in MalT activity caused a concomitant change in the concentration of MalK, the MalT-dependent promoters that drive the expression of the operons encoding the maltose transporter (*malEFG* and *malKlamBmalM*) were replaced by constitutive promoters (23). Notably, the *malKp* promoter was replaced by a weak, constitutive promoter (see Table 1) in order to obtain the low level of *malK* expression that prevails in a wild-type strain in the absence of maltodextrins in the growth medium, i.e., under conditions of MalT repression by MalK. As shown in Fig. 2A, substitution H131A severely impairs MalT inhibition by the MalK repressor. Whereas the activity of wild-type MalT is $\sim$ 30-fold lower in the presence of MalK, MalT-H131A was only $\sim$ 3-fold less active in the presence of the repressor (Fig. 2A).

To determine whether substitution H131A also interferes with MalT inhibition by MalY, we measured the amount of β -galactosidase made by an isogenic ΔmalK *malI::Tn10* reporter strain (pop7186). The inactivation of *malI*, which encodes the repressor of the *malX malY* operon, causes constitutive expression of the operon, thereby rendering the strain MalY⁺ (32). As observed with MalK, substitution H131A strongly decreased MalT inhibition by MalY (Fig. 2A).

Western blot analyses of the MalK⁺ and MalY⁺ derivatives assayed showed that substitution H131A scarcely changes the cellular concentrations of MalT in the MalK⁺ and MalY⁺ strains. The cellular concentration of MalT-H131A is increased by 35% compared to the wild-type level in the MalK⁺ strain, while it is similar to the wild-type level in the MalY⁺

FIG. 2. Effect of various substitutions on the transcriptional activity of MalT *in vivo*. (A) Levels of β -galactosidase in *malT* derivatives of isogenic $\Delta malK$ (no repressors), *malK*⁺ (MalK⁺ MalY⁻), and $\Delta malK malT::Tn10$ (MalK⁻ MalY⁺) reporter strains (pop7175, pop7255, and pop7186, respectively) grown in minimal medium plus glycerol. The values are the means $\pm$ SD of results from at least two independent experiments. w. t., wild type. (B) As in panel A except that the bacteria were grown in the presence of glucose. The low level of β -galactosidase activity observed here compared to that obtained with bacteria grown in the presence of glycerol is due to the fact that the glucose effect reduces the activity of *malEp92*, the promoter assayed, both indirectly through an effect on the *malT* promoter (i.e., on the MalT level) and directly because *malEp92* is also controlled by CRP (48). (C) Immunoblots. The strains used for the β -galactosidase assays were grown in minimal medium plus glycerol as described in Materials and Methods. Total cell extracts were analyzed by SDS-PAGE and developed with anti-MalT and anti- σ^{70} antibodies. The relative MalT levels were quantified and normalized with respect to σ^{70} levels, as described in Materials and Methods. The standard deviation of the relative MalT level values is $\pm 10\%$.

strain (Fig. 2C and D). These data hence confirm that substitution H131A renders MalT largely insensitive to MalK and MalY.

The phenotypes conferred by the H131A substitution, hyperactivity and reduced sensitivity to MalK and MalY, are strikingly similar to those conferred by the MalT-D129A substitution, which alters the catalytic aspartate and specifically abolishes ATP hydrolysis (23). We therefore inferred that H131 replacement by an alanine was likely to abolish MalT ATPase activity and lock the protein in an ATP-bound form, as previously observed with substitution D129A.

To test this hypothesis, we purified the H131A variant and measured its ATPase activity both in the absence and in the presence of maltotriose, which is known to stimulate MalT ATPase activity by a factor of 5 (23). As expected, MalT-H131A displayed little ATPase activity (less than 2% of the wild-type level) regardless of inducer presence (Fig. 3). We

FIG. 3. Substitution H131A abolishes MalT ATPase activity. ATPase assays were performed in the presence of 2 μ M MalT in the absence and in the presence of 1 mM maltotriose.

TABLE 2. ADP and ATP contents of purified MalT variants

Variant	Molar ratio $\pm$ SD ^a	
	ADP + ATP (+ pyruvate kinase)	ATP (- pyruvate kinase)
Wild-type MalT	1.16 $\pm$ 0.04	0.05 $\pm$ 0.01
MalT-H131A	1.03 $\pm$ 0.01	0.97 $\pm$ 0.08
MalT-R171A	1.11 $\pm$ 0.04	0.10 $\pm$ 0.06
MalT-R171E	0.84 $\pm$ 0.03	0.07 $\pm$ 0.03

^a The amounts of ADP plus ATP and of ATP present in the purified MalT fractions were measured by performing luciferase assays in the presence or absence of pyruvate kinase, respectively. The values represent the molar ratio of nucleotide to MalT protomer. The data are the means $\pm$ SD from results of two independent experiments performed in duplicate.

also determined the nucleotide binding state of purified MalT-H131A by performing luciferase assays in the absence and presence of pyruvate kinase, which measures the ATP and ATP plus ADP contents, respectively. Purified MalT-H131A contained one ATP per protomer, whereas the purified wild-type protein was predominantly in an ADP-bound form, as routinely observed (Table 2) (23). Altogether, MalT inability to hydrolyze ATP and its ATP-bound state indicate that substitution H131A does not prevent ATP binding but freezes the protein in an ATP-bound form at a prehydrolytic step.

If the MalT-H131A variant is frozen in an activated form, it should multimerize in the absence of ATP and maltotriose. This prediction was tested by performing gel filtration assays in the absence of effectors. Injection of a 20 μ M MalT-H131A sample gave a major peak of self-associated species and a

FIG. 4. Effect of substitution H131A on MalT self-association. (A) Wild-type MalT and MalT-H131A (20 μ M) were preincubated and filtered through a Superdex 200 column in the absence of effectors, as described in Materials and Methods. The retention volumes (ml) of the peaks are indicated. (B) MalT-H131A (5 and 10 μ M samples) was preincubated and filtered in the presence of ATP alone or ATP plus maltotriose. (C) Wild-type MalT and MalT-H131A (20 μ M) were preincubated and filtered in the presence of ATP and maltotriose. (D) As in panel C except that MalT concentration in the injected samples was varied. Note that the elution volumes obtained in panels A, C, and D cannot be compared to those obtained in panel B because the Superdex 200 column used was not the same.

minor peak of monomers eluting at 0.97 and 1.36 ml, respectively, whereas wild-type MalT was monomeric under the same conditions (Fig. 4A). As observed for activated wild-type MalT (see Fig. 4C), the MalT-H131A multimer peak (apparent molecular weight [MW_{app}], ~775,000) is asymmetric and its position is shifted toward larger retention volumes as the concentration of the protein in the injected sample is decreased (see Fig. S1 in the supplemental material). MalT-H131A hence forms polydisperse homopolymers in rapid equilibrium to the time scale of chromatography like wild-type MalT (20, 39). From these data, we conclude that the ATP/MalT-H131A complex is frozen in a multimerization-competent form.

We then tested whether the variant still responded to the inducer. Gel filtration experiments performed in the presence of maltotriose and ATP revealed that, for a given concentration of protein, the MalT-H131A self-associated forms reproducibly eluted earlier than observed in the presence of ATP alone (Fig. 4B). This indicates that the variant still responds to the inducer, but the stimulatory effect is small. Five μ M MalT-H131A showed the same degree of multimerization in the presence of ATP plus inducer as 10 μ M MalT-H131A filtered in the presence of ATP alone (Fig. 4B). The same results were obtained when investigating the effect of maltotriose on MalT-H131A multimerization in the absence of ATP (see Fig. S2 in the supplemental material). The observation that, in the absence of maltotriose, a 2-fold-higher concentration of MalT-H131A was required to achieve the same degree of multimerization as in the presence of inducer might suggest that at most half of the purified ATP-trapped variant is under an inducer-bound form. Alternatively, the purified ATP-trapped variant might be inducer free. In any case, the weak stimulatory effect of maltotriose on self-association is most likely due to the fact that the auto-inhibition exerted by the sensor domain has already been relieved. Last, in the presence of both effectors, MalT-H131A eluted reproducibly earlier than wild-type MalT (MW_{app}, 900,000 versus 670,000), suggesting that, in the presence of inducer, MalT-H131A is slightly more prone to multimerization than wild-type MalT (Fig. 4C and D). The higher degree of self-association most likely results from the loss of ATPase activity. As shown in reference 39, the multimers formed by wild-type MalT in the presence of maltotriose are also stabilized when ATP is replaced by AMP-PNP, a nonhydrolysable ATP analog. The presence of transient ADP-bound forms generated by ATP hydrolysis are expected to decrease the average degree of multimerization observed in the presence of inducer because they are less prone to self-association (39) (see below).

The sensor I motif. The conserved arginine that is located immediately after the β_4 strand in the NBD of STAND proteins (21, 36, 51, 52) may functionally correspond to the AAA⁺ sensor I (most often an asparagine), which is thought to position the catalytic water molecule for the hydrolytic attack (12). Often preceded by a serine or a threonine, this arginine is well conserved in most STAND clades, including the MalT clade (Fig. 1C). To assess the roles of S159 and R160 in signaling by MalT, we analyzed the effects of substitutions S159A and R160A on the transcriptional activity of the protein *in vivo*. The S159A variant behaved exactly as wild-type MalT, both in the absence of repressors and in the presence of MalK or MalY (Fig. 2A). In contrast, MalT-R160A was inactive: in the

absence of repressors, its activity level was $\leq 2\%$ of the wild-type level (Fig. 2A). As shown by immunoblots (Fig. 2C and D), the cellular concentrations of both variants were either similar to or only slightly increased compared to the levels of wild-type MalT in the three reporter strains (increases of 15 to 30% and 10 to 60% were measured for MalT-S159A and MalT-R160A, respectively). Based on these results, we conclude that Arg160 is essential for MalT activity, whereas Ser159 is dispensable.

Arg171, an arginine finger? Two STAND clades, the MalT and the MNS clades, have a conserved arginine finger in the loop preceding β_5 in the NBD (Fig. 1C). This residue is surface exposed at the protomer interface in the crystal structure of the ADP-bound, open form of SSO1545, a MNS clade representative (51). It might hence correspond to the AAA⁺ arginine finger which plays a key role in ATP hydrolysis by interacting *in trans* with the γ -phosphate of the ATP bound to the adjacent protomer (15, 28). To investigate this possibility, we analyzed the *in vivo* and *in vitro* effects of alanine and glutamate substitutions of the corresponding residue, R171, in MalT.

In the absence of any repressors, the MalT-R171E variant was inactive *in vivo*, whereas MalT-R171A was 16% as active as wild-type MalT (Fig. 2A), although the cellular concentrations of both variants were slightly higher than that of wild-type MalT (Fig. 2C and D). The reduced activity conferred by the substitutions was further reduced to basal levels in the presence of MalK or MalY (Fig. 2A).

To delineate the role of the R171 residue, we purified and characterized MalT-R171A and -R171E. Both proteins were purified in a monomeric, ADP-bound form like the wild type (Fig. 5A and Table 2), indicating that they were correctly folded. Their abilities to multimerize in the presence of ATP and maltotriose were assessed by gel filtration of 20 μ M MalT samples. Wild-type MalT gave a major peak of self-associated species at 0.99 ml (MW_{app}, ~715,000). In contrast, MalT-R171E eluted at 1.35 ml, whereas MalT-R171A eluted at an intermediary position, at 1.17 ml (MW_{app}, ~300,000). We verified that, under these conditions, MalT-R171A apparent molecular weight varies the concentration of the protein (data not shown), thereby indicating that the variant forms polydisperse homopolymers in rapid equilibrium to the time scale of the chromatography like wild-type MalT. The fact that MalT-R171E eluted slightly earlier than the monomeric species and the asymmetry of the peak suggest some residual ability to multimerize. The degrees of multimerization of the variants that were observed in these assays correlate with their *in vivo* activities. Altogether, these data strongly suggest a direct role of the arginine 171 side chain in MalT self-association.

The ADP-bound, open forms of MalT multimerize *in vitro* in the presence of maltotriose, albeit with a reduced efficiency compared to that of ATP-bound forms (39). As shown in Fig. 5B, substitution R171A also impaired the ADP- and inducer-dependent multimerization, thereby indicating that the effect of substitution R171A on MalT self-association is not specific to the presence of a γ -phosphate in the catalytic pocket.

Finally, ATPase assays performed in the presence of maltotriose revealed that substitution R171E dramatically reduced ATP hydrolysis in the presence of inducer, while substitution R171A had a milder effect (6-fold versus 2-fold) (Fig. 6). These substitutions also decreased the basal levels of ATPase activity

FIG. 5. Effect of substitutions R171A and R171E on MalT self-association. (A) MalT (20 μ M) was preincubated and filtered through a Superdex 200 column in the absence of effectors or in the presence of ATP and maltotriose, as described in Materials and Methods. (B) MalT (20 μ M) was preincubated and filtered in the presence of ADP and maltotriose.

measured in the absence of inducer, albeit to a lesser extent than observed in the presence of inducer (Fig. 6). Again, substitution R171E had a stronger effect than substitution R171A. Stimulation of the ATPase activity by maltotriose is hence decreased for both variants, with MalT-R171E scarcely responding to the inducer. The weak stimulatory effect of the inducer on MalT-R171E ATPase activity is consistent with the very low tendency of the variant to self-associate in the presence of ATP and maltotriose. That R171 substitutions reduce MalT abilities to hydrolyze ATP and self-associate to the same extent points at a tight coupling between the two reactions.

DISCUSSION

Role of the conserved MalT histidine 131 in the hydrolytic attack. One important finding of this work is that, besides the Walker B catalytic aspartate, another residue, His131, plays a

crucial role in the catalysis of the β - γ bond scission. This conclusion is based on the observation that alanine substitution of His131 freezes the protein at a prehydrolytic step. As revealed by *in vitro* studies, the H131A variant, which scarcely shows any detectable ATPase activity, is trapped in an active, ATP-bound form, i.e., in a form highly competent for oligomerization and downstream signaling regardless of inducer presence. Consistent with the variant inability to recycle into an ADP-bound form, MalT-H131A is hyperactive and largely escapes inhibition by the negative effectors MalK and MalY *in vivo*. Based on our model, MalT-H131A would be characterized by an open, ATP-bound NOD and because NOD would be trapped in an open state, the sensor domain could not exert any auto-inhibitory effect, thereby accounting for the weak stimulatory effect of maltotriose (Fig. 1B).

In some respects, *malT-H131A* resembles other *malT* muta-

FIG. 6. Effect of substitutions R171A and R171E on MalT ATPase activity. ATPase assays were performed in the presence of 2 μ M MalT in the absence and in the presence of 1 mM maltotriose. The values are the means $\pm$ SD of results from two independent experiments in which the three variants were assayed in parallel. The differences observed between the values obtained for wild-type MalT in these experiments and those obtained in the Fig. 3 experiment are due to the use of different salt and ATP solutions.

tions in NOD that also confer constitutive signaling and render the protein less sensitive to negative effectors (38). This reflects the fact that the resting form is at the heart of the mechanism integrating negative and positive controls of protein activity. Any mutation that specifically destabilizes the resting form or prevents its adoption will render signaling both inducer independent and repressor insensitive, such as mutations impairing NOD closure or ATP hydrolysis (8).

The unsuspected role of H131 in ATP hydrolysis that is revealed by this study is consistent with the high conservation of this histidine residue across the MalT clade. Furthermore, in most of the other STAND proteins whose catalytic acidic aspartate or glutamate is located immediately after the Walker B motif, the residue corresponding to MalT histidine 131 is noticeably a polar residue, Asp, His, or Gln, depending on the clade (see reference 21), which suggests a conserved function. Interestingly, most of the AAA⁺ proteins also have a polar residue (Asp, Asn, His, or Glu) at the same position (12, 27), but to our knowledge, its role has not been investigated. Based on the structural relationship between the two protein classes, this polar residue might also play a key role in ATP hydrolysis by AAA⁺ and its substitution might freeze AAA⁺ proteins in an ATP-bound state, as observed for AAA⁺ variants in which the catalytic acidic residue was replaced by an alanine (18, 50).

Whether the conserved polar residue at the +2 position downstream from the catalytic residue is directly or indirectly involved in the hydrolytic attack by MalT is unclear, since no structures are yet available for ATP-bound forms of STAND with a polar residue at this position. However, as suggested by the X-ray structures of AAA⁺ that seem to be primed to hydrolyze ATP, notably based on the proximity of the γ -phosphate to the side chains of the sensor I and the catalytic residues, the conserved polar residue might play a crucial, though indirect, role in the hydrolytic attack by maintaining the proper spatial relationship between the catalytic residue and the sensor I. The side chain of the conserved polar residue is indeed hydrogen bonded to a backbone amine of the sensor I region and/or to the hydroxyl of the S/T residue located upstream from the sensor I in the crystal structures of the yeast ATP γ S-bound clamp loader (PDB code 1SXJ) (4), the ATP-bound NSF D2 domain (PDB code 1NSF) (53), and the ADP.BeF₃-bound γ subunit of the *E. coli* clamp loader (PDB code 3GLF) (41) (see Fig. S3 in the supplemental material). Interestingly, the comparison of AMP-PCP/DnaA and AMP-PNP/Orc to the cognate ADP-bound forms (12, 13, 42) indicates that the hydrogen-bonding pattern of the polar residue depends on the presence of a γ -phosphate, since it is hydrogen bonded to the catalytic residue in the ADP-bound forms, which further argues for a crucial role in ATP hydrolysis. That such an architectural role may apply as well to the conserved polar residue in STAND is supported by the observation that it is also hydrogen bonded to the catalytic residue in the open, ADP-bound form of SSO1545 (51).

The STAND sensor I motif. As pointed out by Leipe et al. (21), the well-conserved arginine that is located right after the β 4 strand in the NBDs of STAND proteins might be functionally equivalent to the AAA⁺ sensor I asparagine, which senses the presence of ATP, most likely by positioning the catalytic water molecule for the hydrolytic attack (12). Consistently, the corresponding arginine in CED4 (R273) is close to the γ -phos-

phate in the crystal of the ATP-bound form (52). Our observation that alanine substitution of the corresponding arginine in MalT (R160) abolishes protein activity further supports the above hypothesis; the sensor I residue is indeed expected to play a key role in the adoption of the proper conformation following ATP binding. Alanine substitution of the corresponding arginine (R313) of another STAND, the pathogen resistance protein I-2 from tomato, also causes a loss-of-function phenotype (47).

In STAND proteins (21), the so-called sensor I arginine is often preceded by a serine or a threonine, as observed for AAA⁺ proteins (27). Alanine substitution of the corresponding residue in MalT (S159A) alters neither MalT activity nor its sensitivity to repressors *in vivo*. Intriguingly, the corresponding change (T1230A) in the pathogen resistance Prf protein from tomato, another STAND, confers a loss-of-function phenotype (37). Although it is conserved, the importance of this Ser/Thr may hence vary with the proteins.

R171, an arginine finger? Substitution R171A alters a conserved residue that is predicted to be located at the protomer interface, possibly close to the γ -phosphate of the ATP bound to the adjacent protomer in multimers, based on the structure of the ADP-bound form of SSO1545, another STAND protein (51). The substitution reduces both MalT self-association and the ATPase activity observed in the presence of maltotriose, which is consistent with an arginine finger function. Alanine substitution of the arginine finger has also been shown to impair both ATP hydrolysis and self-association in the case of simian virus 40 (SV40) large T antigen and VCP, two AAA⁺ proteins (16, 49). The mildness of the effect of substitution R171A contrasts, however, with the dramatic effect of arginine finger alanine substitutions on AAA⁺ ATPase activities (16, 31, 43). In the case of MalT, the arginine finger might play a minor role in the hydrolytic attack.

Alternatively, arginine 171 might not be an arginine finger but merely an element of the NBD surface involved in inter-protomer interaction. The effects of R171 substitutions on MalT ATPase activity would then result from the absence of ATP stabilization by MalT self-association, i.e., an increase in the *K_m* for ATP. Such a possibility is supported by the observation that substitution R171A reduces MalT self-association in the presence of inducer whether the protein is ADP or ATP bound. The ADP-bound multimers would indeed be expected to be insensitive to the removal of a bona fide arginine finger unless the latter has differential roles depending on the nucleotide bound.

ATP hydrolysis is primarily catalyzed by the MalT multimeric forms. In a previous work (23), we inferred that MalT multimers are the catalytically competent forms based on two observations: (i) that maltotriose both promotes self-association in the presence of ATP and stimulates ATP hydrolysis and (ii) that alanine substitution of the catalytic aspartate (D129), which is known to freeze P-loop ATPases at a prehydrolytic state, traps MalT in an ATP-bound, multimeric form. The tight coupling between the ATPase activity and multimerization that was observed here for the R171A and R171E variants further supports this conclusion. Together with the aforementioned observations, it provides compelling evidence that the ATP hydrolysis observed in the presence of inducer is primarily catalyzed by MalT multimers. This ensures signal extinction

and MalT resetting into ADP-bound state, with the relative concentrations of maltotriose (whether it is endogenous or exogenous) and negative effectors determining whether the protein will be stabilized as a resting form or undergo another round of nucleotide exchange and multimerization. Most likely, the low rate of ATP hydrolysis that characterizes MalT has been evolutionarily set so that the rate of recycling will be optimum with respect to the physiology of the bacterium.

In this respect, MalT behaves differently from APAF1 (*H. sapiens* proapoptotic factor), the sole other STAND whose functioning as an ADP/ATP switch has been studied *in vitro*. Intriguingly, in the case of APAF1, the catalytically competent forms are the ATP-bound monomers (33), thereby indicating that the timing at which ATP hydrolysis occurs may vary with the STAND depending on the physiological constraints. The inability of APAF1 multimers to hydrolyze ATP might be related to the absence of an arginine finger in APAF1 and might also possibly involve an inhibitory finger.

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