ATP Hydrolysis and Pristinamycin IIA Inhibition of the Staphylococcus aureus Vga(A), a Dual ABC Protein Involved in Streptogramin A Resistance

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In Gram-positive bacteria, a large subfamily of dual ATP-binding cassette proteins confers acquired or intrinsic resistance to macrolide, lincosamide, and streptogramin antibiotics by a far from well understood mechanism. Here, we report the first biochemical characterization of one such protein, Vga(A), which is involved in streptogramin A (SgA) resistance among staphylococci. Vga(A) is composed of two nucleotide-binding domains (NBDs), separated by a charged linker, with a C-terminus in regulation of the SgA antibiotic resistance mechanism conferred by dual ATP-binding cassette (ABC) proteins, such as Vga(A) involved in resistance to streptogramin A (SgA) antibiotics, remains so far elusive.

Like other MLS antibiotics, streptogramins inhibit protein translation (3). They consist of two components, produced simultaneously by several strains of streptomyces. The SgA components are cyclic polyunsaturated macrolactones such as pristinamycin IIA (PIIA). The SgB components are cyclic depsipeptides such as pristinamycin IA (PIA). Both components target the ribosomal peptidyl transferase cavity as observed from the crystal structure of the streptogramin-bound 50S subunit (4). The two components are separately bacteriostatic, but they act synergistically when combined, becoming bactericidal. The synergism between SgA and SgB is related to the ability of SgA to enhance the affinity of SgB for the ribosome (5), inducing a conformational rearrangement of the U2585 nucleotide of the 23 S rRNA (4). This synergism was exploited for manufacturing mixtures usable in human medicine, such as Pyostacine® or Synercid® (6–8). A high level of resistance to the synergic mixtures is observed in Gram-positive bacteria when both SgA and SgB resistance determinants are combined (9). Resistance to SgB is due to ermm (MLSgB resistance phenotype) (10) or vgb (SgB resistance phenotype) (11) genes. Resistance to SgA is achieved by ribosome modification as described for Cfr (12), or by drug acetylation as described for Vat(D) (13), or by a putative efflux mechanism, as recently suggested for the ABC protein Vga(A) (14).

ABC proteins constitute one of the most abundant family of proteins in living organisms. They are mainly involved in transport processes, but very few of them have been characterized as antibiotic exporters (15, 16). Typical ABC transporters consist of two transmembrane domains (TMDs), which determine substrate specificity, and two highly conserved nucleotide-
binding domains (NBDs), which couple ATP hydrolysis to substrate transport. The four domains may be expressed as separated polypeptides or fused in a variety of configurations (17). Among ABC antibiotic exporters, substrate specificity can be wide, as illustrated by LmrA or VcaM that can extrude a plethora of antibiotics (18, 19). On the contrary, substrate specificity can be restricted to a subclass of antibiotics, exemplified by MacB that confers resistance only to 14- and 15-membered ring macrolides (20, 21).

Vga(A) belongs to the antibiotic resistance (ARE) subfamily of ABC proteins. ARE proteins comprise two NBDs fused into the same polypeptide chain with no identified TMD partner to constitute a canonical transporter (15, 22). Many ARE subfamilies are found in MLS antibiotic-producing actinomycetes (23). The most studied ARE proteins are Ole(B), from the oleanomycin producer Streptomyces antibioticus (24), and two proteins of distinct specificities: Msr(A) and Vga(A), both of which are coded by mobile genetic elements in staphylococci. Msr(A) confers a high level resistance to 14- and 15-membered ring macrolides and to SgB components (25), whereas Vga(A) confers a low level resistance to lincosamides and a high level resistance to SgA components (14).

In this work, we report the first biochemical characterization of an ARE protein. We have produced full-length Vga(A) in Escherichia coli and purified it as a soluble protein under native conditions. We have characterized its ATPase activity and investigated the effect of different antibiotics targeting the 50 S ribosomal subunit on this activity. We demonstrate the specific interaction between Vga(A), a dual ABC protein devoid of TMDs, and PIIA, its cognate MLS substrate.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Antibiotics**—Plasmid pIP1845 (14), a derivative of the pR8474 shuttle vector carrying the wild-type vga(A) gene, allowing expression of this gene in both *E. coli* and staphylococci, was used as a DNA template in PCR experiments. *E. coli* strain TOP10 and plasmid pCR4 (Invitrogen) were both used for cloning the blunt-ended PCR products. Screening for plasmid DNA mutagenesis was achieved by using *E. coli* XL1-Blue supercompetent cells (Stratagene). *E. coli* strain AG100A, susceptible to SgA by disruption of the AcrAB pump (26), and *Staphylococcus epidermidis* strain BM3302 (27) were used as recipients for testing the functional integrity of the genes cloned into pIP1840, as described previously (14). Overexpression and purification of the recombinant proteins were carried out using BL21 (DE3) pDia17 (28) transformed with vga(A) constructs cloned into the pL1VEX2.3 vector (Roche Applied Science). Antibiotics (ampicillin, lincomycin, and chloramphenicol) were purchased from Sigma or were gifts from Aventis (pristinamycin IA and IIA) and Abbott (erythromycin). Antibiotics were dissolved in water, except for the pristinamycin compounds and chloramphenicol, which were dissolved in methanol and ethanol, respectively. PIIA was qualitatively controlled before and after the enzymatic tests by mass spectrometry.

**Antibiotic Susceptibility**—The minimal inhibitory concentrations of SgA were determined using Mueller-Hinton agar plates (Bio-Rad) in the presence or absence of orthovanadate (Sigma) with a 2-fold increase in antibiotic concentration from 0.125 μg·ml⁻¹ to 128 μg·ml⁻¹. Overnight cultures of *E. coli* AG100A or *S. epidermidis* BM3302 harboring pIP1840 and its derivatives contained 100 μg·ml⁻¹ ampicillin or 10 μg·ml⁻¹ chloramphenicol, added to Luria-Bertani or brain-heart infusion media, respectively. 10 μl of a 1000-fold dilution of these cultures was spotted onto Mueller-Hinton agar plates. Incubation was done for 24 h at 37 °C.

**Plasmid Constructions**—Full-length and 3' truncated versions of the vga(A) gene were obtained by PCR from plasmid pIP1845 using NcoI sense primers VGA5 and VGA8, in combination with antisense primers VGA3-Smal, and VGA2-EcoRV, respectively (Table 1). Amplification was done in both cases with PfX DNA polymerase (Invitrogen). PCR products were cloned into pCR4 with Zero Blunt TOPO kit (Invitrogen). Inserts of expected sizes were completely sequenced to check the correctness of the genes: the longest one encoded a full-length version of the wild-type Vga(A) protein modified only by the insertion at +2 of an alanine (called thereafter Vga(A)), whereas the shortest one contained an insertion at +2 of a glycine coupled with a deletion at the C terminus of the last 18 amino acids (called thereafter Vga(A)ΔCter). Subcloning of the recombinant vga(A) genes into pL1VEX2.3 vector cut with NcoI and Smal enzymes yielded the pL1VEX-derived expression plasmids: pIP1884 carried the NcoI-Smal fragment that codes for Vga(A), whereas pIP1834 carried the NcoI-EcoRV fragment that codes for Vga(A)ΔCter. Both recombinant Vga(A) proteins contain a C-terminal 6× histidine tag provided by the vector. Functional *in vivo* testing of the recombinant proteins, i.e. the capacity to confer bacterial resistance against SgA, was achieved by cloning the full-length and the truncated histagged versions of Vga(A) into the *E. coli*-S. aureus expression plasmid pIP1840 as previously described (14). Two single mutations downstream of the Walker B motifs, E105Q and E410Q (Fig. 1), were obtained by site-directed mutagenesis using oli-

### TABLE 1

| Oligonucleotide name | Used for | Sequence |
|----------------------|----------|----------|
| VGA5                 | Cloning of a full-length version of Vga(A) | CTCCATGGCAAAAATAATGTTAGAGGGAC |
| VGA5-Smal            | Cloning of a full-length version of Vga(A) | CTCCATGGCAAAAATAATGTTAGAGGGAC |
| VGA8                 | Cloning of a ΔCter version of Vga(A) | CTCCATGGCAAAAATAATGTTAGAGGGAC |
| VGA2-EcoRV           | Cloning of a ΔCter version of Vga(A) | CTGAAAGCTCTCCGAAGGTTCAATACCTC |
| E105QForward         | Glu replaced by Gln at position 105 | CTGCTTATAGCAGTTCACCAACACAACTAACCT |
| E105QReverse         | Glu replaced by Gln at position 105 | AGTTAGTTGTTGGTTACCT |
| E410QForward         | Glu replaced by Gln at position 410 | AACCAACAAAACAAACAACTAACCTC |
| E410QReverse         | Glu replaced by Gln at position 410 | GAAAGTTTTCTGTGATTGATCCTAGTACCAACAGT |
godeoxyribonucleotides listed in Table 1 and the QuikChange kit (Stratagene) as recommended by the manufacturer. Sequencing reactions and synthesis of the oligodeoxyribonucleotides were provided by Genome Express.

Production of the Recombinant Vga(A) Proteins—Strain BL21(DE3) pDIA17 was transformed with the pIVEX-derived expression plasmids and grown at 37 °C in yeast extract tryptone (2YT) medium (Difco) containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) until the absorbance at 600 nm reached a value of 0.4 unit. Production of the Vga(A) recombinant proteins was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Promega, Madison, WI) for 16 h at 16–18 °C (final cell density of 3–4 units at 600 nm). Then, cells were harvested by centrifugation (10,000 × g for 15 min) at 4 °C, frozen in liquid nitrogen, and stored at −80 °C until use. Mutated proteins were expressed and overproduced in the same culture conditions as the wild type. Protein overexpression was controlled by 10% SDS-PAGE analysis of total cell extracts.

Protein Purification—The same protocol was followed for all recombinant Vga(A) proteins. All purification steps were performed at 4 °C. The cell pellet from a 2-liter culture was resuspended in 40 ml of buffer A (50 mM sodium/potassium phosphate, pH 7.0, 20 mM NaCl, 1 mM MgCl2, 1 mM imidazole, and 0.5% Triton X-100) with 1 mM Pefabloc-SC (Roche Applied Science). Cells were lysed by sonication (5 s on/5 s off, 3 cycles) in a Vibro-cell sonicator (Fisher Bioblock Scientific, Illkirch Cedex, France). The cell lysate was incubated for 30 min at room temperature under mild shaking with 1500 units of benzonase (Sigma). Four milligrams of protamine sulfate (Sigma) were then added, and the cell lysate was incubated for further 30 min under the same conditions. The cell extract was centrifuged at 200,000 × g for 1 h, and imidazole was added to the supernatant to a final concentration of 25 mM. The extract was applied onto a 1-ml HisTrap nickel-affinity column (GE Healthcare Life Sciences) connected to an AKTA-Explorer system (GE Healthcare Life Sciences) at a flow rate of 1 ml/min. The resin was washed with buffer B (50 mM sodium/potassium phosphate, pH 7.0, 400 mM NaCl) and 50 mM imidazole until the baseline (A280) was reached. Proteins were eluted from the column with a 50–250 mM imidazole gradient in buffer B. Vga(A)-containing fractions were controlled by SDS-PAGE analysis, pooled, and concentrated to a final volume of 10 ml. This pool was loaded onto a 50-ml HiPrep 26/10 desalting column (GE Healthcare Life Sciences) equilibrated with buffer C (50 mM sodium/potassium phosphate, pH 7.0, 1 mM EDTA, 10% glycerol, 7 mM β-mercaptoethanol) with 20 mM NaCl, at a flow rate of 3 ml/min. Vga(A)-containing fractions were applied onto a 1-ml Resource S cationic exchanger resin (GE Healthcare Life Sciences) and eluted with a linear gradient of 20–500 mM NaCl in buffer C. Vga(A) fractions were collected and concentrated on Amicon Ultra 10K membranes (Millipore) to a final volume of 400 μl. The last purification step was performed on a Superdex 200 HR10/30 column (GE Healthcare Life Sciences) equilibrated with buffer D (25 mM Tris–HCl, pH 7.0, 150 mM NaCl, 7 mM β-mercaptoethanol). The Vga(A) protein was finally stored at −80 °C at 30–100 μM. Protein concentration was determined with the Bio-Rad protein assay using bovine serum albumin as a standard. SDS-PAGE was carried out using a 10:25 acrylamide/bisacrylamide gel and stained with Coomassie Blue. Molecular mass standards for SDS-PAGE and for gel filtration chromatography were purchased from GE Healthcare Life Sciences. Immune detection of Vga(A) proteins was carried out as previously described (14).

ATPase Activity—The ATPase activity of Vga(A) proteins was determined at 30 °C by following the amount of radiolabeled inorganic phosphate released during [γ-33P]ATP hydrolysis. The [33P]Pi produced was isolated from [γ-33P]ATP (GE Healthcare Life Sciences) using the charcoal method (29). Standard reaction mixtures (100 μl) contained, unless otherwise indicated, 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl2, and 0.2–1 μM of purified Vga(A). The reaction was started by adding [γ-33P]ATP (200 μM, 4 Bq/μmol−1) and stopped at the indicated time at 4 °C by withdrawing 10–μl aliquots of the reaction mixture and adding them to 400 μl of a 4% activated charcoal suspension in 20 mM H3PO4. After centrifugation (10,000 × g for 5 min), 200 μl of supernatant was mixed with 3 ml of Optiphase HiSafe3 (PerkinElmer Life Sciences) and counted in a Wallac 1414 liquid-scintillation counter. When antibiotics were used, the amount of methanol never exceeded 2% of the final reaction mix and was kept constant for all the experimental conditions that were tested. To probe inhibition by PIIA, kinetic assays were performed as previously described with 1 μM purified Vga(A), ATP concentrations ranging from 50 to 800 μM, and PIIA from 100 to 800 μM. To test reversibility of PIIA inhibition, 10 μM Vga(A) was preincubated for at least 10 min at 30 °C in the reaction buffers with or without 1 mM PIIA, and then diluted 10 times in the same buffers with or without 1 mM PIIA. When buffers without PIIA were used for the sample dilution, the final PIIA concentration dropped down to 0.1 μM. [γ-33P]ATP was added to start the reaction kinetics. Freshly boiled orthovanadate solutions were prepared as described (30).

RESULTS

Expression and Antibiotic Resistance Properties of Vga(A) in Staphylococci and in E. coli—To analyze the biochemical properties of Vga(A) and to simplify its purification, a tag consisting of 11 residues, including 6 histidines, was added to the carboxyl end of the full-length protein (Fig. 1). The histidine tag did not modify the minimal inhibitory concentration values of PIIA in S. epidermidis (Table 2), demonstrating that it does not alter the biological function of Vga(A). By contrast, Vga(A) was unable to confer SaA resistance in E. coli AG100A, a strain rendered susceptible to SaA compounds by disruption of the AcrAB pump. On Western blots probed with a specific antisera, a band of ~66 kDa, corresponding to Vga(A), was present in the soluble extracts of E. coli cells (not shown). This band was not detected in membrane fractions, contrary to what occurred within the original hosts, S. aureus or S. epidermidis (14). This result suggests that the absence of resistance of E. coli toward SaA is not due to defective expression of the protein in the heterologous host. Obviously at least one protein partner targeting Vga(A) to the membranes and/or other factor that contributes to the functionality of Vga(A) in staphylococci is not present in E. coli.
ATPase Activity of Vga(A) and Pristinamycin IIA Inhibition

Purification of Vga(A) and Its Mutated Derivatives—Because the tag did not affect Vga(A) functionality in the native host, we expressed and purified recombinant Vga(A) from E. coli. Using pIVEX vector, Vga(A) was significantly overproduced at 16–20 °C and extracted mainly in a soluble form. Elution of Vga(A) from an immobilized metal ion affinity chromatography column yielded several peaks containing the protein with high amounts of nucleic acids that prevented binding to the subsequent cationic exchanger column. Removal of contaminating nucleic acids, by using benzonase and protamine sulfate precipitation, highly increased the efficiency of the second purification step (Resource S). The third and final purification step, performed on a size-exclusion chromatography column (Superdex 200), allowed us to obtain Vga(A) purified to homogeneity (supplemental Fig. S1A). The purification yields ranged from one to two mg liter⁻¹ culture.

The three mutated derivatives, Vga(A)ΔCter, Vga(A)-E105Q, and Vga(A)-E410Q (Fig. 1), were overproduced and purified following the same protocol. However, we were unable to obtain significant soluble amounts of the Vga(A)-E105Q-E410Q double mutant. All Vga(A) recombinant proteins behave as monomers with the expected molecular weight (supplemental Fig. S1B), and no dimer formation could be evidenced by gel filtration experiments, even in the presence of Mg²⁺ and/or ATP in all buffers (not shown). All proteins were stored at −80 °C for months, without loss of catalytic activity, which remained homogeneous among various preparations.

Intrinsic ATPase Activities of Vga(A) and Its Mutants—The ATPase activity of Vga(A) was linear during the time of kinetics (Fig. 2A). The high catalytic activity of Vga(A) allowed us to work with final protein concentrations that ranged from 0.1 to 1 μM. Similarly to all other ABC proteins, the catalytic activity of Vga(A) was strictly dependent on the presence of Mg²⁺ ions. Because the initial velocity of hydrolysis decreased only by ~50% at 500 mM NaCl, we concluded that Vga(A) is not salt-sensitive at physiological concentrations. This stands in sharp contrast to HlyB ATPase domains, whose ATPase activity drops to <25% in the presence of 100 mM NaCl (31). Vga(A) exhibited a weak sensitivity to orthovanadate, because 50% inhibition of the catalytic activity was achieved with 2 mM of this compound and 90% inhibition with 10 mM (not shown). At the latter concentration, there is no effect of this compound on the minimal inhibitory concentration values of SgA in S. epidermidis, indicating that both ATPase activity and biological function of Vga(A) are quite resistant to orthovanadate.

The two NBDs of Vga(A) have distinct sizes, the N-terminal one (NBD1) being smaller than the C-terminal (NBD2), with a large deletion between the Q-loop and the signature motif (Fig. 1). To evaluate the contribution of each NBD to the ATPase activity of Vga(A), the catalytic glutamate residues downstream of the Walker B motifs were mutated, and the ATPase activities of the mutants were measured. Compared with Vga(A), the catalytic activities of Vga(A)-E105Q and Vga(A)-E410Q represent 70 and 25% of the total ATPase activity, respectively, when measured in the standard reaction mixture (200 μM ATP) (Fig. 2B). The summed ATPase activities of the two mutants nearly account for the entire activity of the wild type. We conclude that ATP hydrolysis can take place at one site independently of
This region, a deletion encompassing the last 18 residues was made and the properties of this Vga(A)ΔCter protein were analyzed. When compared with the wild type, Vga(A)ΔCter conferred a 4-fold reduced level of SgA resistance (Table 2), despite similar expression levels in the host (not shown). Because the resistance phenotype is dependent on the ATPase activity of the protein (14), we investigated whether this Cter deletion, which does not include any known NBD structural element, would cause a modification in the ATP hydrolysis rate. A precise determination of the kinetic parameters indicated that the affinity for ATP of the Vga(A)ΔCter appears to be slightly lower (102 μM) than that of Vga(A) (78 μM). The maximum velocity was also slightly affected by the deletion (4.6 versus 6.8 pmol-pmol⁻¹-min⁻¹) (Fig. 3). Thus, the deletion of the 18 C-terminal residues only weakly affected the ability of Vga(A) to hydrolyze ATP. The tenuous differences detected in vitro cannot by themselves explain the decrease in antibiotic resistance levels determined in staphylococci. Therefore, the deleted Cter sequence would contain residues important for biological activity but not essential for intrinsic ATPase activity.

**ATPase Activity of Vga(A) Is Specifically Inhibited by Pristinamycin IIA**—The sole presence of Vga(A) in staphylococcal membranes confers SgA resistance (14). In the absence of characterized TMDs, we tested whether the antibiotic specificity of the putative transporter could be, at least in part, determined by Vga(A) itself. We therefore tested the influence of several antibiotics from the MLS family on the intrinsic ATPase activity of Vga(A). We also used as a control, the non-MLS antibiotic chloramphenicol, whose binding site overlaps that of SgA antibiotics in the ribosomal peptidyl transferase center (4). No effect on the catalytic activity of Vga(A) was detected when erythromycin (macrolide), lincomycin (lincosamide), PIA (streptogramin B), and chloramphenicol were added to the reaction mix (Fig. 4A), in line with the inability of Vga(A) to confer a high level of resistance against these antibiotics (14). In contrast, a strong inhibition of the ATPase activity was conferred by PIIA, the antibiotic against which Vga(A) confers a high level resistance. Such an inhibition suggests that the purified ABC protein directly interacts with PIIA, whose structure is shown in Fig. 4B. An average value of 450 μM PIIA was necessary to inhibit 50% of the ATPase activity of Vga(A) (Fig. 5A). The affinity for the antibiotic is therefore somewhat lower than that determined for the nucleotide. To understand the mechanism of PIIA inhibition of Vga(A) ATPase activity, we also set up kinetic experiments with various concentrations of PIIA and ATP. Data for PIIA inhibition of Vga(A) ATPase activity at different ATP concentrations strongly support a non-competitive mechanism (Fig. 5B). This suggests that the ATP and the PIIA binding sites are distinct.

Given the high chemical reactivity of PIIA and to rule out a possible covalent bond between PIIA and the protein, Vga(A) was incubated with the drug in the conditions found to inhibit its ATPase activity by at least 50%. No molecular adduct of the protein could be detected by electrospray mass spectrometry analysis (not shown). This demonstrates that the drug is not covalently bound to Vga(A). To investigate the reversibility of inhibition by PIIA, Vga(A) was preincubated with a high inhibitor concentration (1 mM PIIA). Such
In vitro inhibition values of ATPase activity by PIIA were the same for Vga(A)ΔCter and Vga(A) at all antibiotic concentrations (Fig. 7, A and B). Such results show unambiguously that the C terminus of Vga(A) is not involved in PIIA recognition. Moreover, because both Vga(A)ΔCter and Vga(A) are detected in membrane fractions of S. epidermidis (not shown), the C terminus apparently does not interfere with the functional anchoring of Vga(A). However, Vga(A)ΔCter is particularly less efficient in vivo than Vga(A) to confer SgA resistance (Table 2), suggesting that the last 18 amino acids of the protein are important for biological activity, while they are not involved in the specific SgA interaction.

**DISCUSSION**

We report the first biochemical characterization of a full-length dual ABC protein of the ARE subfamily, which comprises a large set of proteins characterized by two fused NBDs and no evidence for TMDs. Proteins of this subfamily are involved in MLS antibiotic resistance. To the best of our knowledge, only a fragment of Ole(B), a protein belonging to this subfamily, has been produced and purified successfully (24). However, this construct consisted of a fusion of the E. coli maltose-binding protein to the N-terminal ABC domain of Ole(B). Our results, obtained with a full-length protein modified only by addition of a histidine tag that does not influence its biological activity, constitute a framework that might be generalized to the other proteins of the ARE subfamily.

Native molecular weight determinations by gel filtration suggest that Vga(A) is exclusively monomeric and that ATP does not induce formation of protein dimer. It is generally accepted that NBDs of ABC systems dimerize upon binding of ATP. In the presence of this nucleotide, substantial levels of dimeric forms have been recorded by gel filtration. Stable dimers in solution and in crystals could be obtained in some cases by introducing mutations that disrupted the ATP hydrolytic activity (33, 34).

Purified Vga(A) protein displays a strong basal ATPase activity, and its kinetic parameters for ATP hydrolysis compares well with those available for other ABC ATPases. Purified Vga(A) is weakly inhibited by orthovanadate since a concentration of 10 mM is needed to achieve 90% inhibition of ATPase activity. Vanadate, which mimics inorganic phosphate (Pi), inhibits ABC ATPases in the micromolar range by forming a stable trapped complex with ADP after the first round of ATP hydrol-

**FIGURE 4. Influence of antibiotics on the ATPase activity of Vga(A).** A, the ATP hydrolysis rate measured as a function of PIIA concentration (100–800 μM) with 1 μM Vga(A) and 200 μM [γ-32P]ATP from 15-min kinetics. Indicated values represent the relative ATPase activity as compared with that measured in the absence of PIIA. B, molecular structure representation of pristinamycin IIA.

**FIGURE 5. Inhibition of Vga(A) ATPase activity by PIIA.** A, ATP hydrolysis rate measured as a function of PIIA concentration (100–800 μM) with 1 μM Vga(A) and 200 μM [γ-32P]ATP from 15-min kinetics. Indicated values represent the relative ATPase activity as compared with that measured in the absence of PIIA. B, non-competitive inhibition of ATPase activity of Vga(A) by PIIA. Vga(A) was assayed in standard conditions as previously described with 200–800 μM PIIA and 50–800 μM ATP. A direct representation was used to determine the K<sub>m</sub> and V<sub>m</sub> values for ATP with Michaelis-Menten equation at each concentration of PIIA. V<sub>m</sub> and K<sub>m</sub> correspond to values obtained in the absence of PIIA. K<sub>m</sub> was not significantly modified by PIIA. K<sub>i</sub> was determined using linearization of the equation defining V<sub>m</sub> for non-competitive inhibition: V<sub>m</sub> = V<sub>m</sub>/[(PIIA)/K<sub>i</sub>]), and the intercept was set to 1.
ATPase Activity of Vga(A) and Pristinamycin IIA Inhibition

**FIGURE 6. Reversibility of the PIIA-induced inhibition of Vga(A) catalytic activity.** ATPase kinetics were performed for 20 min in the standard buffer with 1 μM Vga(A) and 500 μM [γ-32P]ATP. The experiments were done in the absence (A) or with PIIA final concentrations of 0.1 mM (B) and 1 mM (C). Prior to the addition of [γ-32P]ATP to start the reaction, samples D, E, and F were first incubated for 10 min with 1 mM PIIA (E and F), or without (D) as a control. As described under “Experimental Procedures,” reaction mixtures were subsequently diluted and final concentrations of PIIA were 0 mM (D), 0.1 mM (E), or 1 mM (F).

**FIGURE 7. Comparison of the sensitivities of Vga(A) and Vga(A)ΔCter to PIIA.** ATPase activity (total cpm) of 0.5 μM Vga(A)wt (A) and 1 μM Vga(A)ΔCter (B) with 200 μM [γ-32P]ATP in the presence of different concentrations of PIIA: 0.16 μM (•), 0.5 μM (□), 1 μM (○). Control without PIIA (△) and blank values with no enzyme (×) are also indicated.

ysis, thereby preventing subsequent cycles of catalysis (35). In this respect, Vga(A) behaves similarly to isolated ATPase subunits of ABC transporters, like MalK whose ATPase activity is resistant to vanadate but highly sensitive to this inhibitor when present in complex with its cognate membrane proteins MalF and MalG (36). However, as tested here, vanadate is clearly not an inhibitor of the SgA resistance mechanism in staphylococci. Insensitivity to vanadate might be a general feature of the ART family of ABC systems that comprise homologues to both Insensitivity to vanadate might be a general feature of the ART family of ABC systems that comprise homologues to both MalG (15), an *E. coli* maltose transporter. In contrast, the ATPase activity of ABC domains produced alone is insensitive to transported substrates, with few exceptions such as the ATPase subunits of the *E. coli* galactose (MglA) and spermidine (PotA) transporters, respectively. Activation (MglA) as well as inhibition (PotA) of basal ATPase activity were reported in presence of their substrate (44, 45). We propose that, in the case of Vga(A), the observation of substrate-mediated inhibition of ATPase activity of the purified protein may reflect the lack of an essential partner, possibly a transmembrane protein, that would be present in staphylococci and absent in *E. coli*.

The antibiotic binding site in Vga(A) is probably different from or non-overlapping with the ATP-binding site, as suggested by the non-competitive inhibition pattern of ATPase activity by PIIA. Contrary to recent findings reported for the Wzm/Wzt lipopolysaccharide exporter where the substrate binding site was identified in a C-terminal extension of the Wzt NBD (46), our biochemical characterization of the Vga(A)ΔCter protein allowed us to conclude that the C-terminal sequence of Vga(A) is not responsible for PIIA recognition. Indeed, our *in vitro* results demonstrated the same sensitivity of Vga(A) and Vga(A)ΔCter toward PIIA. This deletion has clearly a greater impact *in vivo*, because it confers a reduced level of resistance to PIIA without affecting the normal (40–42). The fact that overall ATPase activity of Vga(A) tolerates mutations in individual ABC domains suggests, at least *in vitro*, that the ATPase activities of the two NBDs are not strictly coordinated, i.e. an altered catalytic site in the NBD dimer allows ATP hydrolysis at the other site. The mutation affecting NBD2 is more detrimental than the other, revealing some asymmetry in NBD function. This behavior might be a consequence of the large dissymmetry observed in the sequence of Vga(A), where the N-terminal domain is shorter than the C-terminal one (Fig. 1). More precisely, the helical subdomain of NBD1 lacks at least two predicted helices compared with most ABC NBDs, because it contains only the helix down-stream of the ABC signature. The particular topology around this catalytic site should have an incidence on ATP hydrolysis, because the structural rearrangements, occurring in NBDs on ATP binding and ADP release and consisting of rotations of the helical subdomain relative to the RecA-like subdomain, have been described as being key points in the catalytic cycle among ABC transporters (reviewed in Ref. 43).

Each protein of the ARE subfamily confers specific resistance to different antibiotics of the MLS class. In ABC transporters, the substrate specificity is usually dependent on substrate binding sites present in the TMDs. However, no such integral membrane proteins have been identified so far to interact with ARE subfamily ATPases. Moreover, antibiotic resistance can be obtained in sensitive staphylococci by introducing the gene encoding such ABC alone. Our observation that Vga(A) ATPase activity is strongly affected by PIIA demonstrates a direct interaction of purified Vga(A) with the SgA antibiotic. Consequently, this ABC ATPase contains a substrate recognition site.

In canonical ABC transporters, activation of ATPase activity by the substrate seems to be the rule, as shown for P-glycoprotein and for the *E. coli* maltose transporter. In contrast, the ATPase activity of ABC domains produced alone is insensitive to transported substrates, with few exceptions such as the ATPase subunits of the *E. coli galactose* (MglA) and spermidine (PotA) transporters, respectively. Activation (MglA) as well as inhibition (PotA) of basal ATPase activity were reported in presence of their substrate (44, 45). We propose that, in the case of Vga(A), the observation of substrate-mediated inhibition of ATPase activity of the purified protein may reflect the lack of an essential partner, possibly a transmembrane protein, that would be present in staphylococci and absent in *E. coli*.

The antibiotic binding site in Vga(A) is probably different from or non-overlapping with the ATP-binding site, as suggested by the non-competitive inhibition pattern of ATPase activity by PIIA. Contrary to recent findings reported for the Wzm/Wzt lipopolysaccharide exporter where the substrate binding site was identified in a C-terminal extension of the Wzt NBD (46), our biochemical characterization of the Vga(A)ΔCter protein allowed us to conclude that the C-terminal sequence of Vga(A) is not responsible for PIIA recognition. Indeed, our *in vitro* results demonstrated the same sensitivity of Vga(A) and Vga(A)ΔCter toward PIIA. This deletion has clearly a greater impact *in vivo*, because it confers a reduced level of resistance to PIIA without affecting the normal

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membrane localization in staphylococci. Altogether, our data strongly suggest that the Vga(A) C terminus might be rather involved in regulation and/or energy coupling of the SgA resistance mechanism.

The relatively long linker between the two NBDs constitutes another attractive potential drug-binding site. Very recently, a Vga(A)C, variant conferring in vivo an increased resistance to lincomycinides and a reduced resistance to SgA, was isolated from Staphylococcus haemolyticus (47). As compared with Vga(A), this variant displays four sequence changes that affect amino acids located within the linker region and responsible for the altered substrate specificity. This observation, in line with our in vitro results, suggests a role of this linker in substrate recognition. Site-directed mutagenesis will be next used within the linker region of Vga(A) to alter the PIIA substrate specificity of the protein and therefore to confirm its importance in the specificity of the antibiotic resistance phenotype of ARE proteins.

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