Interaction between ATP, oleandomycin and the OleB ATP-binding cassette transporter of *Streptomyces antibioticus* involved in oleandomycin secretion

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The OleB protein of *Streptomyces antibioticus*, oleandomycin (OM) producer, constitutes an ATP-binding cassette transporter containing two nucleotide-binding domains and is involved in OM resistance and its secretion in this producer strain. We have characterized some properties of the first nucleotide-binding domain of OleB using an overexpressed fusion protein (MBP–OleB') between a maltose-binding protein (MBP) and the first half of OleB (OleB'). Extrinsic fluorescence of the base-modified fluorescent nucleotide analogue 1,*N*⁶-ethenoadenosine 5'-triphosphate (ϵ ATP) and 2'(3')-*o*-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate was determined in the presence of MBP and the fusion protein MBP–OleB', and it was found that ϵ ATP binds to MBP–OleB' with a stoichiometry of 0.9. Measurements of the intrinsic fluorescence of the MBP–OleB' fusion protein indicated

that ATP induces a decrease in the accessibility of the MBP–OleB' tryptophans to acrylamide, an indication of a folding effect. This conclusion was confirmed by the fact that ATP also induces considerable stabilization against guanidine chloride denaturation of MBP–OleB'. Two effects were found to be associated with the presence of Mg²⁺ ions: (1) an increase in the quenching of MBP–OleB' intrinsic fluorescence by ATP; and (2) an increase in the accessibility of MBP–OleB' tryptophans to acrylamide. Significant changes in the intrinsic fluorescence of the fusion protein were also observed in the presence of OM, demonstrating the existence of interaction between the transporter and the antibiotic in the absence of any hydrophobic membrane component.

INTRODUCTION

The ABC transporters (ATP-Binding Cassette) [1,2], also referred to as traffic ATPases [3], are a large family of membrane-associated export and import systems which share a well-conserved amino acid region of approx. 200 residues. This region represents the ATP-binding domain and has two characteristic motifs known as Walker A and B motifs [4]. They participate in the incorporation into, and secretion from, the cells of many different molecules, including sugars, amino acids, oligopeptides, ions, etc. Some of the most important transporters of this family, because of their clinical implications, are the eukaryotic multidrug resistance proteins responsible for multiresistance to chemotherapeutic drugs [5], and the cystic fibrosis transmembrane regulator found to be defective in patients with cystic fibrosis [6].

Antibiotic resistance in producer organisms can also be mediated by ABC transporters. Genes encoding ATP-binding proteins have been found to confer resistance to the macrolide antibiotics carbomycin, spiramycin [7] and tylosin [8] in the respective producer strains, and also to the antitumour drugs daunorubicin [9] and mithramycin [10] and to the ionophore tetracosin [11]. From *Streptomyces antibioticus*, the producer of the macrolide oleandomycin (OM), we have cloned and characterized two OM resistance genes, both encoding ATP-binding proteins: the *oleC* [12] and the *oleB* genes [13]. In addition, it has been demonstrated that both OM and its inactive glucosylated form can be secreted outside the mycelium by the OleB protein [13]. Analysis of the deduced amino acid sequence of OleB shows that it contains two nucleotide-binding domains (NBDs) but only the first NBD is sufficient to confer resistance to OM [13].

We have recently overexpressed this first NBD of OleB as a fusion protein (MBP–OleB') with a maltose-binding protein (MBP) [14]. In this paper we present experimental evidence that the interaction between the cofactor (ATP), the substrate (OM) and the transporter induces some conformational changes in the fusion protein.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade. All solutions were made with distilled and deionized (Milli-Q) water. ATP, guanidine chloride, acrylamide and 1,*N*⁶-ethenoadenosine 5'-triphosphate (ϵ ATP) were from Sigma. 2'(3')-*o*-(2,4,6-Trinitrophenyl)-adenosine-5'-triphosphate (TNP-ATP) was from Molecular Probes.

Fluorescence measurements

Fluorescence measurements were carried out in a Perkin-Elmer MFF-44A fluorimeter at 25 °C. The ATP fluorescence was determined by using an excitation wavelength of 340 nm and an emission wavelength of 410 nm (in the case of ϵ ATP), and an excitation wavelength of 410 nm and an emission wavelength of 530 nm (in the case of TNP-ATP). The slit widths at excitation and emission were 10 and 5 nm respectively. Binding of ATP analogues was determined by the difference between the fluores-

Abbreviations used: ABC, ATP-binding cassette; ϵ ATP, 1,*N*⁶-ethenoadenosine 5'-triphosphate; MBP, maltose-binding protein; NBD, nucleotide-binding domain; OM, oleandomycin; TNP-ATP, 2'(3')-*o*-(2,4,6-trinitrophenyl)adenosine-5'-triphosphate.

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cence of ATP alone and in the presence of the protein. Inner-filter effect was corrected using a procedure previously described [15]. Stoichiometric analysis was performed as previously described [16,17]. In this method I_0/α is plotted as a function of $1/1-\alpha$ where I_0 is the protein concentration and α the ratio $\Delta F/\Delta F_{\max}$, giving both dissociation constant and concentration of bound substrate. Tryptophan intrinsic fluorescence was measured by using an excitation wavelength of 295 nm and an emission wavelength of 340 nm. The slit widths were 16 and 6 nm for excitation and emission respectively. Samples were measured in 1 cm rectangular quartz cuvettes in 1 ml of 20 mM Tris/HCl, pH 7.4, 0.2 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM sodium azide. The protein solutions and solutes to be tested were added to the buffer during the experiments as microvolume additions of concentrated solutions. The fluorescence values were corrected for the dilution effect and for background light intensity. Apparent dissociation constants were determined from the substrate concentrations causing a change of 50% in extrinsic or intrinsic fluorescence. Quenching by acrylamide and guanidine chloride denaturation was calculated by measuring the intrinsic fluorescence of the proteins. Protein concentrations were from 0.6 μM (for extrinsic fluorescence) and 0.3 μM (for intrinsic fluorescence). All the values given are the averages of at least three (in some cases five) determinations using three different preparations of proteins.

Protein determinations

Protein concentrations in the different samples were calculated by the protein-dye binding assay [18].

RESULTS

Binding of ATP to MBP–OleB'

Different fluorescent analogues of ATP have the property of increasing their fluorescence when bound to a macromolecule. Therefore, the measurement of their fluorescence in the presence of a putative ATP-binding protein can be used as a very convenient method to determine the interaction between ATP and the nucleotide-binding protein. Fluorescent analogues such as ϵATP and TNP-ATP have been successfully used by different authors in several systems [16,19–23]. We therefore decided to characterize the interaction between such ATP analogues and OleB. To analyse the binding of ATP to the OleB ABC transporter of *S. antibioticus* we used a hybrid protein (MBP–OleB') overexpressed and purified from *Escherichia coli* containing plasmid pMALORB' [14]. In this construction the first half of *oleB* (from the starting codon to a *Bgl*II site located between the two NBDs) [14] was fused in-frame to the 3'-end of the *malE* gene. The resultant hybrid protein (MBP–OleB') is therefore made from the first NBD of OleB and the MBP. Titration of MBP with ATP (Figure 1) did not show any relevant increase in fluorescence. This important control emphasizes that the nucleotide-binding properties of the fusion protein (see below) are due to interaction of ATP with OleB' itself and not with the MBP. Fluorescence of ATP showed a clear increase with respect to ATP concentration in the presence of 0.6 μM MBP–OleB' (Figures 1A and 1B). The apparent K_d was 10 μM ($\pm 2 \mu\text{M}$) for ϵATP and 4 μM ($\pm 1 \mu\text{M}$) for TNP-ATP. A convenient method of determining the stoichiometry (ATP:OleB') is to plot the titration data, using the method described by Stinson and Holbrook [17]. Since results obtained with this method are not significant when the protein concentration is lower than the K_d [17], we repeated the titration with 6 μM MBP–OleB' using TNP-ATP. The data plotted as described by Stinson and Holbrook

Figure 1 Titration of MBP ($\circ$) and MBP–OleB' ($\bullet$) by ϵATP (A) and by TNP-ATP (B) and determination of the stoichiometry of TNP-ATP on MBP–OleB' (C) using a previously described method [23]

Protein concentration was 0.6 μM . ΔF is represented in arbitrary units (a.u.). I_0 is the nucleotide concentration, α is the ratio $\Delta F/\Delta F_{\max}$.

indicate a K_d of 3.7 μM and a stoichiometry for ATP:OleB' of 0.9 (Figure 1C). In order to determine if ATP could bind to the same site as its fluorescent analogues, the titrations were carried out in the presence of different concentrations of ATP. Because ATP does not significantly modify the fluorescence of TNP-ATP (results not shown), titration of MBP–OleB' with TNP-ATP, in the presence of a range of ATP concentrations (0–16 mM), was carried out (Figure 2A). The apparent K_d of TNP-ATP in the presence of ATP was evaluated as described [24], assuming a hyperbolic binding curve reaching the same saturation value for all curves. The apparent K_d for TNP-ATP, plotted as a function of ATP concentration, showed a linear correlation, indicating a competitive inhibition between ATP and TNP-ATP (Figure 2B), and extrapolation to apparent $K_d = 0$ gave an estimated K_d for ATP of 4 mM. We also observed that fluorescence of ATP analogues in the presence of the MBP–OleB' fusion protein was significantly reduced by Mg^{2+} (from 50 μM to 2 mM) (results not shown). This observation is in agreement with that reported for a sarcoplasmic reticulum Ca^{2+} -ATPase [23] and for the bacterial protein translocator HlyB [16], where a similar reduction of the

Figure 2 (A) Titration of MBP-OleB' with TNP-ATP, and (B) apparent K_d of TNP-ATP as a function of ATP concentration

(A) MBP-OleB' was titrated with TNP-ATP in the presence of different concentrations of ATP: 0 mM (●); 4 mM (○); 8 mM (▲); 16 mM (△). Protein concentration was 0.6 μM .

Figure 3 Effect of ATP on the intrinsic fluorescence of MBP-OleB'

Tryptophan intrinsic fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 340 nm. S.D. did not exceed 5%.

TNP-ATP fluorescence was observed when $MgCl_2$ was present. The finding that the fusion protein binds to the amylose resin and also binds ATP demonstrates that within the fusion complex both the MBP and OleB' protein moieties have folded correctly in order to allow their respective substrate binding sites to function. All these experiments demonstrate that the OleB' protein binds ATP.

Figure 4 Effect of $MgCl_2$ on the intrinsic fluorescence of MBP-OleB' in the presence (●) or in the absence (○) of 2 mM ATP

Measurements of ATP-induced changes in intrinsic fluorescence of MBP-OleB'

Fluorescence of tryptophan (and to a lesser extent tyrosine) in proteins depends significantly on their environment. Consequently, the study of variations in the intrinsic fluorescence of proteins can be used to obtain information about protein conformation. The emission spectrum of fluorescence of MBP-OleB' was recorded at an excitation wavelength of 295 nm. At the 295/340 nm excitation/emission wavelength pair, the only significant contribution to the intrinsic fluorescence comes from the tryptophan residues [25]. Since tryptophan fluorescence is a sensitive marker of the conformational changes of proteins [25], we examined whether ATP and Mg^{2+} induce any change in this property of MBP-OleB'. As Figure 3 shows, ATP induces a decrease in the intrinsic fluorescence of MBP-OleB'. The ATP concentration corresponding to 50% quenching was 5 mM, which is in good agreement with the K_d value of 4 mM observed using ATP as a competitor of TNP-ATP (Figure 2). We also observed that ATP induces partial fluorescence quenching of MBP (results not shown). However, because MBP-OleB' fluorescence is approx. three times more important than MBP fluorescence alone, we can reasonably assume that the effect of ATP on MBP-OleB' results essentially from its interaction with OleB'. In the presence of $MgCl_2$ (but in the absence of ATP) no significant changes in the intrinsic fluorescence of MBP-OleB' were observed (Figure 4). However, these conditions significantly increased the effect of ATP on the intrinsic fluorescence of MBP-OleB'. No relevant effect of $MgCl_2$, either in the presence or absence of ATP, on the intrinsic fluorescence of MBP, was observed (results not shown).

Acrylamide quenching and guanidine denaturation

The pattern of the acrylamide quenching of tryptophan intrinsic fluorescence of MBP did not show any significant alteration in the presence of 1 mM ATP or 1 mM ATP plus 2 mM $MgCl_2$ (Figure 5A). In contrast, 1 mM ATP induced a considerable decrease in MBP-OleB' quenching by acrylamide, and this effect was increased in the presence of 2 mM $MgCl_2$ (Figure 5B). Moreover, ATP induced considerable stabilization against the

Figure 5 Acrylamide quenching of the intrinsic fluorescence of MBP (A) and MBP-OleB' (B)

Tryptophan intrinsic fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 340 nm. F_0 and F represent intrinsic fluorescence in the absence and in the presence of acrylamide respectively: (●) protein alone; (○) protein plus 1 mM ATP; (×) protein plus 1 mM ATP plus 2 mM $MgCl_2$.

Figure 6 Denaturation profile of MBP-OleB' induced by guanidine chloride

The unfolding at 25 °C of MBP-OleB' by different concentrations of guanidine chloride was determined in the presence (○) or absence (●) of 5 mM ATP. Tryptophan fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 340 nm. S.D. did not exceed 5%.

denaturing effect of guanidine chloride on MBP-OleB' (Figure 6).

Effect of ATP on the intrinsic fluorescence of MBP-OleB' at basic pH

At basic pH (pH 10.1) we observed an increase in the intrinsic fluorescence of MBP-OleB' at very low concentrations of ATP (Figure 7A), which is in contrast to the decrease in fluorescence observed at neutral pH (Figure 3). However, at higher ATP concentrations (above 2 mM) the intrinsic fluorescence decreased (Figure 7A). For up to 1 mM ATP, the maximum effect of an increase in ATP concentration is observed near pH 10.1 (Figure 7B), and above 1 mM ATP the maximal effect shifts progressively to pH 11 (Figure 7C). Up to pH 12 no increase in turbidity was observed, as determined by following the absorbance at 400 nm,

Figure 7 Influence of pH on the intrinsic fluorescence of the MBP-OleB'

(A) Effect of ATP on intrinsic fluorescence at pH 10.1. S.D. did not exceed 5%. (B) and (C) Variation of the intrinsic fluorescence of MBP-OleB' as a function of pH at different concentrations of ATP: (B) 1 mM (●); 2 mM (○); 3 mM (×); and (C) 5 mM (●); 7 mM (○); 10 mM (×).

Table 1 Influence of different nucleotides on intrinsic fluorescence of MBP-OleB' fusion protein

*Nucleotide concentration at half quenching effect. †These values were obtained using 2 mM of each nucleotide.

Nucleotide	IC ₅₀ (mM)*	Fluorescence decrease (%)†
ATP	5 ± 0.5	6 ± 1
ADP	2.5 ± 0.3	22 ± 3
GTP	3 ± 0.3	32 ± 2
GDP	1 ± 0.1	84 ± 1

thus suggesting that no protein precipitation perturbed the measurements (results not shown).

Interaction with other nucleotides and OM

The change in intrinsic fluorescence of MBP-OleB' was also measured by titration with ADP, GDP and GTP. The titration curves obtained were compared with those from titration with ATP (Table 1), where both the IC₅₀ (nucleotide concentration corresponding to half quenching of intrinsic fluorescence) and the percentage of fluorescence decrease induced by the presence of 2 mM nucleotide are indicated. Guanine nucleotides showed more affinity for the fusion protein than adenine nucleotides. Similarly, dinucleotides showed greater affinity than trinucleotides.

Figure 8 Effect of OM (●), erythromycin (○) and inorganic phosphate (▲) on the intrinsic fluorescence of MBP-OleB'

S.D. did not exceed 3%.

We also studied the effect of OM on intrinsic fluorescence at a concentration range which did not exceed 2 mM, since higher concentrations interfered with the determinations due to the high absorbance of OM. Consequently, no IC_{50} value was determined but, as Figure 8 shows, 2 mM OM significantly decreased (20%) the intrinsic fluorescence of MBP-OleB'. This effect was approx. four times higher than the effect of the structurally closely related macrolide erythromycin, suggesting that MBP-OleB' has more affinity for OM than for erythromycin. Since OM was supplied as OM phosphate, we also tested (as a control) the effect of NaH_2PO_4 on the intrinsic fluorescence, and the results (Figure 8) indicate that this salt only produced a slight decrease in the fluorescence.

DISCUSSION

ABC transporters are characterized by containing four membrane-associated domains: two hydrophobic and two hydrophilic. The hydrophilic component is presumed to bind ATP and couple its hydrolysis to the transport process [2]. Several ABC transporters have been reported to be involved in drug resistance in antibiotic producers [7–13] but, in none of these ABC transporters, has experimental evidence been given to show that the presumptive ATP-binding protein binds this nucleotide. In this paper we present, for the first time, evidence showing that an ABC transporter involved in antibiotic resistance in a producer organism (the OleB transporter of *S. antibioticus*) binds ATP and also interacts with the substrate (OM), inducing structural changes in the transporter. Although it has not yet been demonstrated that a consequence of this interaction is ATP hydrolysis, the fusion protein (MBP-OleB') has ATPase activity [14].

As shown in the present study, MBP possesses a poor ability to modify the fluorescence of two ATP analogues (ϵ ATP and TNP-ATP), suggesting that MBP cannot specifically bind these compounds. On the other hand, the MBP-OleB' hybrid protein significantly increased fluorescence of the ATP analogues, indicating the fixation of these compounds on the OleB' protein with apparent K_d values of 10 μ M and 4 μ M for ϵ ATP and TNP-ATP respectively. The Stinson and Holbrook analysis [17], using

TNP-ATP and high protein concentration (6 μ M), suggested an ATP:binding site stoichiometry close to 1:1.

OleB' contains tryptophan residues which confer an intrinsic fluorescence on the protein, making it possible to test whether ATP could induce some structural effects on the protein. We have observed that ATP quenched MBP-OleB' fluorescence with an IC_{50} of 5 mM. This strongly suggests that ATP binds OleB' with lower affinity than ATP fluorescent analogues. Similar observations using TNP-ATP fluorescence are well documented [16,23,24]. Using the ability of acrylamide to quench tryptophan fluorescence we observed that ATP decreases access of acrylamide to the MBP-OleB' fluorophores, but not to the MBP fluorophores. We can therefore conclude that ATP induces folding of MBP-OleB'. This conclusion was confirmed by the observation that ATP strongly protects MBP-OleB' against guanidine chloride denaturation. Interestingly, we observed that the MBP-OleB' fusion protein was also sensitive to the presence of other mononucleotides, with guanine nucleotides showed a greater affinity than adenine nucleotides. This does not necessarily mean that the protein has GTPase activity. In fact guanine nucleotides could bind the protein on a non-catalytic site(s). However, this observation raises the interesting question as to whether or not guanine-nucleotide binding could be an important modulating factor of ATP binding and ATPase activity of OleB'. This question is presently under study in our laboratory. Moreover, the presence of $MgCl_2$ significantly increases the effect of ATP on the intrinsic fluorescence of MBP-OleB', while no significant effect was found on MBP alone (results not shown). A similar effect of $MgCl_2$ in the presence of ATP was observed on the intrinsic fluorescence of the sarcoplasmic reticulum ATPase [26].

Structural changes upon addition of ATP or MgATP are well documented for other ATP-binding proteins. From structural models of the ATP-binding component of different transport systems, it has been proposed that a helical loop domain connecting Walker motifs A and B might be involved in transducing the free energy of ATP hydrolysis to the transmembrane domain components [1,3]. Moreover, from crystallographic analyses of ATP-binding proteins, it has been proposed that the lysine/arginine residue of the Walker A motif interacts with ATP, while the aspartic acid/glutamic acid residue of Walker B interacts with Mg^{2+} either directly or via a water molecule [27]. In the case of the MBP-OleB' fusion protein, and to gain insight into the amino acid with which ATP mainly interacts, we studied the ability of ATP to modify intrinsic fluorescence as a function of pH. The optimum pH for this effect was between 10.1 and 11. The pK values of the epsilon amine of lysine is 10.5, suggesting that ATP could interact preferentially with this residue. However we observed that at alkaline pH (pH 10.1) the ATP effect is a two-step process with an increase in fluorescence at low ATP concentrations and a decrease at high ATP concentrations (above 2 mM ATP). This suggests that more than one amino acid that can be titrated at alkaline pH must be involved in the interaction with ATP. Other potential amino acids could be tyrosine or arginine with pK values of 9.5 and 12 respectively. Interestingly, a similar two-step process was also observed at neutral pH for an *E. coli* phosphofructokinase [28]. In the same way, an increase induced by ATP of the intrinsic fluorescence of sarcoplasmic reticulum ATPase was observed from 1 μ M to 100 μ M ATP [26]. Experiments now in progress in our laboratory are focused on the site-directed mutagenesis of specific Walker A residues (particularly lysine) and the assay of their ability to fix ϵ ATP.

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