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Reconstitution in liposome bilayers enhances nucleotide binding affinity and ATP-specificity of TrwB conjugative coupling protein

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ABSTRACT

Bacterial conjugative systems code for an essential membrane protein that couples the relaxosome to the DNA transport apparatus, called type IV coupling protein (T4CP). TrwB is the T4CP of the conjugative plasmid R388. In earlier work we found that this protein, purified in the presence of detergents, binds preferentially purine nucleotides trisphosphate. In contrast a soluble truncated mutant TrwBΔN70 binds uniformly all nucleotides tested. In this work, TrwB has been successfully reconstituted into liposomes. The non-membranous portion of the protein is almost exclusively oriented towards the outside of the vesicles. Functional analysis of TrwB proteoliposomes demonstrates that when the protein is inserted into the lipid bilayer the affinity for adenine and guanine nucleotides is enhanced as compared to that of the protein purified in detergent or to the soluble deletion mutant, TrwBΔN70. The protein specificity for adenine nucleotides is also increased. No ATPase activity has been found in TrwB reconstituted in proteoliposomes. This result suggests that the N-terminal transmembrane segment of this T4CP interferes with its ATPase activity and can be taken to imply that the TrwB transmembrane domain plays a regulatory role in its biological activity.

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1. Introduction

TrwB is an integral membrane protein that plays a crucial role in bacterial conjugation [1] and belongs to the coupling protein family of type IV protein secretion systems (T4CP) [2]. Several members of this family have been described as essential elements for conjugative DNA transfer (i.e., TraD of F-like plasmids, TraG of RP4, or TrwB of R388), or T-DNA transport to plant cells (VirD4 of the Ti plasmid of *Agrobacterium tumefaciens*) [3]. They might be present in all conjugative systems, presumably connecting the relaxosome (the protein complex that properly processes the DNA molecule to be transferred) and the transfer apparatus (a multiprotein complex that forms the type IV transport channel). Also, it has been proposed that coupling proteins could have an active part in pumping the single-stranded DNA during cell mating, using chemical energy from ATP hydrolysis to transport the DNA molecule through the mating channel [4]. Although other members of this family have been studied to some extent [5,6], up to now TrwB is the only T4CP purified to homogeneity [1] and characterized in detail [7–9]. Sequence analysis predicts an integral membrane protein of 507 residues [10] with a transmembrane domain (TMD) comprising the first 70 N-terminal residues [11]. It also contains characteristic NTP-binding motifs, reminiscent of those of the α and β subunits of F_1-ATPase [12] in its bulky cytosolic domain.

We previously reported the purification of native TrwB in monomeric and hexameric forms, in the presence of n-Dodecyl-β-D-maltoside (DDM) [1] as well as the purification of TrwBΔN70, a soluble mutant of TrwB lacking the TMD [10]. Comparative studies regarding protein stability and nucleotide binding specificity of both proteins revealed important differences among them [7,9]. While TrwB was biologically active and formed hexamers [1], TrwBΔN70 was conjugative defective and monomeric in solution, its hexameric form having only been reported under crystallization conditions [13]. TrwBΔN70 hexamer formation is enhanced by the presence of TrwA and/or DNA [8,14]. Moreover, wild-type TrwB purified in the presence of detergents is significantly more stable against temperature and denaturing agents than its soluble counterpart [7]. In addition, TrwB purified in the presence of detergents binds preferentially purine over pyrimidine nucleotides trisphosphate. Conversely, TrwBΔN70 binds uniformly both purine and pyrimidine nucleotides [9] although its nucleotide affinity is higher than that of TrwB purified in DDM. A DNA-dependent ATPase

Abbreviations: 5-IAF, 5-iodoacetamide fluorescein; AmdiS, 4-acetamido-4′-maleidyl-stibene-2,2′-disulphonic acid; NEM, N-ethylmaleimide; T4CP, type IV coupling protein; TMD, transmembrane domain; TNP-ATP, 2′(or 3′)-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate

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activity has been reported for TrwBΔN70 [8,14] while this activity remains elusive in TrwB purified in the presence of detergents. These important discrepancies among TrwB and its soluble mutant could be directly related to the amphipathic nature of the protein. There are evidences in our previous research pointing to a regulatory role of the TMD over the biological activity of TrwB [1,7,9]. Besides, elements from the membrane, such as lipids or other conjugative membrane proteins could be necessary for the true catalytic properties of TrwB to manifest. Finally, since detergents inhibit ATPase activity of TrwBΔN70 (our unpublished data), it could also be possible that the detergent present in TrwB preparations could alter the characteristics of TrwB, specially inhibiting ATPase activity.

Therefore for TrwB, as for many membrane proteins that express their activities only when correctly oriented and inserted in a lipid bilayer, reconstitution in phospholipid vesicles seemed to be the right choice for a meaningful study. Moreover, reconstitution of membrane proteins into liposomes provides a powerful tool in structural as well as functional areas of membrane protein research [15,16]. In this work we report the detergent-mediated reconstitution of TrwB into liposomes. We have studied some of the characteristics of the reconstituted protein in comparison with the protein purified in the presence of detergents, among them the nucleotide binding activity. We show that reconstituted TrwB binds ATP in a selective manner and with higher affinity than the soluble mutant (TrwBΔN70) or the whole protein solubilized in the presence of detergents suggesting a gating control mechanism for its TMD. However, the reconstituted protein remains unable to hydrolyze ATP.

2. Materials and methods

2.1. Materials

L-α-phosphatidylethanolamine (PE) from *Escherichia coli*, cardiolipin (CL) from bovine heart, and L-α-phosphatidylethanolamine-N-(lissamine rhodamine) (PE-Rh) were purchased from Avanti Polar Lipids. Phosphatidylglycerol (PG) was obtained from Lipid Products. Bio-Beads SM-2 (BB) were obtained from BioRad. n-octyl β-D-glucopyranoside (OG), Triton X-100, cholate, ATP, GTP, ADP, TTP, Ni–NTA–Atto 647 N conjugate and routine chemicals were purchased from Sigma. n-Dodecyl β-D-maltoside (DDM) and octaethylene glycol monododecyl ether ($C_{12}E_8$) were from Anatrace. TNP-ATP and the fluorescent reagents 5-iodoacetamide fluorescein (5-IAF), 4-acetamido-4′-maleidylstibene-2,2′-disulphonic acid (AmdiS) and N-ethylmaleimide (NEM) were purchased from Molecular Probes.

2.2. Overexpression and purification of TrwB

The purification protocol was similar to that previously reported [9], except for the fact that both TrwB monomers and hexamers were eluted from the phosphocellulose column simultaneously with buffer A [50 mM Tris–HCl (pH 7.8), 0.1 mM EDTA] supplemented with 0.5 mM DDM and 1 M NaCl. Next, TrwB eluted from the phosphocellulose column was loaded onto 5 ml Hi-Trap Chelating column (GE Healthcare) equilibrated with buffer A supplemented with 0.3 mM DDM, 200 mM NaCl, and 50 mM imidazole. Proteins were eluted from the column with 225 mM imidazole in buffer A supplemented with 0.3 mM DDM and 200 mM NaCl. The protein was concentrated using an ultrafiltration cell with a YM-50 ultrafiltration membrane of regenerated cellulose (Amicon). Imidazole was removed from the sample by PD-10 desalting column (GE Healthcare) in buffer A supplemented with 20% (v/v) glycerol, 200 mM NaCl and 0.2 mM DDM. Protein determinations were performed by the Bradford method [17]. The whole process was carried out at 4 °C. Protein was frozen in liquid nitrogen and stored at −80 °C.

2.3. Preparation of large unilamellar vesicles

Large unilamellar vesicles (LUVs) were prepared from a mixture of PE:PG:CL (molar ratio 76.3:19.6:4.1) that emulates the composition of the bacterial inner membrane [18]. LUVs for reconstitution were prepared by the extrusion method as described by Hope et al. [19]. The solvent was evaporated with a stream of nitrogen and the sample was completely dried under vacuum for 2 h. The dry lipid film was hydrated with buffer B [50 mM Tris–HCl (pH 7.8) and 200 mM NaCl] and stirred vigorously until the lipid film was completely resuspended. The large multilamellar vesicle suspension was disrupted by ten freeze–thaw cycles and then extruded ten times through a 100-nm pore filter using a Mini Extruder (Avanti Polar Lipids, Inc.) to prepare LUVs.

2.4. Reconstitution of TrwB into liposomes

First, protein-free liposomes were solubilised by adding increasing amounts of detergents to the vesicle suspensions (2.5 mM lipid). Different detergents (i.e., OG, DDM, Triton X-100, cholate and $C_{12}E_8$) at various concentrations were tested for their suitability for liposome solubilisation. Then, solubilisation induced by detergent addition was analyzed following turbidity changes at 520 nm [20] in a Cary 3 Bio (Varian) spectrophotometer.

For TrwB reconstitution, 100 μl liposome solution (31 mM) was solubilised with 48 μl OG (500 mM) to reach the "onset" solubilisation point [21]. After equilibration of the detergent–phospholipid mixture for 30 min at room temperature, purified TrwB was added at a lipid: protein molar ratio of 250:1 in a total volume of 0.5 ml and incubated for 30 min at room temperature. Then, the detergent was removed in three steps by adsorption on BB (pre-treated according to Holloway [22]). In the first step, 100 mg of pre-treated BB was added to the lipid–protein–detergent solution and incubated for 3 h at 4 °C. Then, the same amount of beads was added and incubated for 1 h at the same temperature. Finally, another 200 mg of beads was added and incubated overnight at 4 °C for the complete removal of the detergent. Quantification of the remaining DDM and OG was performed by the sugar detection method described by Urbani and Warne [23]. To separate proteoliposomes from liposomes and aggregated protein, a discontinuous sucrose gradient was used. The sample was loaded at the bottom of a discontinuous 2.5–60% (w/v) sucrose gradient (3 ml) in buffer B and centrifuged for 3 h, at 100,000×*g* at 4 °C. For the appropriate diffusion of the sample, the 60% sucrose layer was supplemented with 0.05% (w/v) Triton X-100. Liposomes and proteoliposomes banded separately, while unincorporated protein remained in the lower 60% sucrose layer. Subsequently, 0.5 ml aliquots were taken and protein and lipid content in each fraction was analyzed. Finally, the fractions containing both lipid and protein were diluted to 8 ml with buffer B and centrifuged for 2 h at 100,000×*g* at 4 °C. The resulting pellet containing the proteoliposomes was resuspended in buffer B and stored on ice.

2.5. SDS-PAGE and immunoblot analysis

For protein quantification after reconstitution each fraction was loaded onto a 12.5% SDS-PAGE gel stained with Coomassie Brilliant Blue and the amount of protein was quantified by densitometry using the Quantity One Software in a GS-800 Calibrated Densitometer (Bio-Rad). TrwB was used as the standard protein for quantification. For this purpose, known amounts of purified TrwB were loaded in different lanes and used to build the standard curve.

2.6. Determination of total phosphorous

Phospholipid quantification was performed following the method described by Bartlett [24] and modified by Bottcher et al. [25].

2.7. Conjugate binding to TrwB proteoliposomes analysed by flow cytometry

The binding of Ni–NTA–Atto conjugate to TrwB proteoliposomes was analyzed by flow cytometry. Liposomes [PE:PG:CL (molar ratio 76.3:19.6:4.1)] were labelled with rhodamine by adding 0.2 mol% PE-Rh. TrwB was then reconstituted into proteoliposomes following the above-described protocol. Subsequently, proteoliposomes were incubated in buffer B supplemented with both 0.5% (w/v) BSA and fluorescently labelled conjugate at 1:100 (v/v) for 1 h at 4 °C. The proteoliposomes were then washed by diluting them up to 8 ml in buffer B and centrifuging for 2 h (100,000×*g*, 4 °C). The pellet was resuspended in buffer B. Control liposomes were prepared following the same protocol. Liposomes and proteoliposomes were analyzed on a FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA, EEUU). Data acquisition was stopped when 10,000 events were counted for each sample. Data analysis was carried out with Cell Quest software (BD Biosciences) by plotting the fluorescence of the different samples in a histogram.

2.8. TrwB orientation in proteoliposomes

TrwB orientation was determined by using membrane-permeable and -impermeable thiol-reactive reagents as described by Sanowar and Le Moual [26]. Proteoliposomes (1.5 μM TrwB) were subjected to one of the following treatments at 22 °C with an incubation time of 10 min between reagent additions except for solubilisation with OG where incubation time was 30 min. Total protein labelling: proteoliposomes were permeabilized with 220 mM OG and subsequently labelled with 0.33 mM 5-IAF (a fluorescent membrane-impermeable thiol-reactive reagent). Outside-oriented protein labelling: proteoliposomes were incubated with 0.33 mM 5-IAF to label solely the external protein. Inside-oriented protein labelling: First, proteoliposomes were incubated with 0.33 mM AmdiS (a non-fluorescent membrane-impermeable thiol-reactive reagent) to block externally exposed cysteine residues. Subsequently, proteoliposomes were solubilised with 220 mM OG and then incubated with 0.33 mM 5-IAF to label solely the internally exposed cysteine residues. Unspecific labelling: proteoliposomes were incubated with 15 mM NEM (a non-fluorescent membrane-permeable reagent) and then incubated with 0.33 mM 5-IAF. After incubation with the first reagent, proteoliposomes were washed by diluting them up to 8 ml in buffer B and centrifuging for 2 h (100,000×*g*, 4 °C). The pellet was resuspended in buffer B. All reactions were stopped by addition of 6× Laemmli loading buffer [250 mM Tris-HCl (pH 7.0), 10% (w/w) SDS, 20 mM EDTA, 50% glycerol, 0.1% Bromophenol Blue and 9% (v/v) 2-mercaptoethanol] and analyzed by SDS-PAGE. Fluorescence of proteins labelled with 5-IAF was visualized using the vision compact imaging system (Scie-Plas, Southam, UK). After fluorescence measurements, the same gel was stained with Coomassie Brilliant Blue for protein quantification.

2.9. Measurement of nucleotide binding activity in proteoliposomes

The nucleotide binding properties of TrwB proteoliposomes were analysed using a fluorescent ATP analogue, namely TNP-ATP [9]. Experiments were performed at 25 °C using a Fluoromax-3 spectrofluorometer (Jobin Ivon-Horiba, Longjumeau, France) with spectral bandwidths of 4 and 8 nm for excitation and emission, respectively. TrwB proteoliposomes were suspended in buffer B. The apparent dissociation constants of proteoliposome–TNP-ATP complex and proteoliposome–nucleotide complex were calculated as described before by Hormaeche et al. [9]. All spectra were corrected for buffer fluorescence that contained all the components needed except for TrwB. When the extrinsic fluorescence of TNP-ATP was studied, excitation was performed at 410 nm, and emission was scanned in the 470–650 nm range. TNP-ATP binding was determined by calculating the increase of the curve area between 500 and 600 nm in the presence of TrwB with the following quadratic equation in the case of increasing TNP-ATP fluorescence:

$$F = F_{\min} + \left\{ (F_{\max} - F_{\min}) \left[\left(E_t + L + K_d^{TNP-ATP} \right) - \left(\left(E_t + L + K_d^{TNP-ATP} \right)^2 - 4E_t L \right)^{\frac{1}{2}} \right] \right\} \Big/ 2E_t \quad (1)$$

where F is the relative fluorescence intensity, $F_{\min}$ is the relative fluorescence intensity at the start of titration, $F_{\max}$ is the fluorescence intensity at saturating concentration of TNP-ATP (L), E_t is the total concentration of TrwB in proteoliposomes, and $K_d^{TNP\text{-}ATP}$ is the apparent dissociation constant of TrwB proteoliposomes–substrate complex.

In the case of displacement of bound TNP-ATP by ATP, the following quadratic equation was used:

$$F = F_{\max} - \left\{ (F_{\max} - F_{\min}) \left[(E_t + L + K_{0.5}) - \left((E_t + L + K_{0.5})^2 - 4E_t L \right)^{\frac{1}{2}} \right] \right\} \Big/ 2E_t \quad (2)$$

where $F_{\max}$ is the fluorescence intensity at start of titration, $F_{\min}$ is the fluorescence at saturating concentration of ATP, $K_{0.5}$ represents the amount of ATP necessary to displace half the amount of bound TNP-ATP, and L represents ATP concentrations. Therefore, the apparent dissociation constant of TrwB proteoliposomes–ATP complex (K_d^{ATP}) can be calculated by using $K_{0.5}$ value obtained from the displacement experiments and the following equation:

$$K_d^{ATP} = K_{0.5} \Big/ \left\{ 1 + \left[L / K_d^{TNP-ATP} \right] \right\} \quad (3)$$

where L represents TNP-ATP concentration at start of titration.

When other nucleotides were used, the nomenclature for the corresponding dissociation constants was $K_d^{nucleotide}$. In all cases, average values of at least three closely similar experiments are taken.

2.10. Analysis of ATPase activity of TrwB proteoliposomes

In TrwB proteoliposomes ATPase activity was measured using either a coupled-enzyme assay or a colorimetric assay as described previously [8].

3. Results

3.1. Reconstitution of TrwB into proteoliposomes

Most membrane protein reconstitution procedures involve the use of detergents to disintegrate the structure of liposomes before protein insertion [27]. Thus, the first step for TrwB reconstitution was to evaluate solubilisation of liposomes through changes in absorbance at 520 nm [20]. The rather unusual composition of these liposomes [PE:PG:CL (76.3:19.6:4.1 mol:mol)] intended to mimic the bacterial inner membrane lipid composition [18]. Since the nature of the detergent during protein insertion is one of the parameters critically affecting the process [27], liposome suspensions containing 2.5 mM phospholipid were solubilised by stepwise addition of detergents (OG, DDM, Triton X-100, cholate and $C_{12}E_8$). As observed in Fig. 1, except for DDM, all detergents tested were efficient for the solubilization of PE:PG:CL liposomes because they displayed characteristic solubilisation curves that could be interpreted using a “three-state” model for the solubilisation process [16,28] showing clearly the onset (detergent-saturated liposomes) and total solubilisation (small mixed micelles) points. In contrast, DDM did not cause full solubilisation even at the highest detergent concentration tested (50 mM). As we were interested in achieving a unidirectional insertion of TrwB into liposomes and since among the detergents tested only OG and DDM have been described to ensure a unidirectional insertion of proteins

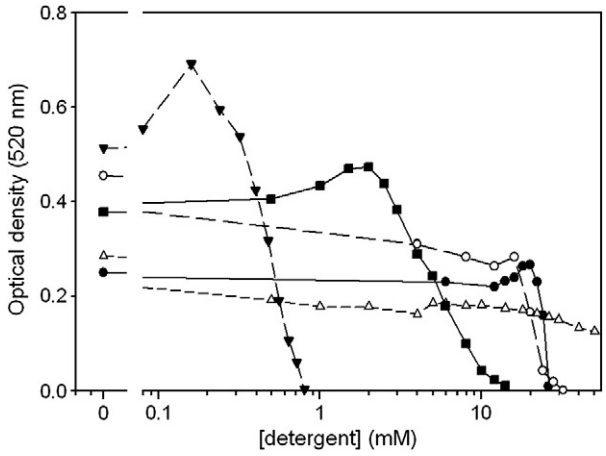
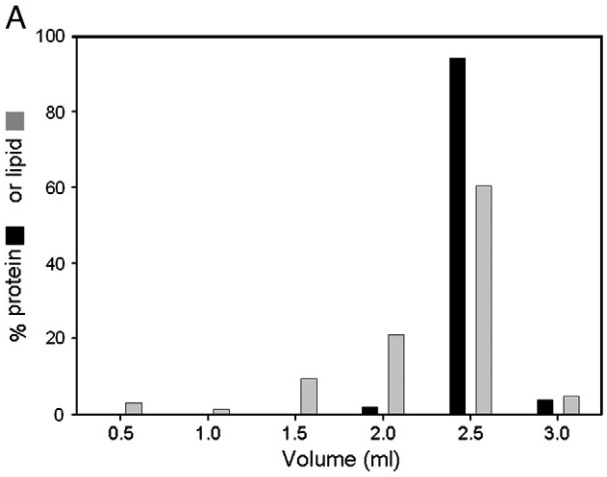


Fig. 1. Solubilization of liposomes by different detergents. Turbidity changes induced by stepwise addition of detergents on liposome suspensions containing 2.5 mM phospholipid: OG (●), DDM (Δ), Triton X-100 (○), cholate (▼) and $C_{12}E_8$ (■). The turbidity value was obtained measuring optical density at 520 nm for each detergent concentration.



B

Lipid:protein ratio	PE:PG:CL (76.3:19.6:4.1)
Initial mol:mol	250:1
Final mol:mol	99:1 ± 11
% protein recovery	40% ± 4.8

Fig. 2. (A) Analysis of protein and lipid content of the fractions obtained from the sucrose density gradients for proteoliposome separation. The percentages of protein (black bar) and lipid (grey bar) in the fractions after the sucrose gradient ultracentrifugation were quantified as described in the Materials and methods section. (B) Lipid:protein molar ratio and protein incorporation of TrwB into proteoliposomes.

into the membrane [15,29,30], OG was the option to accomplish TrwB detergent-mediated reconstitution.

The results indicated that OG concentration to reach the "onset" solubilisation point of liposomes at 2.5 mM phospholipid was 19 mM (Fig. 1). When solubilisation experiments were carried out at different lipid concentrations it was found that for proteoliposomes containing 6.25 mM phospholipid solubilization was completed at 48 mM OG. Therefore, in all the TrwB reconstitution experiments the detergent: lipid molar ratio used was 8.

After liposome solubilisation and protein insertion into the phospholipid bilayer, efficient removal of detergent from the reconstituted proteoliposomes is absolutely necessary because even traces of detergent can affect the activity of the reconstituted protein [16,31–33]. In particular for TrwB the majority of detergents tested inhibited the ATPase activity of its soluble mutant TrwBΔN70 (data not shown). A powerful method for detergent removal is detergent adsorption onto hydrophobic BB [34]. Different studies have demonstrated that amount of beads added, temperature and incubation time influence detergent removal [31,35,36]. In particular, optimal temperature of detergent removal is a controversial question because, although detergent adsorption efficiency is higher at higher temperatures (i.e., 25 °C) [35], in general membrane proteins, and TrwB in particular, require low temperatures when manipulated. Consequently, for TrwB reconstitution, different incubation temperatures (i.e., 25 °C and 4 °C), BB amounts (i.e., 11.4, 22.8, 45.7 and 57.1 mg BB/mg detergent) and incubation times (i.e., 4.5 h and 16 h) were used and the percentage of detergent removal was measured (Table 1). After analysing all the above mentioned parameters, the best conditions for detergent removal were addition of 57.1 mg of BB/mg of detergent for 16 h at 4 °C. In fact, under these conditions almost all of the detergent (99.8%) was removed (Table 1). In this regard, we have evidence that at those detergent concentration values TrwBΔN70 ATPase activity is not inhibited (data not shown). Note that in the proteoliposome formation protocol, once the proteoliposomes are formed, sucrose density gradient and subsequent washing steps lead to much lower detergent concentrations than those obtained just after BB-mediated removal.

For TrwB reconstitution, we prepared LUVs with a PE:PG:CL (76.3:19.6:4.1) composition, with the aim of reconstituting TrwB

Table 1
Parameters describing BB-mediated detergent removal in TrwB reconstitution.

Sample	BB amount[a] (mg)	Incubation time[b] (h)	Incubation temperature (°C)	% Detergent removal	Residual detergent (mM)
Detergent Sol[1]	80	4.5 (3/1/0.5)	4	<95 %	<2
Detergent Sol[1]	160	4.5 (3/1/0.5)	4	98.4	0.74
Detergent Sol[1]	320	4.5 (3/1/0.5)	4	99.8	0.12
Detergent Sol[1]	320	4.5 (3/1/0.5)	25	99.8	0.09
Detergent Sol[1]	400	4.5 (3/1/0.5)	4	99.8	0.1
Detergent Sol[1]	400	O/N (3/1/O/N)	4	99.9	0.07
Liposomes[2]	320	4.5 (3/1/0.5)	4	99.6	0.17
Liposomes[2]	400	4.5 (3/1/0.5)	4	99.7	0.16
Liposomes[2]	400	O/N (3/1/O/N)	4	99.9	0.04
Proteoliposomes[3]	400	O/N (3/1/O/N)	4	99.8	0.11

[a] BB-mediated detergent removal was achieved in three different additions as follows: the amount of BB added in the first and second additions corresponded to one quarter of the total amount of BB, in the third addition one half of the total amount of BB was added.

[b] Incubation times were different for the three additions: first incubation time was 3 h, second incubation time was 1 h and the third incubation time corresponded to the rest of the time indicated in the table.

[1] Detergent solution. BB-mediated detergent removal was done over detergent in solution. Initial detergent concentration was 48 mM = 7 mg of detergent.

[2] Lipid–detergent solution. BB-mediated detergent removal was done over a liposome preparation. Initial detergent concentration was 48 mM = 7 mg of detergent.

[3] Protein–lipid–detergent solution. BB-mediated detergent removal was done over a proteoliposome preparation. Initial detergent concentration was 48 mM = 7 mg of detergent (plus a maximum of 0.14 mM of DDM from the protein preparation).

under near-native conditions. Purified TrwB in 0.2 mM DDM was added to liposomes partially solubilised with OG and detergents were removed by adsorption to BB as described previously. Next, the proteoliposomes were separated from liposomes and unincorporated TrwB, using sucrose density gradient centrifugation. After centrifugation liposomes and proteoliposomes banded separately, while unincorporated protein remained in the lower 60% sucrose layer (bottom part of the tube). In order to optimize the separation method, different gradient volumes (i.e., 7.5 and 3 ml) were used. It was found that volumes of 3 ml rendered better results because the proteoliposomes remained in a

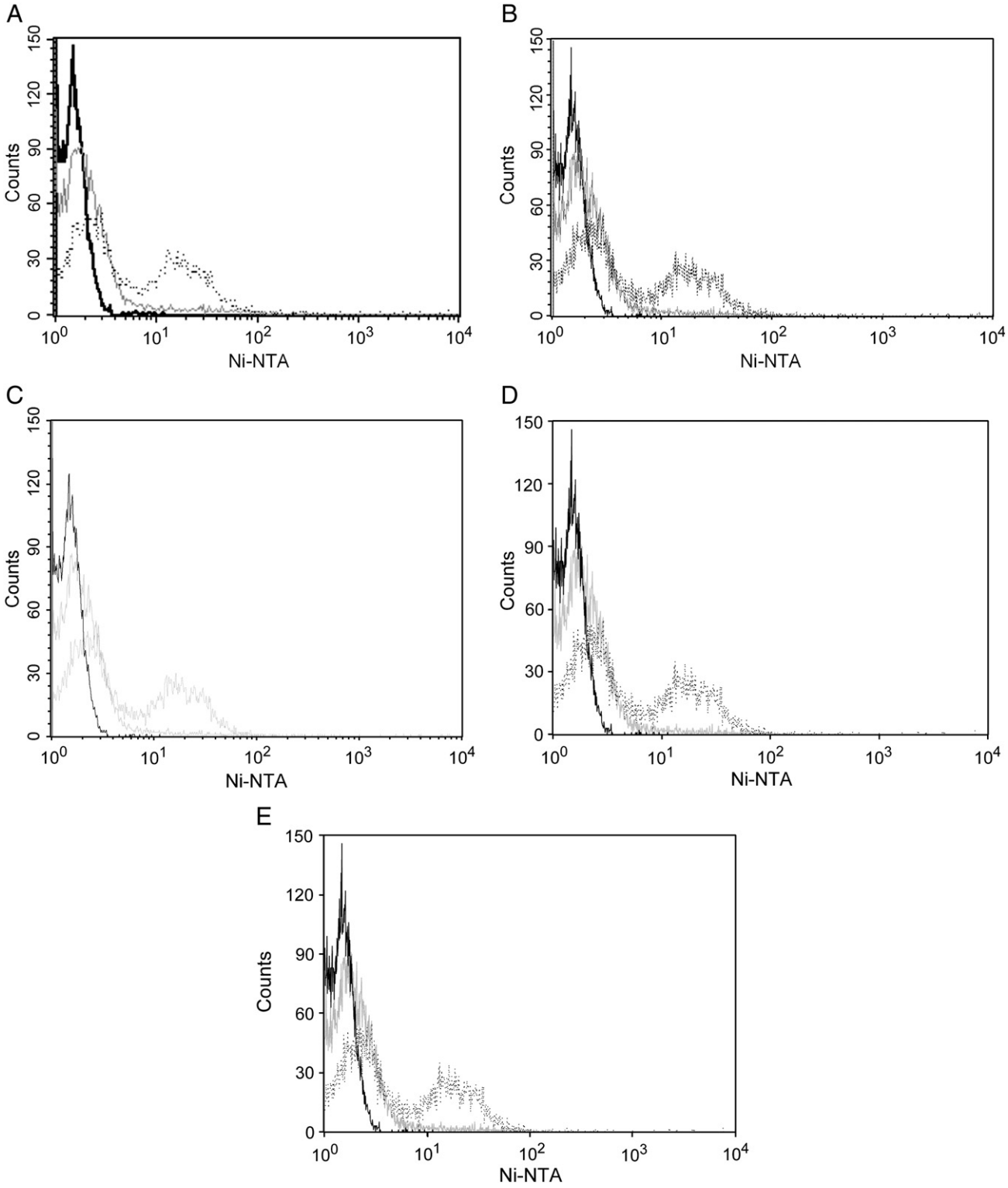


Fig. 3. Detection of TrwB proteoliposomes by flow cytometry. Liposomes or proteoliposomes were incubated with Ni–NTA conjugate in a 1:100 (v/v) ratio for 1 h at 4 °C, centrifuged at 100,000×*g* for 1 h at 4 °C, suspended in buffer B and analysed by flow cytometry. Representative profiles showing the reactivity of Ni–NTA conjugate with liposomes or proteoliposomes were represented by histograms of cell counts *vs* FL4 fluorescence. Liposomes (black line), liposomes incubated with Ni–NTA conjugate (grey line) and TrwB proteoliposomes incubated with Ni–NTA conjugate (dotted line).

smaller volume and this facilitated separation. Therefore, from this point onwards, 3 ml sucrose gradients were used in all the experiments. A completely different behaviour was observed between the control sample (i.e., protein-free liposomes that had undergone the complete reconstitution process) and TrwB proteoliposomes. Although in both cases a turbid band corresponding to lipid was observed, its position moved to higher densities (bottom half of the gradient) in the proteoliposomes sample, indicating that TrwB was reconstituted into the lipid bilayer, a typical behaviour of reconstituted proteins when analyzed by sucrose discontinuous gradients [34,37,38]. To further confirm that TrwB protein was reconstituted into vesicles, the fractionated bands were recovered in 500 µl aliquots and the percent lipid and protein were calculated for each fraction. As seen in Fig. 2A, proteins and lipids essentially co-migrated down in the fraction corresponding to 2.5 ml (30% sucrose layer) from the top of the gradient and negligible amounts of TrwB were detected in the higher density fraction where aggregated proteins would accumulate. There was a strict correspondence between maximal protein and phosphate amounts (i.e., 90 and 60% of protein and phosphate, respectively) in the same fraction (Fig. 2A). This result strongly suggests that TrwB was successfully reconstituted into PE:PG:CL liposomes. The efficiency of TrwB reconstitution was assessed by measuring the protein and lipid content in the different fractions of the gradient. The lipid:protein molar ratio of the fraction containing proteoliposomes (Fig. 2B) was 99:1 and this fraction yielded 40% protein recovery.

3.2. Flow cytometry analysis of Ni–NTA–Atto conjugate binding to TrwB proteoliposomes

Flow cytometry is a technique for counting, examining, and sorting microscopic particles suspended in a stream of fluid. This technique is especially interesting when used with fluorescence tagged conjugates to detect the corresponding antigen on the cell surface and then analyse its characteristics. In order to obtain additional evidence for the reconstitution of TrwB into liposomes, we analysed proteolipomes using flow cytometry with TrwB specific detection. Besides, this method would give us qualitative information about protein orientation since only inside-out oriented protein would cross-react with the fluorescent conjugate. Because TrwB was purified as a His-tagged fusion protein, it was possible to use Ni–NTA–Atto conjugate which is a synthetic molecule that provides

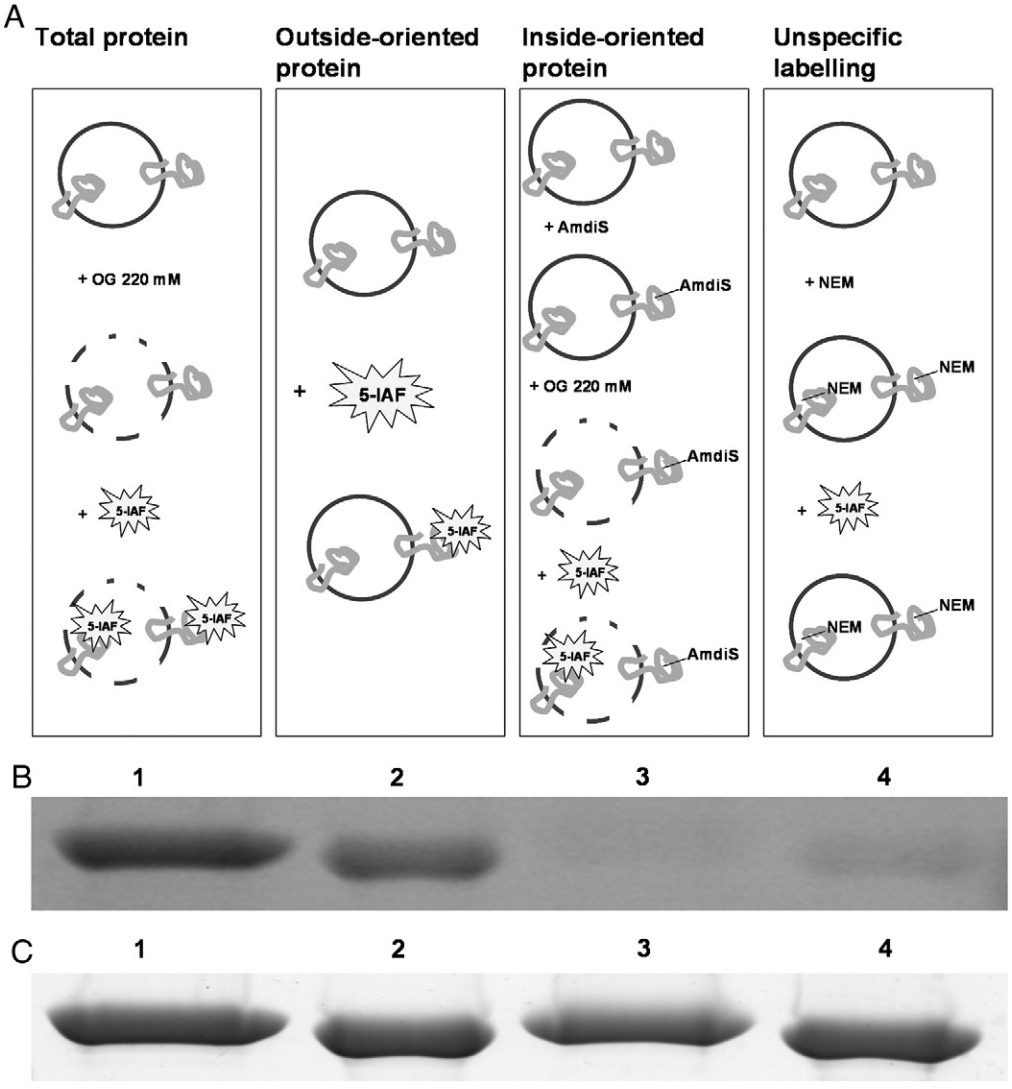


Fig. 4. Orientation of TrwB into liposomes. (A) Schematic representation of the orientation determination procedure. Total protein labelling: solubilization of proteoliposomes with OG and incubation with 5-IAF. Outside-oriented protein labelling: proteoliposomes were incubated directly with the impermeable probe 5-IAF. Inside-oriented protein labelling: outside-oriented TrwB was blocked with the membrane-impermeable reagent AmdiS. This was followed by solubilisation with OG and then incubation with 5-IAF to label the residual inside-oriented TrwB. Unspecific labelling: proteoliposomes were incubated successively with NEM and then with 5-IAF. The reactions were stopped by treating the samples with SDS, for SDS-PAGE electrophoresis. (B) Fluorescence of 5-IAF-labelled TrwB. (C) Total (Coomassie-stained) TrwB in proteoliposomes.

specific detection for His-tagged fusion proteins. The Atto dye delivers a fluorescent signal that is used for protein detection. Additionally, liposomes were marked with PE-rhodamine because their size ranges within the detection limit of the technique and otherwise they could be taken as noise. After incubating the proteoliposomes with Ni–NTA–Atto conjugate as described in the Materials and Methods section, the binding of the fluorescent conjugate to TrwB proteoliposomes was examined. Histograms in Fig. 3 show that Ni–NTA–Atto conjugate efficiently bound the TrwB proteoliposomes (dotted line) but not liposomes (grey line). Thus TrwB was in fact reconstituted into liposomes and at least partly in an inside-out orientation.

3.3. TrwB asymmetric orientation in proteoliposomes

TrwB contains a unique cysteine at position 337 in its cytoplasmic domain. Therefore, the method of detection based on differential binding of membrane-permeable and -impermeable thiol-reactive reagents described by Sanowar and Le Moual [26] appeared suitable for determination of TrwB orientation in proteoliposomes (Fig. 4A). 100% labelling was obtained by solubilising proteoliposomes with 220 mM OG and labelling the cysteine residue with 5-IAF (Fig. 4B, lane 1). To label only the outside-oriented protein, proteoliposomes were incubated directly with the impermeable probe 5-IAF (Fig. 4B, lane 2). According to these results, more than 90% of the protein reconstituted into liposomes was outside-oriented. To confirm this result, the percentage of TrwB molecules facing the intraluminal cavity was determined by blocking the outside-oriented TrwB with the membrane-impermeable reagent AmdiS, solubilising the proteoliposomes with OG and then incubating them with 5-IAF to label the residual inside-oriented TrwB (Fig. 4B, lane 3). Very little, if any, fluorescence was detected, suggesting that virtually no TrwB was inside-oriented in proteoliposomes. Finally, to measure non-specific labelling, proteoliposomes were incubated with 15 mM NEM and then incubated with 0.33 mM 5-IAF (Fig. 4B, lane 4). Very little non-specific fluorescence was found in the analysis. Consequently, it can be concluded that TrwB insertion was asymmetric with the cytosolic domain facing out. To ensure that the amount of protein was equivalent in all the experiments, the same gel was Coomasie Blue stained and showed similar amounts of protein in all lanes (Fig. 4C).

3.4. Nucleotide binding activity of TrwB proteoliposomes

As previously reported [1,10], TrwB nucleotide binding activity can be measured by following the increase of fluorescence emission intensity of the fluorescent ATP analogue TNP-ATP when binding to the protein. Classically, this fluorescent analogue has been used to characterize ATP binding proteins [39]. Previous studies carried out in our laboratory [1,9] described a different behaviour of the nucleotide binding activity between TrwB purified in the presence of DDM and its soluble mutant TrwBΔN70. But neither TrwB purified in the presence of detergent nor TrwBΔN70 represents the protein natural environment, i.e., the lipid bilayer. Therefore, nucleotide binding activity was assayed in TrwB proteoliposomes through the emission intensity of the fluorescent ATP analogue TNP-ATP. Subsequently, bound TNP-ATP could be dislodged from its binding site(s) by excess ATP. As depicted in Fig. 5A, reconstituted TrwB maintained its ability to bind nucleotides. The fluorescence emission spectrum of TNP-ATP of proteoliposomes (Fig. 5A, spectrum 1) was similar to the one recorded for TrwB purified in DDM [1,9] or TrwBΔN70 [10]. Pure liposomes devoid of TrwB protein did not exhibit significant TNP-ATP binding (data not shown). Moreover, when TrwB proteoliposomes were studied, bound TNP-ATP could also be displaced from its binding site(s) by excess ATP (Fig. 5A, spectrum 2). In all cases, TNP-ATP fluorescence emission increased in the presence of TrwB proteoliposomes and 10 mM ATP dislodged the fluorescent analogue from its binding site(s). The displacement obtained by TrwB proteoliposomes in the presence of 10 mM ATP was 74%.

3.5. Substrate specificity of TrwB proteoliposomes

When TrwB proteoliposomes were titrated with increasing concentrations of TNP-ATP saturation was observed (Fig. 5B). The dissociation constant obtained after fitting experimental data (average values of three closely similar experiments) to Eq. (1) was $K_d^{TNP\text{-}ATP} = 0.18$ μM for the proteoliposome–TNP-ATP complex. This value is over ten-fold lower than the corresponding one for TrwB purified in DDM (3.35 μM) or for TrwBΔN70 (3.27 μM) described by Hormaeche and co-workers [9], indicating a higher affinity for TNP-ATP of membrane-reconstituted TrwB.

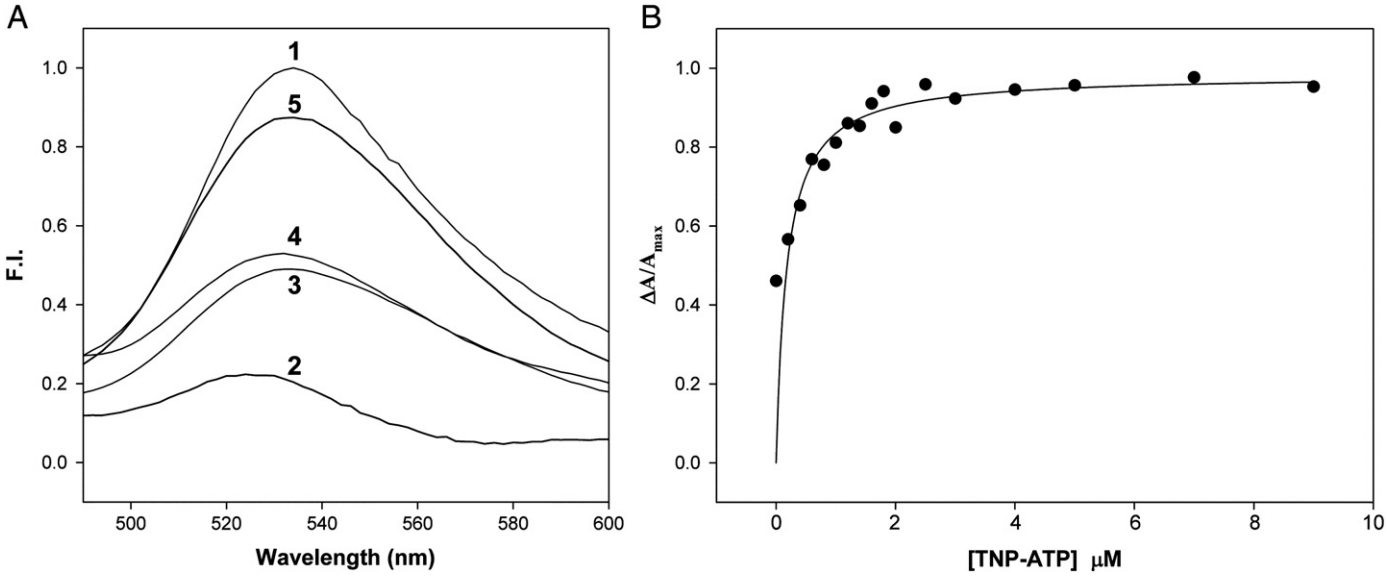


Fig. 5. Nucleotide binding of TrwB proteoliposomes. (A) Fluorescence spectra of TrwB-bound TNP-ATP in the absence and presence of 10 mM of natural nucleotides. Details of the experiment are described under Materials and methods section. Spectrum 1, TrwB proteoliposomes (1 μM TrwB) in the presence of TNP-ATP (10 μM); spectrum 2, conditions of spectrum 1 plus ATP; spectrum 3, conditions of spectrum 1 plus GTP; spectrum 4, conditions of spectrum 1 plus ADP; spectrum 5, conditions of spectrum 1 plus TTP. (B) Fluorescence-monitored titration of TNP-ATP binding to TrwB proteoliposomes. Successive aliquots of TNP-ATP stock solutions were added to a 0.4 ml sample of TrwB proteoliposomes (1 μM TrwB). The line represents the best fit to the data generated using Eq. (1).

Subsequently, TNP-ATP was used in chase experiments to measure the affinity constants of different unmodified nucleotides. First, 10 μM TNP-ATP was used to saturate the protein binding site(s) and then 10 mM of unmodified nucleotides was added to test the TNP-ATP displacement. As seen in Fig. 5A, when ADP or GTP was tested 48% of the TNP-ATP was displaced. In contrast, TTP could only displace 15% of the fluorescent analogue.

In view of these results, we proceeded to calculate the apparent dissociation constant of the unmodified nucleotides. In this set of experiments 10 μM TNP-ATP was used to saturate the protein binding site(s) and back-titration with the appropriate unlabelled nucleotide was performed. When proteoliposomes saturated with TNP-ATP were back-titrated with ATP, fluorescence decreased gradually (Fig. 6A, filled triangles). The experimental points fitted well to a curve generated from Eq. (2), which allows an estimation of $K_{0.5}$. From this value, Eq. (3) allows computation of the apparent dissociation constant of the proteoliposome–ATP complex (K_d^{ATP}) [40]. From these results a $K_d^{ATP} = 4.8 \times 10^{-5}$ M for TrwB proteoliposomes was obtained (Table 2). The corresponding values for TrwB in detergent were 2.6×10^{-3} M (monomers) (Fig. 6B) and 1.0×10^{-3} M (hexamers) and for TrwBΔN70 K_d^{ATP} was 2.2×10^{-4} M (Fig. 6C) [9]. Similar experiments of TNP-ATP displacement were performed with other natural nucleotides. Table 2 summarizes the values corresponding to the apparent dissociation constants ($K_d^{nucleotide}$) obtained from the displacement titration curves (Fig. 6A). It can be observed that TrwB proteoliposomes have the ability to discriminate ATP from GTP or TTP. When GTP was analyzed data rendered $K_d^{nucleotide}$ values of 8×10^{-5} M, higher than the one corresponding to ATP, indicating that TrwB proteoliposomes displayed lower affinity for GTP than for ATP. Finally, data corresponding to TTP indicate that this nucleotide was not able to efficiently release bound TNP-ATP from TrwB proteoliposomes. It should be noted that when TrwB was assayed in the presence of detergent there was a clear discrimination between purine and pyrimidinine nucleotides triphosphate [9], but not a clear specificity for ATP.

Moreover, regarding K_d^{ATP} values in the reconstituted system, there were two orders of magnitude lower than TrwB purified in DDM (2.63 mM) and one order of magnitude lower than the ones observed for TrwBΔN70 (0.22 mM) [9], indicating that the affinity of TrwB for nucleotides was much higher when the protein was inserted into the lipid bilayer. These results demonstrate that the lipidic environment has a dual effect on TrwB nucleotide binding activity, increasing its overall affinity for NTPs and providing specificity for ATP.

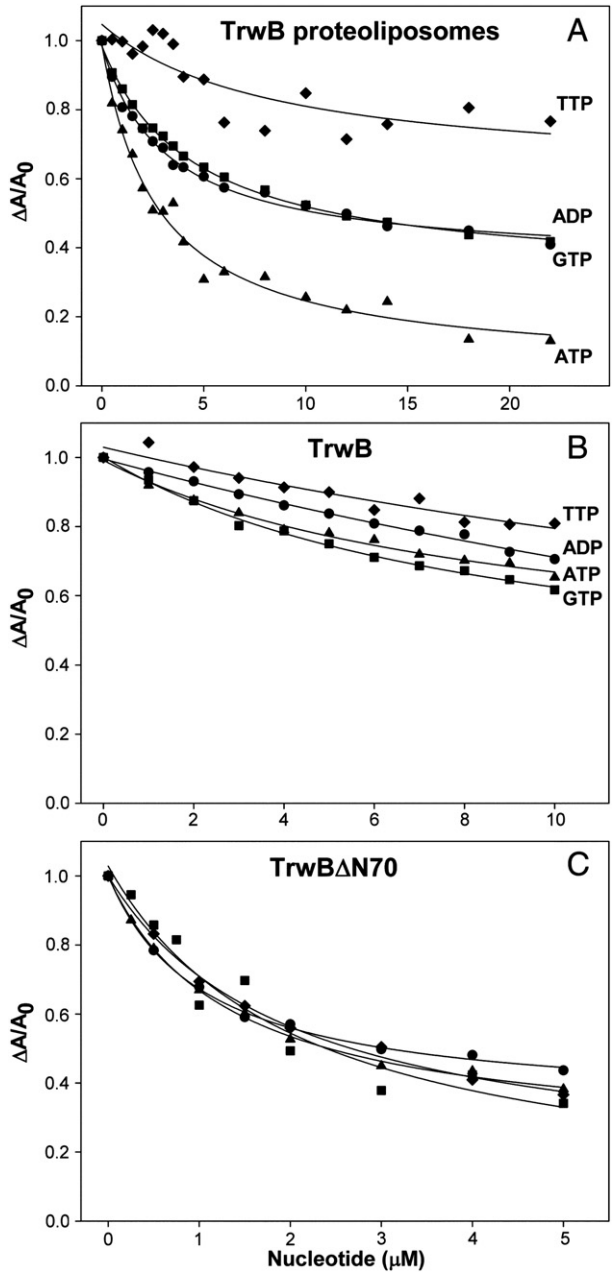


Fig. 6. (A) Displacement of bound TNP-ATP by unmodified nucleotides in TrwB proteoliposomes. Successive aliquots of nucleotide stock solutions were added to TrwB proteoliposomes (1 μM TrwB) saturated with TNP-ATP (10 μM) in buffer B. The solid line represents the best fit to the data generated using Eq. (2). (B) Displacement of bound TNP-ATP by unmodified nucleotides in TrwB in detergent. (C) Displacement of bound TNP-ATP by unmodified nucleotides in TrwBΔN70. Unmodified nucleotides used as competitors were ATP (▲), GTP (■), ADP (●) and TTP (♦). Data for panels B and C have been obtained from Hormaeche et al. [9].

3.6. ATPase activity of TrwB proteoliposomes

The ATPase activity of TrwB proteoliposomes was assayed as previously described by two methods: a coupled-enzyme assay and a colorimetric assay [8], as described in the Materials and methods section. Since TrwBΔN70 has a DNA-dependent ATPase activity enhanced by the protein TrwA [8,14], TrwB proteoliposomes (3 or 6 μM protein) were incubated with or without 6 nM ssDNA and 2 μM TrwA. Since temperature, pH, ionic strength or ATP concentration could modify the protein ATPase activity, different experimental conditions were established to study the putative ATPase activity of TrwB proteoliposomes. Among them, different temperatures between 4 °C and 37 °C, pH values between 6.2 and 7.8, ionic strength corresponding to 36 or 200 mM NaCl, and different ATP concentrations (i.e., 5, 20 and 26 mM) were investigated. Nevertheless, TrwB proteoliposomes did not show any ATPase activity under any of the conditions assayed.

4. Discussion

TrwB, as all members of the T4CP family, is a membrane protein that plays a crucial role in the conjugative process of plasmid R388. It has been extensively studied in our laboratories [1,7,9] but, due to its amphipathic nature, biochemical and biophysical studies are often difficult to carry out.

Table 2
$K_d^{nucleotide}$ values for natural nucleotide binding to TrwB proteoliposomes. Results were compared using a non-parametric Kruskal–Wallis test (Statistical significance $P<0.05$). Differences between nucleotides were statistically significant, $P=0.041$.

Nucleotide	$K_{0.5}$ (mM)	$K_d^{nucleotide}$ (mM)
ATP	2.73 ± 0.36	0.048
GTP	4.5 ± 0.31	0.080
ADP	3.18 ± 0.28	0.056
TTP	8.54 ± 6.89	0.15

Consequently, a soluble deletion mutant lacking the TMD (TrwBΔN70) was constructed, hoping that it would retain the characteristics of the whole protein [10]. Nevertheless, when TrwB and TrwBΔN70 were comparatively studied, significant differences were found [7,9]. Among them, although a high DNA-dependent ATPase activity was reported for the soluble mutant [8,14] up to date no ATPase activity has been reported for TrwB. Moreover, when nucleotide binding activities were compared, TrwB in the presence of detergent showed a lower affinity for all the nucleotides tested but a higher selectivity for purine nucleotides trisphosphate than the soluble mutant TrwBΔN70 [9].

Protein reconstitution provides a model membrane system that can be used to carry on detailed biochemical and biophysical studies to obtain functional and structural data for membrane proteins. Therefore, in order to elucidate the effect of the bilayer and also the role of the TMD in the in vivo activity of the protein, in the present work TrwB was reconstituted into liposomes and its nucleotide binding and ATPase activities were analyzed. Optimal conditions for TrwB reconstitution into liposomes were evaluated following the detergent-mediated method [27]. After analyzing the effect of different detergents, OG was chosen to achieve TrwB reconstitution. The choice of detergent is a crucial step in protein reconstitution as reported by various authors [41–44].

The correct insertion of the protein into the bilayer depends on the rate of detergent removal [16]. In this work a BB-based method was used [34]. Classically, this procedure is performed at room temperature but since this can be deleterious for membrane proteins, the method was modified so that at 4 °C and after overnight incubation times almost the total amount of detergent (99.8%) was eliminated. These same conditions have been found useful in other studies [44,45].

After density gradient centrifugation of the proteoliposome preparation the protein incorporation yield was 40%. In similar studies on the reconstitution of other membrane proteins incorporation yields ranging from 62% to less than 10% had been described [44,46,47]. Nevertheless, on some occasions, yields of recovery can be around 90% [26].

The orientation of the incorporated protein was determined through flow cytometry analysis. The results of these experiments, along with the analysis based on membrane-permeable and -impermeable thiol reagents, indicated that TrwB was uniformly reconstituted with the NBD oriented towards the external side of the lipid bilayer vesicle. We considered that these proteoliposome preparations were appropriate for functional studies of TrwB. The unidirectional reconstitution of different transport proteins has been previously reported in different protein reconstitution studies [43,44,48,49].

Nucleotide binding activity of TrwB proteoliposomes was analyzed by TNP-ATP binding and displacement by natural nucleotides [9]. TrwB proteoliposomes showed a displacement of the fluorescent nucleotide analogue by ATP as shown by TrwB in the presence of detergents [9]. In this regard, when comparing the nucleotide binding activity of both TrwBΔN70 and TrwB purified in DDM with TrwB reconstituted into proteoliposomes, the latter showed a marked preference for ATP over the other natural nucleotides analyzed (i.e., GTP, ADP, TTP) while TrwB purified in the presence of detergent showed equal preference for ATP and GTP [9]. Moreover, when TrwBΔN70 was studied no preference for any particular nucleotide was observed [9]. Concerning the affinity for natural nucleotides, TrwB reconstituted into proteoliposomes showed a two orders of magnitude higher affinity than TrwB in the presence of detergent and one order of magnitude higher than that observed for TrwBΔN70 [9].

In sum, studies of nucleotide binding to TrwB purified in DDM and reconstituted into proteoliposomes show that the process of reconstitution favours the binding of ATP over other nucleotides and increases its affinity considerably, not only when compared with the protein in the presence of detergent, but also when compared to the affinity shown by the soluble mutant TrwBΔN70. These results lead us to the conclusion that the lipid environment is important in the modulating role of the TMD, suggesting that the membrane provides the environment for the putative DNA pumping activity of TrwB where a selective high affinity for ATP would be needed if the energy for such a biological activity would come from ATP hydrolysis. Nevertheless, up to now no ATPase activity has been found in TrwB proteoliposomes.

The fact that even under these conditions ATPase activity remains elusive suggests that the TMD could control the activity of TrwB in vivo, presumably because other proteins from the T4SS would be necessary to interact with the TMD, indicating the start of the conjugative process and, therefore, the need for DNA pumping system activation. In this regard, the study of possible interactions with proteins belonging to the T4SS is necessary to obtain information about how to "switch on" the activity of TrwB in the cell. TrwE is a plausible candidate because an interaction with TrwB has been already described [50] and also because this protein has been described as an energy sensor [51]. TrwE could be a signaling molecule between the outside of the cell and TrwB for this one to switch on its activity of ATP-dependent DNA pumping. In this regard, protein co-reconstitution would be a good method to elucidate the components necessary for the modulation of TrwB activity. Devesa and co-workers [45] developed a method, in theory applicable to all membrane proteins that allows simultaneous incorporation of proteins and thus could be used to study the role of TrwE on the ATPase activity of TrwB.

It is not easy to compare results from studies carried out on different membrane proteins, since little is known about the effects of differing preparations of proteoliposomes on the structure and function of membrane proteins [52]. Nevertheless, in most cases, the lipid environment is necessary to provide the appropriate structural arrangements required for protein activity. In this regard, changes in the activity of different reconstituted proteins have been observed in the literature. For instance, MacB, an ABC-type transporter of the type 1 secretion systems, displayed an inhibition of its ATPase activity upon incorporation into the bilayer [53]. In turn, after losing its activity during the purification process, the PhoQ histidine kinase sensor recovered its initial activity when reconstituted [26].

To summarize, our study investigates the reconstitution and the characterization of the nucleotide binding activity from inside-out reconstituted TrwB. To the best of our knowledge, this is the first reconstitution of a T4CP. This work provides data which can be used as the first step to elucidate the activity of TrwB in the cell, in particular the role of the TMD in the protein function. This is an example of reconstitution as the best approach for studying membrane proteins. In fact, through this system we demonstrate that TrwB when inserted into the membrane shows some of the putative properties necessary to hydrolize ATP to be used as the driving force for DNA pumping (i.e., high affinity and specificity for ATP binding).

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