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Membrane insertion stabilizes the structure of TrwB, the R388 conjugative plasmid coupling protein

Ana J. Vecino [a], Igor de la Arada [a,1], Rosa L. Segura [a], Félix M. Goñi [a], Fernando de la Cruz [b], José L.R. Arrondo [a], Itziar Alkorta [a,*]

[a] Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain
[b] Departamento de Biología Molecular, Universidad de Cantabria, C/Herrera Oria s/n, 39011 Santander, Spain

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ABSTRACT

TrwB is an integral membrane protein that plays a crucial role in the conjugative process of plasmid R388. We have recently shown [Vecino et al., Biochim. Biophys. Acta 1798(11), 2160–2169 (2010)] that TrwB can be reconstituted into liposomes, and that bilayer incorporation increases its affinity for nucleotides and its specificity for ATP. In the present contribution we examine the structural effects of membrane insertion on TrwB, by comparing the protein in reconstituted form and in the form of protein/lipid/detergent mixed micelles. TrwB was reconstituted in PE:PG:CL (76.3:19.6:4.1 mol ratio) with a final 99:1 lipid:protein mol ratio. This lipid mixture is intended to mimic the bacterial inner membrane composition, and allows a more efficient reconstitution than other lipid mixtures tested. The studies have been carried out mainly using infrared spectroscopy, because this technique provides simultaneously information on both the lipid and protein membrane components. Membrane reconstitution of TrwB is accompanied by a decrease in β-sheet contents and an increase in β-strand structures, probably related to protein–protein contacts in the bilayer. The predominant α-helical component remains unchanged. The bilayer-embedded protein becomes thermally more stable, and also more resistant to trypsin digestion. The properties of the bilayer lipids are also modified in the presence of TrwB, the phospholipid acyl chains are slightly ordered, and the phosphate groups at the interface become more accessible to water. In addition, we observe that the protein thermal denaturation affects the lipid thermal transition profile.

1. Introduction

Bacterial conjugation is a plasmid-mediated process aimed at transferring DNA in a unidirectional way between bacteria from a donor to a recipient cell. The conjugative plasmid R388 has the shortest known mobilization region [1]. Only three plasmid-encoded proteins, TrwA, TrwB, and TrwC, together with *ori*T, are involved in R388 mobilization [2]. In particular, TrwB is the type IV coupling protein (T4CP) encoded by R388 and it appears to connect the relaxosome (properly processed DNA molecule to be transferred) with the transport apparatus (a multi-protein structure). TrwB is an integral membrane protein consisting of 507 residues, that contains characteristic nucleotide binding domains, reminiscent of those of F_1-ATPase α and β subunits [3]. The protein consists of a large (ca. 440 residues) extramembranous domain, and a transmembrane domain comprising the 70 N-terminal residues that includes two transmembrane helices and a small periplasmic domain in between [2]. Different studies carried out in our laboratory [4–6] demonstrate that the transmembrane domain plays a major role in protein stabilization [5] and also increases TrwB specificity for nucleotide binding, making the nucleotide binding site(s) less accessible but more selective to purine nucleotide triphosphates [6].

In a previous work [7], our group has successfully reconstituted TrwB into liposomes, with the protein asymmetrically oriented towards the outside of the vesicles. The functional analysis of TrwB proteoliposomes demonstrates that when the protein is inserted into the lipid bilayer TrwB is selective for ATP and the affinity is enhanced as compared to the protein purified in detergent [7]. One important effect of protein insertion into lipid bilayers is assumed to be the thermodynamic stabilization of its structure. However there are few, if any, data addressing experimentally the stabilization of bacterial conjugation proteins by reconstitution in membranes. In the present contribution, we have compared the thermal stability of TrwB in lipid/protein/detergent mixed micelles [5] and reconstituted in lipid bilayers [7]. In our previous work reconstitution was performed in PE:PG:CL bilayers, we have now extended the reconstitution work using either a more complex (*E. coli* total lipid extract) or a more simple (egg PC) bilayer

Abbreviations: T4CP, type IV coupling protein; TMD, transmembrane domain; IR, infrared spectroscopy; TNP-ATP, 2′(or 3′)-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; OG, n-octyl β-D-glucopyranoside; DDM, n-Dodecyl-β-D-maltoside

* Corresponding author. Tel.: + 34 94 601 2568; fax: +34 94 601 35 00.
E-mail address: itzi.alkorta@ehu.es (I. Alkorta).
[1] Present address: Institut für Biophysik, Johann Wolfgang Goethe-Universität, Max-von-Laue-Strasse 1, 60438, Frankfurt am Main, Germany.

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composition. Thermal perturbation has been monitored by IR spectroscopy, a technique that provides quantitative assessment of protein secondary structure and its modifications [8–10]. Protein accessibility to proteases has also been tested on detergent-solubilized and reconstituted TrwB. Finally, an ATP-hydrolase activity that has been detected in a soluble, truncated form of TrwB [11], has been explored, with negative results, in the reconstituted protein.

The data show that reconstituted TrwB is less accessible to proteases, and more stable against thermal challenge than the detergent-solubilized form. These observations are in agreement with the data according to which the reconstituted protein shows higher affinity and specificity for the substrate nucleotide ATP [7], and the lack of ATP hydrolase activity is suggesting the need for TrwB to interact with a different R388 product in order to develop its full activity.

2. Materials and methods

2.1. Materials

L-α-phosphatidylethanolamine (PE) from *E. coli*, cardiolipin (CL) from bovine heart, *E. coli* total lipid extract and egg PC were purchased from Avanti Polar Lipids. Phosphatidylglycerol (PG) was obtained from Lipid Products. Bio-Beads SM-2 (BB) were obtained from Bio-Rad. n-Octyl β-D-glucopyranoside (OG) and routine chemicals were purchased from Sigma. n-Dodecyl-β-D-maltoside (DDM) was from Anatrace.

2.2. Overexpression and purification of TrwB

The purification protocol was similar to that previously reported [6], 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 supplemented with 50 mM imidazole and 0.1 mM PMSF and 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, at a flow rate of 2.5 ml/min. 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 a PD-10 desalting column (GE Healthcare) in buffer A supplemented with 20% (v/v) glycerol, 200 mM NaCl and 0.2 mM DDM. Protein concentration determinations were performed by the Bradford method. 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 either from a mixture of PE:PG:CL (molar ratio 76.3:19.6:4.1) that emulates the composition of the bacterial inner membrane [12], or from *E. coli* total lipid extract, or from egg PC. LUVs were prepared by the extrusion method [7,13]. 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), 200 mM NaCl] and stirred vigorously until the lipid film was completely resuspended. The large multilamellar vesicle suspension was disrupted by 10 freeze–thaw cycles and then extruded 10 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

For TrwB reconstitution, 100 μl liposome solution (31 mM) was solubilized with 48 μl OG (500 mM) to reach the "onset" solubilization point [14]. 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 [15]) at 4 °C. In the first step, 100 mg of pre-treated BB was added to the lipid/protein/detergent solution and incubated for 3 h. Then, the same amount of beads was added and incubated for 1 h. Finally, another 200 mg of beads were added and incubated overnight 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 [16]. 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. 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

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 Böttcher et al. [17].

2.7. Measurement of nucleotide binding activity in proteoliposomes

The nucleotide binding properties of TrwB proteoliposomes were analyzed using a fluorescent ATP analogue, namely TNP-ATP [6]. 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. 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. TrwB proteoliposomes were suspended in buffer B. TNP-ATP (10 μM) was added to a TrwB proteoliposomes (1 μM) solution and spectra were recorded. Subsequently, successive aliquots of ATP were added and the corresponding spectra were recorded. All spectra were corrected for buffer fluorescence that contained all the components needed except for TrwB.

2.8. 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 [11].

2.9. Proteolysis by trypsin and proteinase K

Tryptic digestions of TrwB either in the presence of detergent or in proteoliposomes were performed in buffer B with or without 0.2 mM DDM, respectively. Both digestions were carried out at 25 °C.

Proteolysis was initiated by addition of trypsin to proteoliposomes (5 μM TrwB) at different ratios: 1:10, 1:250, 1:500 and 1:1000 (w/w). Samples were taken at different time intervals, the reaction was stopped by addition of 0.4% (v/v) acetic acid and the mixture was heated for 5 min at 100 °C. When required TrwB proteoliposomes (5 μM TrwB) were treated with 220 mM OG in buffer B for 10 min at 25 °C prior to trypsin addition.

Proteinase K digestion of TrwB proteoliposomes was performed in 50 mM Tris–HCl (pH 7.8), 1 mM $CaCl_2$ buffer and digestion of TrwB purified in the presence of detergent was performed in the same buffer supplemented with 0.2 mM DDM. Digestion was carried out at 25 °C. Proteolysis was initiated by addition of proteinase K to proteoliposomes (5 μM TrwB) at ratios of 1:750 or 1:500 (w/w). Samples were taken at different time intervals, the reaction was stopped by addition of 6 mM PMSF and the mixture was heated for 5 min at 100 °C.

In both cases, degradation products were analyzed in 12.5% SDS-PAGE gels stained with Coomassie Brilliant Blue. Percent degradation was measured by densitometry using the Quantity One Software in a GS-800 Calibrated Densitometer (Bio-Rad).

2.10. Infrared spectroscopy

TrwB samples were measured in buffer B supplemented with 0.2 mM DDM. The H-D exchange was carried out by dialysis at 4 °C against the same buffer in D_2O.

After the sucrose gradient, the fractions with TrwB proteoliposomes were diluted up to 8 ml in 50 mM Tris–DCl (pH 7.8), 200 mM NaCl buffer in D_2O. Then samples were centrifuged for 2 h at 100,000 ×*g* at 4 °C and resuspended in the same buffer for measurements.

Infrared spectra were recorded in a Thermos Nicolet Nexus 5700 (Thermo Fisher Scientific, Waltham, MA) spectrometer equipped with a MCT detector using a Peltier-based temperature controller (Temp-CompTM, BioTools Inc., Florida) and 25 μm optical path excavated windows. Typically 370 scans for each, background and sample, were collected at 2 cm^{-1} resolution and averaged every minute. Temperature was increased at a rate of 1 °C min^{-1}. Data treatment and band decomposition of the original amide I have been described elsewhere [9,18].

3. Results

3.1. Effect of lipid composition on membrane protein incorporation

To examine the influence of lipid composition on TrwB reconstitution and its effects on TrwB activity, either PE:PG:CL (76.3:19.6:4.1) [7], *E. coli* total lipid extract or egg PC liposomes were used for TrwB reconstitution. The former is a defined mixture that mimics the bacterial inner membrane lipid composition [12]. The *E. coli* total lipid extract might include lipids that are necessary for the *in vivo* activity of TrwB. Egg PC was the third option because it is the most common composition in the reconstitution of membrane proteins [19].

Optimal conditions for TrwB detergent-mediated reconstitution into liposomes [20] were described in a previous work [7]. In order to analyze the effect of lipid composition on TrwB reconstitution, LUVs of the above three compositions were prepared. Purified TrwB in 0.2 mM DDM was added to liposomes partially solubilized with 48 mM OG and detergents were removed by adsorption to BB [19] as described [7]. Next, the proteoliposomes were separated from liposomes and unincorporated TrwB using sucrose density gradient centrifugation. Then the different sucrose gradient fractions were analyzed for protein and lipid content.

Sucrose gradients were fractionated in 500 μl fractions and analyzed for protein and lipid content. As reflected in Fig. 1, migration of proteoliposomes through the gradient varied with lipid composition. PE:PG:CL allowed co-migration of most protein and lipid in the 2.5 ml fraction (Fig. 1A). When *E. coli* total lipid extract was used, most of the protein was recovered in the 2.5 ml fraction (30% sucrose layer) (Fig. 1B). Although in this fraction phospholipids and protein coexisted (80% of protein and 20% of lipid), most phospholipids (60%) were found in the 1.5 ml fraction (Fig. 1B). A different behaviour was observed for PC proteoliposomes. In this case the protein banded in the bottom fraction (3 ml) (Fig. 1C), which usually corresponds to aggregated protein, and the maximal amount of lipid (≈50%) was found in the 0.5 ml fraction corresponding to pure lipid. It was concluded that PC did no allow TrwB reconstitution under our conditions, and no further experiments were carried out with the TrwB/PC mixture.

Although the initial lipid:protein ratio was the same in all cases (i.e., 250:1) it changed significantly for the different liposome compositions. Lipid and protein assays indicated that proteoliposomes made out of *E. coli* lipids showed the highest proportion of lipid per protein molecule (140:1), while PE:PG:CL liposomes rendered proteoliposomes with a proportion of 99 molecules of lipid per protein molecule. Protein incorporation yield was 40% and 28% for PE:PG:CL and *E. coli* total lipid extract, respectively.

To investigate whether lipid composition had any effect on the nucleotide-binding activity of TrwB proteoliposomes, the binding capacity of TrwB proteoliposomes of different compositions was analyzed. As shown in Fig. 1D and E, qualitatively similar spectra were obtained when PE:PG:CL and proteoliposomes made out of *E. coli* lipids were examined. In both 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). However PE:PG:CL [7] proteoliposomes showed higher displacements than liposomes made out of *E. coli* lipids.

It has been extensively reported that the activity of membrane proteins can be regulated by the lipid membrane composition [21,22]. Therefore, since TrwBΔN70 has a DNA-dependent ATPase activity enhanced by the protein TrwA [11,23], and although PE:PG:CL TrwB proteoliposomes did not show ATPase activity even when many different conditions were tested [7], proteoliposomes made out of *E. coli* lipids (3 μM protein) were incubated with or without 6 nM ssDNA and 2 μM TrwA to evaluate the existence of ATPase activity in these proteoliposome preparations. Nevertheless, none of samples analyzed showed ATPase activity under any of the conditions assayed. In view of the above results, from this point on experiments were conducted only with TrwB either solubilized as lipid/protein/detergent mixed micelles or reconstituted in PE:PG:CL.

3.2. Secondary structure of solubilized and reconstituted TrwB

Information on the secondary structure of TrwB either in the detergent-solubilized form or inserted into liposomes has been obtained by IR spectroscopy. The infrared amide I band, located between 1700 and 1600 cm^{-1} arises mainly from the C = O stretching vibrations of the peptidic bond. This band is conformationally sensitive and can be used to monitor the protein secondary structure composition and changes thereof induced by external agents [9,24]. Fig. 2 shows the original spectra and their curve-fitting decomposition corresponding to TrwB purified in the presence of detergent (Fig. 2A) and TrwB reconstituted into PE:PG:CL liposomes (Fig. 2B). The original amide I′ envelope (amide I′ instead of amide I to mark that spectra are taken in D_2O buffer) was curve-fitted using the band positions obtained from the deconvolved spectra (see inset), and the results are summarized in Table 1. When TrwB in detergent was analyzed, four bands corresponding to protein structure were observed. The band at 1613 cm^{-1} was not taken into account for protein conformation analysis because it was attributed to tyrosine side chains [25]. Band assignment is not always a straightforward process because band position can be altered by the environment. For instance α-helical components are located around 1650 cm^{-1} [9], but lower wavenumbers have been described for hydrated helices [26].

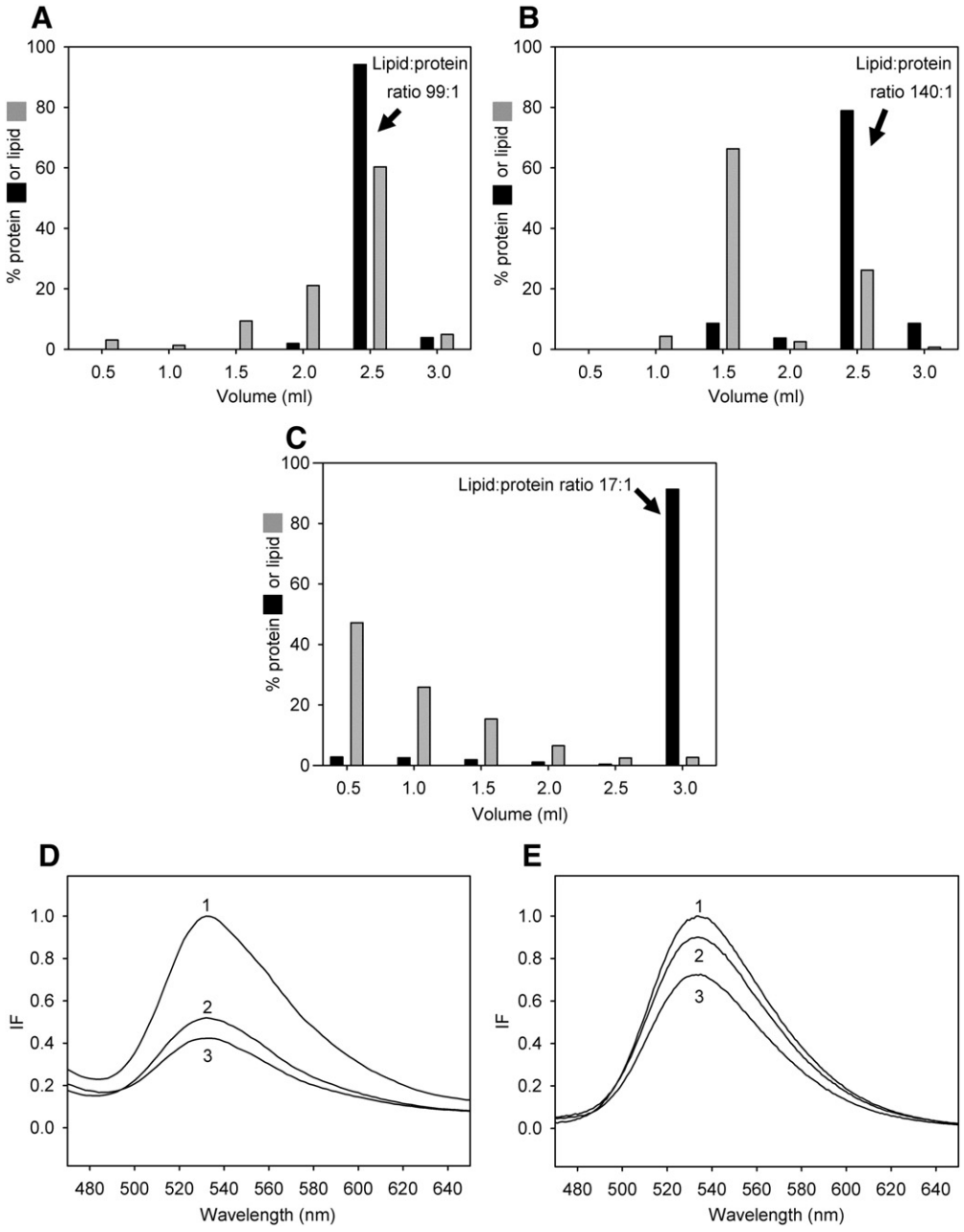

Fig. 1. Effect of lipid composition on TrwB reconstitution. (A–C) Protein and lipid content of the fractions obtained from the sucrose density gradients for proteoliposome separation. The percentages of protein (black bar) and lipid (grey bar) in the fractions after the sucrose gradient ultracentrifugation were quantified as described in the Materials and methods section. (A) PE:PG:CL. (B) *E. coli* total lipid extract. (C) Egg PC. The final lipid:protein molar ratio in the major protein fraction is indicated with an arrow. Nucleotide binding to TrwB proteoliposomes prepared from PE:PG:CL (D) or from *E. coli* total lipid extract (E). Fluorescence spectra of TrwB proteoliposome-bound TNP-ATP in the presence and absence of ATP. All spectra were corrected for buffer fluorescence. Spectrum 1, TrwB proteoliposomes (1 μM TrwB) plus TNP-ATP (10 μM). Spectrum 2, TrwB proteoliposomes (1 μM TrwB) plus TNP-ATP (10 μM) plus ATP (5 mM). Spectrum 3, TrwB proteoliposomes (1 μM TrwB) plus TNP-ATP (10 μM) plus ATP (10 mM).

In fact, in TrwB the component at 1647 cm^{-1} is assigned to hydrated α-helix, whereas the bands at 1631 and 1676 cm^{-1} correspond to the low and high-frequency of β-sheet, the latter also containing a component of β-turns. The band at 1661 cm^{-1} is usually assigned to β-turns [8–10].

When TrwB embedded in lipid bilayers was studied a remarkable change in the shape of the amide I′ envelope was observed, consistent with the appearance of a new band at 1619 cm^{-1}. No significant changes, either in position or area, are seen in the bands corresponding to α-helix or β-turns. However, a decrease from 28% to 17% is observed in the band corresponding to β-sheet at 1632 cm^{-1}. This decrease is concomitant with the increase in the band at 1619 cm^{-1}. The latter band cannot be associated with aminoacid side chains for two reasons: the wavenumber shift is too large to be accounted for by changes in the environment polarity, and the increase in area is not concomitant with an increase in the ratio between the areas of the band at 1515 cm^{-1}, an isolated component due to the C = C vibrational mode band of tyrosine aromatic ring, and the amide I′. The 1619 cm^{-1} band is attributed to β-strands (see Discussion).

3.3. TrwB stability against thermal challenge

In order to obtain a better insight of the changes produced in TrwB when inserted into lipid bilayers, temperature-induced effects on the corresponding IR spectra were recorded. Spectra were retrieved in the 20–80 °C temperature range and the spectral shape as a function of temperature was analyzed. In this way the denaturing process of a

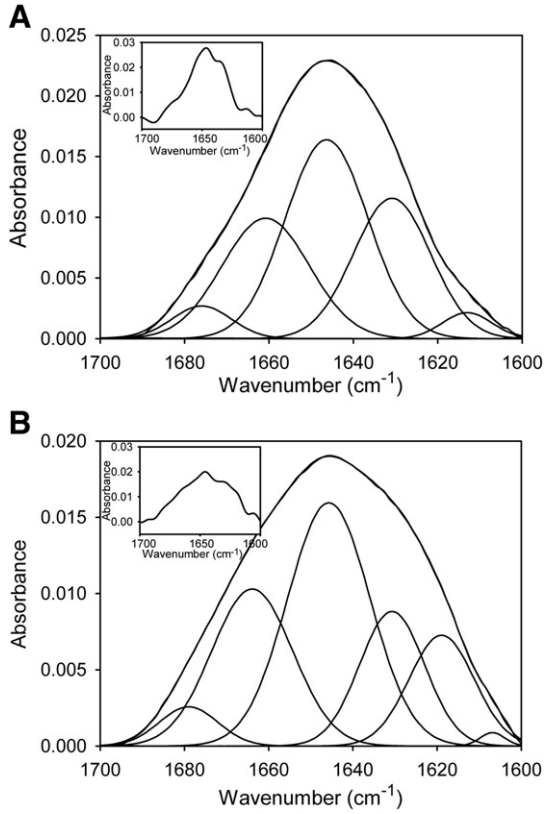

Fig. 2. Amide I′ region of the infrared spectra of TrwB purified in DDM (A) and of PE:PG:CL TrwB proteoliposomes (B). TrwB purified in DDM was dialysed against buffer B in D_2O medium supplemented with DDM 0.2 mM, and the proteoliposomes were ultracentrifuged at 100,000 ×*g* at 4 °C for 2 h and suspended in buffer B in D_2O, and analyzed by IR spectroscopy. The spectra were curve-fitted to show the different secondary structure components as detailed in Table 1. The inset shows the deconvolved spectra of the amide I′ band.

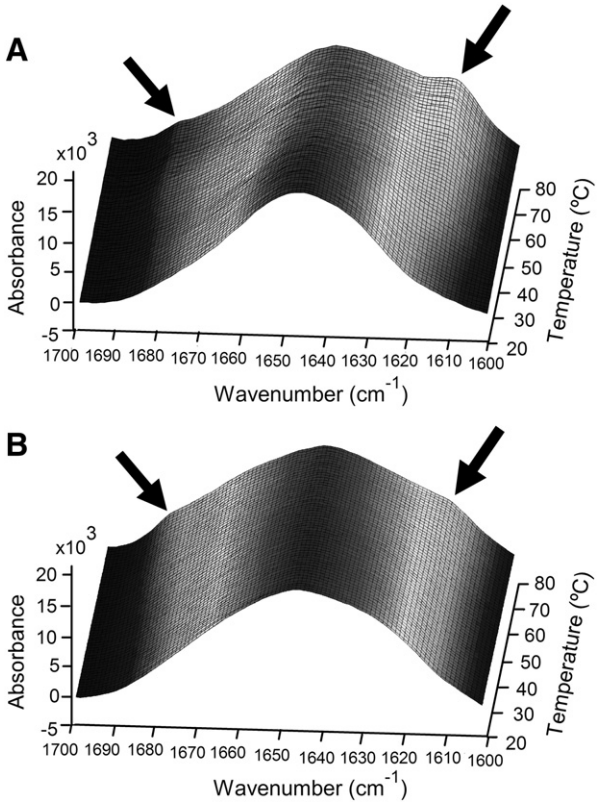

Fig. 3. Deconvolved IR spectra of TrwB purified in DDM (A) and TrwB proteoliposomes of PE:PG:CL (B) at increasing temperatures, showing thermal denaturation of the proteins. The samples were analyzed by IR spectroscopy at different temperatures between 20 and 80 °C. The bands appearing at high temperatures at 1617 and 1685 cm^{-1} (marked with black arrows) are typical of protein aggregation.

sample can be followed and the transition temperature (T_m) estimated. The 3D deconvolved spectra of the protein in detergent or inserted into liposomes of PE:PG:CL are depicted in Fig. 3. In both cases (i.e., TrwB in detergent and TrwB proteoliposomes) the shape of amide I′ changes with temperature and denaturation is marked by the bands corresponding to inter-molecular β-sheet (i.e., bands at 1617 and 1685 cm^{-1}) that indicate protein aggregation (black arrows in Fig. 3). When TrwB was reconstituted in liposomes (Fig. 3B) denaturation occurred at higher temperatures and the bands at 1617 and 1685 cm^{-1} were less prominent than in the presence of detergent (Fig. 3A). This fact would suggest that TrwB in proteoliposomes has a more compact structure, higher temperatures being necessary for protein denaturation than for TrwB in surfactant.

Table 1
Secondary structure components of TrwB in the presence of detergent and of TrwB reconstituted into liposomes as derived from the amide I′ band of infrared spectra obtained in D_2O medium. Both samples were suspended in the same buffer B with or without 0.2 mM DDM, respectively. The spectra are shown in Fig. 1. For details on assignments, see the text.

TrwB in detergent			TrwB proteoliposomes (PE:PG:CL)		
Position (cm^{-1})	Area (%)*	Assignation	Position (cm^{-1})	Area (%)*	Assignation
1676	5	β-Sheet	1679	5	β-Sheet
1661	27	β-Turns	1664	25	β-Turns
1647	41	α-Helix	1645	39	α-Helix
1631	27	β-Sheet	1631	17	β-Sheet
			1619	14	β-Strand

* The figures had been rounded off to the nearest integer.

The process of thermal denaturation can also be followed (in a non-quantitative way) by plotting the half-width of amide I′ vs. temperature. As seen in Fig. 4, when TrwB was reconstituted into liposomes denaturation occurred at temperatures above 65 °C. The midpoint transition temperature of TrwB proteoliposomes is about 17 °C higher than the T_m of TrwB in detergent (48 °C) (Fig. 4). This increase in denaturation temperature appears to be the result of lipid–protein and protein–protein interactions in the bilayer.

Moreover, infrared absorption bands from the different chemical groups of phospholipids can yield information about the structure of the lipid bilayer and protein–lipid interactions [10]. The position around 2850 cm^{-1} due to the antisymmetric C–H stretching vibration

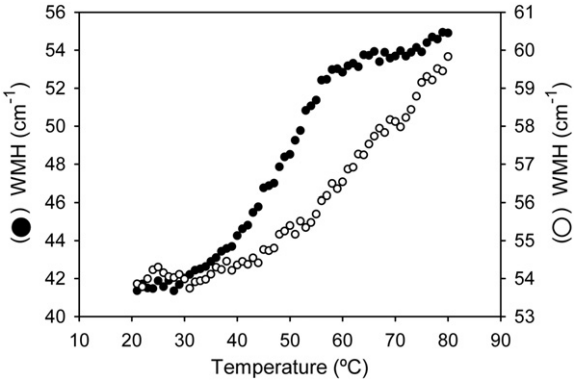

Fig. 4. Thermal denaturation of TrwB reconstituted into liposomes as seen by IR spectroscopy. The widths at half-height (WHH) of the amide I′ bands are plotted as a function of temperature for TrwB proteoliposomes of PE:PG:CL (○) and TrwB purified in DDM (●). Thermal denaturation is marked by a marked increase in bandwidth.

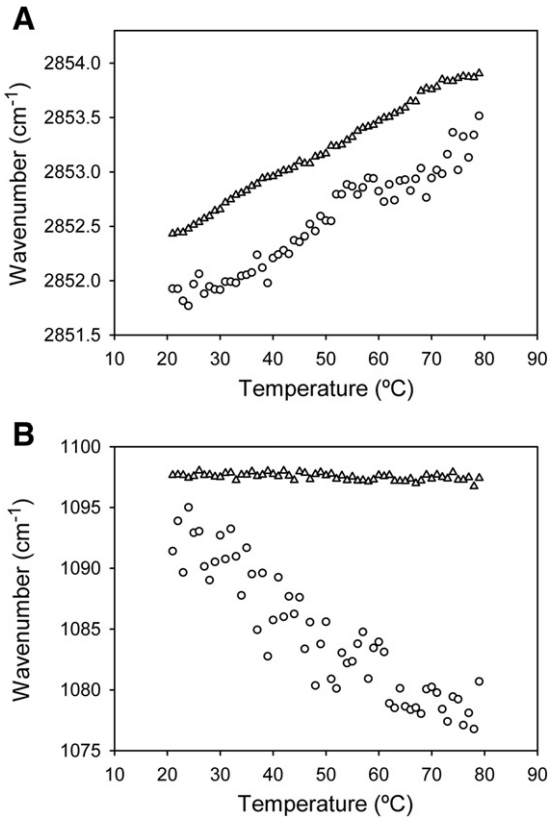

Fig. 5. Protein effects on bilayer lipids, as seen by IR spectroscopy. (A) Band at around 2850 cm^{-1} corresponding to the symmetric stretching of the $-CH_2-$ group in the acyl chains: (○) proteoliposomes of PE:PG:CL, and (Δ) liposomes of PE:PG:CL processed in the absence of protein. (B) Band at 1090 cm^{-1} corresponding to the phospholipid phosphate group: (○) proteoliposomes of PE:PG:CL, and (Δ) liposomes of PE:PG:CL processed in the absence of protein.

gives an idea of the physical state of the bilayer hydrophobic matrix whereas the PO_2^- symmetric band in the 1100–1000 cm^{-1} region monitors the water–membrane interface. Fig. 5A shows the band position due to the antisymmetric methylene stretching as a function of temperature for a liposome preparation of PE:PG:CL in the absence of protein and for PE:PG:CL proteoliposomes. For pure lipids, increasing temperatures cause a slight and rather continuous shift to high wavenumbers. When TrwB is inserted in the bilayer, the thermal pattern for C–H stretching vibrations is modified at the temperature of the protein thermal denaturation in the 50–70 °C range (Fig. 5A), indicating interaction of TrwB with the lipids. Moreover, the band corresponding to proteoliposomes appears at lower wavenumbers at all temperatures when compared to the band in the absence of protein, indicating a higher lipid chain order in the presence of protein.

The IR band corresponding to phosphate group vibrations (Fig. 5B) is temperature-insensitive under our conditions for pure lipid bilayers. In contrast, when the protein is inserted into the bilayer, the phosphate group band shifts gradually to lower wavenumbers, indicating that water accessibility to the phospholipid phosphate groups increases with temperature, irrespective of the protein thermal denaturation.

In summary the IR spectroscopic studies show that TrwB is thermally stabilized when embedded in lipid bilayers, that the protein increases the lipid molecular order and that lipid–protein interactions are detected both in the hydrophobic lipid matrix and at the lipid–water interface.

3.4. TrwB stability against proteolytic digestion

As a further approach to the changes in global protein conformation brought about by bilayer insertion the mixed micelles and the proteoliposomes were subjected to trypsin and proteinase K treatment. Depending on the tertiary structure of the protein, trypsin could have a different accessibility, rendering different trypsinisation patterns. The resulting data could help elucidate the different tridimensional organization of the protein solubilized in detergent in comparison to TrwB proteoliposomes. We analyzed TrwB proteoliposomes and TrwB purified in detergent in order to find the possible differences. As seen in Fig. 6 when the protein is inserted into the lipid bilayer about 40% TrwB is not digested by the protease even at the lowest protein:protease molar ratios described in the Materials and methods section. In contrast, when TrwB solubilized in detergent was digested with trypsin, even after short treatments almost 100% of the protein was digested (Fig. 6C). Digestions with proteinase K rendered similar results in which about 35% of TrwB could not be further proteolyzed (data not shown). These results suggest that the protein is more protected and/or more compactly folded when inserted into the lipid bilayer.

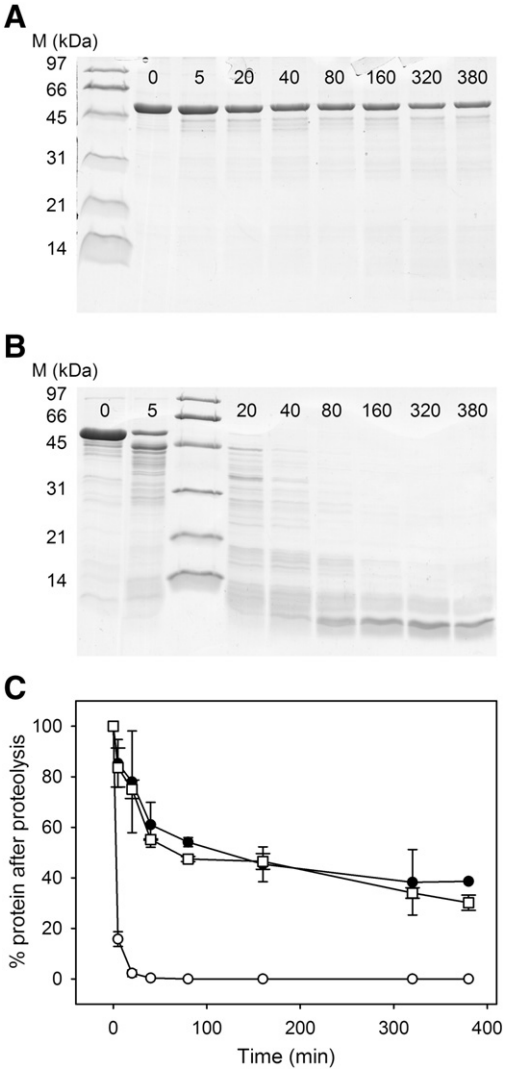

Fig. 6. Trypsin digestion of TrwB and TrwB proteoliposomes. Trypsin digestion (at a 1:500 w:w trypsin:TrwB ratio) was carried out at 25 °C. Aliquots were taken at different times, indicated in minutes above each lane, and analyzed. Coomassie Brilliant Blue-stained SDS-PAGE of tryptic fragments of TrwB proteoliposomes (A) and TrwB in DDM (B). (C) Time course of trypsin digestion of: TrwB proteoliposomes (●), TrwB proteoliposomes treated with 220 mM of OG for bilayer permeabilization before the tryptic digestion (□), and TrwB in DDM (○). Average values ± SD ($n = 3$). Low molecular weight marker from Bio-Rad was used.

4. Discussion

4.1. Structure of TrwB embedded in lipid bilayers

TrwB is an integral membrane protein essential in the R388 bacterial conjugation [2]. This protein belongs to the type IV coupling protein family (T4CP). It is proposed that the proteins of this family connect the relaxosome to the type IV secretion system during bacterial conjugation [27]. The crystal structure of TrwBΔN70 (a deletion soluble mutant) showed that TrwB contains a nucleotide binding domain with α/β topology [28]. Hormaeche et al. [5] observed by IR that in TrwB solubilized in the form of detergent micelles the helicoidal structure was predominant and that TrwBΔN70 had a less compact structure than the native protein. This result suggested that the transmembrane domain (TMD) allows TrwB to fold in a more compact and ordered way, loops and disordered structures being more abundant in the soluble mutant.

In this work, we have studied the structure of TrwB both solubilized in the form of protein/lipid/detergent mixed micelles and reconstituted into liposomes. We observe that TrwB conserves the largely α/β structure previously reported [5]; however, when the protein is inserted into the lipid bilayer an additional band appears at 1619 cm^{-1}. This band can be assigned to an extended structure, a β-strand. A similar situation is found in apoB100, the apoprotein from human serum LDL, where a band at 1617 cm^{-1} has been described in the non-denatured protein in D_2O [29–31], and associated with strands embedded in the membrane, thus less accessible to the solvent [29,31]. Early observations by Valpuesta et al. [32] show that, upon removal of detergent, mitochondrial complex III changed its conformation with an increase in absorbance at ≈1620 cm^{-1}, that today could be attributed to β-strands. Bands at low frequency assigned to extended structures have been described in other proteins; these structures do not form β-sheets with other similar chains but rather interact through hydrogen bonds with similarly extended structures or loops from the same or different polypeptidic chains [8,33]. It has been suggested that in TrwB 15% of the amino acid residues form β-strands [34]. This corresponds very well with the percent β-strands measured by IR in proteoliposomes (Table 1). In the light of the proposed hexameric structure for TrwBΔN70 [28], the β-strand components of TrwB proteoliposome spectra could correspond to structures establishing hydrophobic interactions between protein monomers.

4.2. TrwB stability in micelles and bilayers

Protein thermal denaturation is characterised by the appearance of two IR bands, one below 1620 cm^{-1} and the other above 1683 cm^{-1}, that are associated with aggregation and eventually loss of enzyme activity [35,36]. In the case of TrwB, the bands related to aggregation are 1617 and 1685 cm^{-1}, both for the proteins in micellar and bilayer environments. There is however an important difference in the temperatures, higher for TrwB in bilayer, at which aggregation proceeds. Thermal profiles of TrwB solubilized in detergent and TrwB proteoliposomes (Figs. 3 and 4) indicate that when TrwB is reconstituted into bilayers the protein is more stable than in the presence of detergent, the corresponding T_m values being at least 17 °C higher for TrwB in bilayers. The temperature at which aggregation bands appear is related to the compactness of the protein, less compact proteins tending to melt at lower temperatures [24]. Thus TrwB reconstituted into liposomes would show a more compact structure than in the presence of detergent, insertion into the bilayer being responsible for this change.

Because of the sensitivity of the protein infrared band position to the environment, it has been shown that changes in position of bands assigned to extended structures correspond to changes in the protein topology [37]. Taking into account that aggregation is preceded by unfolding it can be expected that the TrwB unfolding pathway is basically similar in both cases, micelles and bilayers, with the only difference that in proteoliposomes more energy is needed to disrupt the lipid interaction and cause the unfolding and aggregation.

Resistance to proteolysis is a standard procedure to test the proper, compact folding of a protein. Proteolysis assays show that 40% of TrwB in bilayers, but not in micelles, is protected against the proteases tested (Fig. 6). This result could be due to the asymmetric insertion of the protein into the membrane previously shown by Vecino et al. [7]. However the protease assay was repeated in the presence of octylglucoside at a concentration that allows protease permeation through the membrane, and identical results were obtained (Fig. 6C). Thus the combination of thermal denaturation and proteolysis assays confirm that TrwB adopts a more stable conformation when reconstituted into liposomes.

4.3. TrwB interaction with lipids in bilayers

Intrinsic membrane proteins interact with membrane lipids in complex ways that are still far from being understood. IR spectroscopy is a unique tool in that it provides simultaneous information on the lipid and protein membrane components, as well as on the hydrophobic and interface membrane regions [9,10,38,39]. In their pioneering work, Chapman and associates [40–42] described the dissordering effect of intrinsic proteins on fully saturated phospholipid acyl chain. In an early paper [40] the possibility of applying IR spectroscopy to the study of membrane proteins was envisaged, but this was only carried out some years later by Arrondo and co-workers [32,43] and Mantsch and co-workers [44,45].

In the present contribution, our IR spectroscopic data suggest that incorporation of TrwB into lipid bilayers causes: (a) a decrease in β-sheet components of the protein secondary structure, with a concomitant increase in β-strand (Table 1), (b) an increase in lipid chain order (Fig. 5A) and (c) an enhanced water access to phospholipid phosphate groups (Fig. 5B). Moreover, the comparison of protein and lipid data at varying temperatures, replotted in Fig. 7, indicates that the lipid acyl chains "feel" the protein thermal denaturation at ≈50–60 °C. (Note that, although protein methylene groups also contribute to the band at 2852 cm^{-1}, they constitute only a small fraction of the signal).

The bilayer insertion-dependent changes in protein structure, mainly formation of β-strands, have been discussed above. The effects of protein insertion on the lipid structure and dynamics are also interesting, in particular the increase in lipid chain order. This is against the observations by Mendelsohn et al. [46] and Cortijo et al. [41]; however, they were using pure saturated lipids that are intrinsically much more ordered than our composition. Anderle and Mendelsohn [47] showed later that phospholipids of natural origin, as in our study, had their acyl chains slightly oriented by the intrinsic protein Ca^{+2}-ATPase from sarcoplasmic reticulum.

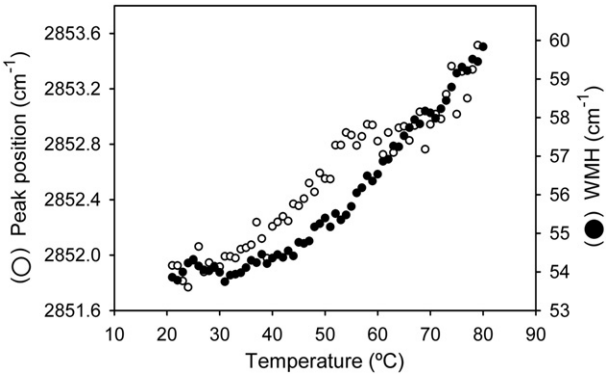

Fig. 7. TrwB thermal denaturation detected through changes in lipid acyl chain vibration. TrwB denaturation is indicated by the large increase in amide I′ bandwith (●) above ≈50 °C. Plotting the C–H stretching band position arising mainly from lipid acyl chains (○) reveals a discontinuity in the temperature region where protein denaturation occurs. Data replotted from Figs. 4 and 5A.

Our observation of protein-dependent changes in phosphate group hydration, or accessibility to water (Fig. 5B), is also interesting, particularly because this is a scarcely studied region in the IR spectrum [48,49]. In general phosphate stretching bands are not modified by intrinsic proteins, at least when zwitterionic phospholipids are used [10] but in our case, in the presence of PG and CL, the situation may be different. Also the fact that our lipid mixture contains PE can make the bilayer rather unstable, and the presence of protein can increase the instability, disrupting the membrane permeability barrier, which would become evident as an increased water accessibility to lipid phosphate groups was observed.

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