New insights into pb5, the receptor binding protein of bacteriophage T5, and its interaction with its Escherichia coli receptor FhuA
Ali Flayhan, Frank Wien, Maïté Paternostre, Pascale Boulanger, Cécile Breyton

To cite this version:

HAL Id: cea-01201918
https://hal-kea.archives-ouvertes.fr/cea-01201918
Submitted on 18 Sep 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Research paper

New insights into pb5, the receptor binding protein of bacteriophage T5, and its interaction with its Escherichia coli receptor FhuA

Ali Flayhana,b,c,d, Frank Wien e, Maïté Paternostref,g, Pascale Boulanger h, Cécile Breytona,b,c,d,*

A R T I C L E   I N F O

Article history:
Received 11 April 2012
Accepted 22 May 2012
Available online 29 May 2012

Keywords:
Phage T5
Conformational changes
Receptor binding protein
SRCD
ATR-FTIR

A B S T R A C T

The majority of bacterial viruses are bacteriophages bearing a tail that serves to recognise the bacterial surface and deliver the genome into the host cell. Infection is initiated by the irreversible interaction between the viral receptor binding protein (RBP) and a receptor at the surface of the bacterium. This interaction results ultimately in the phage DNA release in the host cytoplasm. Phage T5 infects Escherichia coli after binding of its RBP pb5 to the outer membrane ferrichrome transporter FhuA. Here, we have studied the complex formed by pb5 and FhuA by a variety of biophysical and biochemical techniques. We show that unlike RBPs of known structures, pb5 probably folds as a unique domain fulfilling both functions of binding to the host receptor and interaction with the rest of the phage. Pb5 likely binds to the domain occluding the β-barrel of FhuA as well as to external loops of the barrel. Furthermore, upon binding to FhuA, pb5 undergoes conformational changes, at the secondary and tertiary structure level that would be the key to the transmission of the signal through the tail to the capsid, triggering DNA release. This is the first structural information regarding the binding of a RBP to a proteic receptor.

© 2012 Elsevier Masson SAS. All rights reserved.

1. Introduction

Bacterial viruses, bacteriophages, represent the widest group of biological entities on the planet [1]. They have co-evolved with bacteria, turning into virulent killers or prophages, and as such, they have a major impact on the ecology and evolution of their hosts [2]. More than 95% of known bacterial viruses belong to the caudovirales order [3]. The host specificity is determined by the interaction of the Receptor Binding Proteins (RBPs), located at the tip of the tail, with defined receptor(s) at the surface of the bacterium (saccharides and/or proteins). This occurs most often in a two-step process, in which phages initially adsorb reversibly to low affinity receptors, prior to binding irreversibly to secondary sites or receptors [4]. The RBP-receptor interaction triggers conformational rearrangements within the tail structures, which induce capsid opening and cell wall perforation, allowing DNA release and transfer via the tail through the bacterial envelope [5,6]. Deciphering this cascade of events at a molecular level is a major issue for understanding the initial steps of infection.

The last decade has given insights into the structure of phage tail subcomplexes. It was shown that despite infecting different hosts Myoviridae coliphage T4 [5] and the Siphoviridae lacticoccal phage p2 [7] share similar adsorption mechanisms. Other Siphoviridae, such as the Bacillus subtilis phage SPP1 and the coliphages λ and T5, require a specific protein receptor to irreversibly recognise and infect their host [4]. These phages exhibit a limited number of RBPs,

Abbreviations: ATR-FTIR, Attenuated total reflectance Fourier transform infrared spectroscopy; LDAO, N,N dimethyl dodecylamine-N-oxide; RBP, Receptor Binding Protein; SPR, Surface Plasmon Resonance; SDS-PAGE, sodiumdodecylsulfat polyacrylamide gel electrophoresis; SRCD, Synchrotron Radiation Circular Dichroism.

* Corresponding author. CEA, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France. Tel.: +33 3878 3037; fax: +33 3878 5494.
E-mail address: Cecile.Breyton@ibs.fr (C. Breyton).

Contents lists available at SciVerse ScienceDirect
Biochimie
journal homepage: www.elsevier.com/locate/biochi


0300-9084/$ see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2012.05.021
which are located in a unique central tail fibre whose structure remains to be elucidated. An important step in understanding the communication between the phage tail tip and capsid has come from SPPI. Electron microscopy has shown that the tail tip rearrangement following receptor binding of SPPI to its receptor induces domino-type cascade conformational changes of the major tail protein through the tail towards the capsid [8,9].

The Siphoviridae coliphage T5 has proven to be a well-suited model to study phage–host interactions. Its 250 nm-tall ends with three L-shaped fibres attached to a conical baseplate and a straight central fibre [10]. Host recognition is initiated by reversible binding of the L-shaped fibres to the O-antigen of the lipopolysaccharide. The phage then binds irreversibly to the outer membrane iron-ferrichrome transporter FhuA by means of its RBP pb5 [11]. The crystal structure of FhuA has revealed a 22-stranded anti-parallel β-barrel and an N-terminal globular domain that folds inside the barrel and occludes it, referred to as the “cork” [12]. The external loops connecting the β-strands serve as binding sites for the natural substrate ferrichrome and other ligands of FhuA (phages T5, T1, Φ80, N15, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA substrate ferrichrome and other ligands of FhuA (phages T5, T1, and re-concentrated by ultra-centrifugation (HiTrap Chel, 5 ml) and anion exchange (HiTrap Q, 1 ml) columns were performed in 0.1 and 0.05% LDAO, respectively. H6-pb5 was overexpressed and purified FhuA or into liposomes containing FhuA [14]. Furthermore, interaction between purified pb5 and FhuA yields a highly stable, stoichiometric complex, which is not denatured by 2% SDS unless heated to 70 °C [15]. This complex is currently the only complex biochemically available between an outer membrane receptor and a phage RBP. One of the major issues to elucidate the molecular mechanisms that propagate the signal from the RBP to the capsid is to describe the conformational rearrangements that initially take place between the RBP and its receptor. Here, we have further characterised the complex formed between pb5 and FhuA, and determined the strength of interaction between the two proteins. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), near-UV and Synchrotron Radiation Circular Dichroism (SRCD) and limited proteolysis were used to characterise the conformational changes induced by the formation of the complex.

2. Materials and methods

Overexpression and purification—FhuA purification was carried out as described in [16], except that outer membranes were solubilised with 1% N,N dimethyl dodecylamine-N-oxide (LDAO), and the Nickel affinity (HiTrap Chel, 5 ml) and anion exchange (HiTrap Q, 1 ml) columns were performed in 0.1 and 0.05% LDAO, respectively. H6-pb5 was overexpressed and purified as described in [15] with minor modifications: the Nickel affinity (HiTrap Chel, 5 ml) and cation exchange (HighTrap SP, 1 ml) columns were performed in 25 mM MES pH 6.0. All columns were from GE Healthcare. To remove NaCl, FhuA and pb5 were diluted 1000 times with 0.05% NaCl/C14, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA

which are located in a unique central tail fibre whose structure remains to be elucidated. An important step in understanding the communication between the phage tail tip and capsid has come from SPPI. Electron microscopy has shown that the tail tip rearrangement following receptor binding of SPPI to its receptor induces domino-type cascade conformational changes of the major tail protein through the tail towards the capsid [8,9].

The Siphoviridae coliphage T5 has proven to be a well-suited model to study phage–host interactions. Its 250 nm-tall ends with three L-shaped fibres attached to a conical baseplate and a straight central fibre [10]. Host recognition is initiated by reversible binding of the L-shaped fibres to the O-antigen of the lipopolysaccharide. The phage then binds irreversibly to the outer membrane iron-ferrichrome transporter FhuA by means of its RBP pb5 [11]. The crystal structure of FhuA has revealed a 22-stranded anti-parallel β-barrel and an N-terminal globular domain that folds inside the barrel and occludes it, referred to as the “cork” [12]. The external loops connecting the β-strands serve as binding sites for the natural substrate ferrichrome and other ligands of FhuA (phages T5, T1, Φ80, N15, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA

which are located in a unique central tail fibre whose structure remains to be elucidated. An important step in understanding the communication between the phage tail tip and capsid has come from SPPI. Electron microscopy has shown that the tail tip rearrangement following receptor binding of SPPI to its receptor induces domino-type cascade conformational changes of the major tail protein through the tail towards the capsid [8,9].

The Siphoviridae coliphage T5 has proven to be a well-suited model to study phage–host interactions. Its 250 nm-tall ends with three L-shaped fibres attached to a conical baseplate and a straight central fibre [10]. Host recognition is initiated by reversible binding of the L-shaped fibres to the O-antigen of the lipopolysaccharide. The phage then binds irreversibly to the outer membrane iron-ferrichrome transporter FhuA by means of its RBP pb5 [11]. The crystal structure of FhuA has revealed a 22-stranded anti-parallel β-barrel and an N-terminal globular domain that folds inside the barrel and occludes it, referred to as the “cork” [12]. The external loops connecting the β-strands serve as binding sites for the natural substrate ferrichrome and other ligands of FhuA (phages T5, T1, Φ80, N15, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA

which are located in a unique central tail fibre whose structure remains to be elucidated. An important step in understanding the communication between the phage tail tip and capsid has come from SPPI. Electron microscopy has shown that the tail tip rearrangement following receptor binding of SPPI to its receptor induces domino-type cascade conformational changes of the major tail protein through the tail towards the capsid [8,9].

The Siphoviridae coliphage T5 has proven to be a well-suited model to study phage–host interactions. Its 250 nm-tall ends with three L-shaped fibres attached to a conical baseplate and a straight central fibre [10]. Host recognition is initiated by reversible binding of the L-shaped fibres to the O-antigen of the lipopolysaccharide. The phage then binds irreversibly to the outer membrane iron-ferrichrome transporter FhuA by means of its RBP pb5 [11]. The crystal structure of FhuA has revealed a 22-stranded anti-parallel β-barrel and an N-terminal globular domain that folds inside the barrel and occludes it, referred to as the “cork” [12]. The external loops connecting the β-strands serve as binding sites for the natural substrate ferrichrome and other ligands of FhuA (phages T5, T1, Φ80, N15, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA

which are located in a unique central tail fibre whose structure remains to be elucidated. An important step in understanding the communication between the phage tail tip and capsid has come from SPPI. Electron microscopy has shown that the tail tip rearrangement following receptor binding of SPPI to its receptor induces domino-type cascade conformational changes of the major tail protein through the tail towards the capsid [8,9].

The Siphoviridae coliphage T5 has proven to be a well-suited model to study phage–host interactions. Its 250 nm-tall ends with three L-shaped fibres attached to a conical baseplate and a straight central fibre [10]. Host recognition is initiated by reversible binding of the L-shaped fibres to the O-antigen of the lipopolysaccharide. The phage then binds irreversibly to the outer membrane iron-ferrichrome transporter FhuA by means of its RBP pb5 [11]. The crystal structure of FhuA has revealed a 22-stranded anti-parallel β-barrel and an N-terminal globular domain that folds inside the barrel and occludes it, referred to as the “cork” [12]. The external loops connecting the β-strands serve as binding sites for the natural substrate ferrichrome and other ligands of FhuA (phages T5, T1, Φ80, N15, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA

which are located in a unique central tail fibre whose structure remains to be elucidated. An important step in understanding the communication between the phage tail tip and capsid has come from SPPI. Electron microscopy has shown that the tail tip rearrangement following receptor binding of SPPI to its receptor induces domino-type cascade conformational changes of the major tail protein through the tail towards the capsid [8,9].

The Siphoviridae coliphage T5 has proven to be a well-suited model to study phage–host interactions. Its 250 nm-tall ends with three L-shaped fibres attached to a conical baseplate and a straight central fibre [10]. Host recognition is initiated by reversible binding of the L-shaped fibres to the O-antigen of the lipopolysaccharide. The phage then binds irreversibly to the outer membrane iron-ferrichrome transporter FhuA by means of its RBP pb5 [11]. The crystal structure of FhuA has revealed a 22-stranded anti-parallel β-barrel and an N-terminal globular domain that folds inside the barrel and occludes it, referred to as the “cork” [12]. The external loops connecting the β-strands serve as binding sites for the natural substrate ferrichrome and other ligands of FhuA (phages T5, T1, Φ80, N15, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA

which are located in a unique central tail fibre whose structure remains to be elucidated. An important step in understanding the communication between the phage tail tip and capsid has come from SPPI. Electron microscopy has shown that the tail tip rearrangement following receptor binding of SPPI to its receptor induces domino-type cascade conformational changes of the major tail protein through the tail towards the capsid [8,9].

The Siphoviridae coliphage T5 has proven to be a well-suited model to study phage–host interactions. Its 250 nm-tall ends with three L-shaped fibres attached to a conical baseplate and a straight central fibre [10]. Host recognition is initiated by reversible binding of the L-shaped fibres to the O-antigen of the lipopolysaccharide. The phage then binds irreversibly to the outer membrane iron-ferrichrome transporter FhuA by means of its RBP pb5 [11]. The crystal structure of FhuA has revealed a 22-stranded anti-parallel β-barrel and an N-terminal globular domain that folds inside the barrel and occludes it, referred to as the “cork” [12]. The external loops connecting the β-strands serve as binding sites for the natural substrate ferrichrome and other ligands of FhuA (phages T5, T1, Φ80, N15, HK02, the bacterial toxins Colicin M and Microcin J25, and the antibiotic albomycin) [13]. An interesting feature of T5 is that DNA
Data of pb5 above 90 °C had to be omitted due to aggregation of the protein. For FhuA, data were fitted using a three-state transition model, with correction for pre- and post-transition linear changes in ellipticity as a function of temperature. Data was fitted with the following equation: \( \theta_i = a\theta_1 + a\theta_2 + a\theta_3 \), where \( \theta_i \) is the observed ellipticity at any temperature, \( a \) the mole fraction and \( \theta \) the ellipticity, and the subscript F, I and U refer to the fully folded, the intermediate and the unfolded forms, respectively. Finally, for the complex, data were best fitted using a two-state transition model, between a folded dimer and unfolded monomers as described in [23]. Data was treated with SigmaPlot 8.0.

2.4. Near-UV CD

Near-UV CD spectra of FhuA, pb5 and of the FhuA–pb5 complex were recorded on a JOBIN YVON CD6 at 20 °C. All samples were loaded into the same 1 cm quartz cell. Acquisitions (1 nm step 2 s integration) between 240 and 320 nm were performed in triplicates. Averaged sample spectra were subtracted from their corresponding buffer baselines. Spectra were normalised to protein concentration (0.3–0.5 mg/ml) and pathlength. The calculated spectrum of the FhuA–pb5 complex was obtained by adding the normalised spectra of FhuA and pb5.

2.5. ATR-FTIR

Spectra were measured at 4 cm\(^{-1}\) resolution with a Bruker IFS 66 spectrophotometer equipped with a 45° n ZnSe ATR attachment. The buffer signal was removed by subtraction of the sample buffer (concentrator flow through) spectrum recorded before each measurement. FhuA and pb5 samples were in 20 mM Tris pH 8.0, ~200 mM NaCl, 0.1% LDAO and 25 mM MES pH 6.0, ~400 mM NaCl, respectively. Shown spectra resulted from the average of 30 scans, and were corrected for the linear dependence on the wavelength of the absorption measured by ATR. The spectra of each protein and of the complex were normalised to the area between 1550 and 1800 cm\(^{-1}\), i.e. the amide I and II region, and by the mass of each protein. It has been checked that the contribution of LDAO is negligible in the Amide I- II region. To extract the different secondary structure determinants, the spectra (after subtraction of a linear baseline between 1590 and 1710 cm\(^{-1}\)) were decomposed into 4 Gaussian components using Peak Fit Software (4.12 version) and the residuals were minimised for each spectrum.

2.6. Proteolysis

Purified pb5, FhuA and the pb5–FhuA complex were incubated with trypsin (1/1200, 1/600, 1/300 w/w protease/pb5), chymotrypsin (1/500, 1/250, 1/125 w/w) subtilisin (1/500, 1/250, 1/125 w/w) for 40–50 min at room temperature. After the reaction, one half of the proteolyzed proteins was mixed with its unproteolysed partner to form a proteolysed and its unproteolysed partner. It has been checked that the contribution of LDAO is negligible in the Amide I- II region.

3. Results

3.1. Probing secondary structure changes upon FhuA–pb5 complex formation

SRCD is a powerful tool for the characterisation of the secondary structure content of proteins: i) it can resolve CD signals down to 168 nm in the far-UV region due to the high flux of the synchrotron light source, and ii) the signal-to-noise levels are greatly improved when compared to in house CD. This allows high sensitivity, better spectral resolution, and ultimately improves the information content and the secondary structure prediction [24]. Fig. 1A and B shows the spectra of FhuA, pb5 and of the FhuA–pb5 complex. FhuA and pb5 display a large positive peak at 196–197 nm (\( \pi \rightarrow \pi^* \)) and a negative peak at 218–216 nm (\( n \rightarrow \pi^* \)), respectively, which are characteristic of \( \beta \)-sheets, and in agreement with published CD spectra [15,25,26]. The deconvolution into secondary structure identifies a content of 47%, 9%, 6%, 38% for FhuA and of 44%, 11%, 6%, 39% for pb5 of \( \beta \)-sheets, turns, \( \alpha \)-helices and other structures, respectively. These proportions are compatible with the crystal structure of FhuA, and with secondary structure prediction of pb5 (Table 1). The lower intensity of the pb5 CD signal could indicate the presence of less structured regions. The spectrum of the FhuA–pb5 complex displays similarly two peaks, at 199 nm and 218 nm. The comparison of the measured spectrum of the FhuA–pb5 complex with the normalised sum of the individual spectra (calculated spectrum, see Methods) however shows clear differences (Fig. 1B). A shift of the maximum from 196 to 199 nm and an increase of the intensity of the signal, as well as a small change around 220 nm are significant and clearly distinguishable above the noise level. These differences indicate conformational changes and reorganisation at the secondary structure level within the proteins upon complex formation. Deconvolution reveals an increase of the \( \beta \)-sheet at the expense of other structures (Table 1).

These results were further confirmed by ATR-FTIR. This technique also probes secondary structures of proteins. The analysis is focused on amide I vibrations (1600–1700 cm\(^{-1}\)), which mainly arise from the vibration stretching mode of the backbone carbonyl groups. The decomposition assigns wavenumbers to backbone carbonyl groups involved in different strengths and types of hydrogen bonds, and therefore secondary structure types [27,28]. In accordance with the literature, the absorption around
treatments with classical dissociating agents (4 M NaCl, 3 M MgCl₂, 20 mM EDTA, 1 M Na₂SO₄, 4 M Guanidinium-chloride, 1 M diethylenetriamine, 1% acetic acid, 10 mM Glycine pH 2.0, 25 mM HCl, or 100 mM NaOH) (Fig. 2). Dissociation of the complex was observed after injection of 6 M Guanidinium-chloride (Fig. 2), or 50 mM HCl, 100 mM NaOH) (Fig. 2). Dissociation of the complex was detectable after either a night or a day after injection of classical denaturing reagents (20 µl at 20 µl/min). 1. 4 M NaCl, 2. 3 M MgCl₂, 3. 20 mM EDTA, 4. 4 M Guanidinium-HCl and 5. 6 M Guanidinium-HCl. All solutions contained 0.05% LDAO. R.U.: resonance unit.

Table 1
Analyses of the secondary structures of FhuA, pb5 and the FhuA–pb5 complex. Numbers are given in %. SRCD spectra were deconvoluted using the DICHROWEB server [19] with the CURVEFIT analysis program and taking the SP175 as a reference dataset. Pb5 sequence analysis was performed with the Garnier program [44]. FTIR spectra were decomposed into 4 Gaussian using peak Fit (see Fig. S1). For pb5, the % of random structures are converted to b-structure, in total agreement with the SRCD results.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Method</th>
<th>β-sheet</th>
<th>Turn</th>
<th>α-helix</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>FhuA PDB (1QFG)</td>
<td>53</td>
<td>13</td>
<td>7</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>CD [26]</td>
<td>38</td>
<td>12</td>
<td>17</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>SRCD (rmsd – 0.018)</td>
<td>47</td>
<td>9</td>
<td>6</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>FTIR</td>
<td>50</td>
<td>18</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pb5 Sequence prediction</td>
<td>40</td>
<td>24</td>
<td>7</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>CD [15]</td>
<td>51</td>
<td>22</td>
<td>6</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>SRCD (rmsd – 0.028)</td>
<td>44</td>
<td>11</td>
<td>6</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>FTIR</td>
<td>44</td>
<td>24</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRCD (rmsd – 0.024)</td>
<td>52</td>
<td>8</td>
<td>4</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Complex SRCD calculated (rmsd – 0.028)</td>
<td>46</td>
<td>10</td>
<td>6</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

1635–1650 cm⁻¹ is assigned to random and α-helical conformations, the combination of absorption around 1615–1630 and 1675–1685 cm⁻¹ to anti-parallel β-sheet structures, and the vibration around 1663–1670 cm⁻¹ to turn structures.

The ATR-FTIR spectra of FhuA and pb5 (Fig. 1C) confirm the high β-sheet content of both proteins. Indeed, their decomposition identified for FhuA 50%, α-helical 32% and 32% of β-sheet, -turn, and random and α structures, respectively, in very good agreement with the crystal structures (Table 1, Fig. S1). For pb5, respective % of 44, 24 and 32 are found, also in good agreement with the sequence prediction and the SRCD spectrum (Table 1). The comparison of the spectrum measured for the complex with that obtained by addition of the normalised spectra of FhuA and pb5 shows some substantial differences, witnessing secondary structure reorganisation upon complex formation (Fig. 1D). The subtraction of both spectra shows some substantial differences, witnessing secondary structure reorganisation upon complex formation (Fig. 1D). The subtraction of both spectra shows some substantial differences, witnessing secondary structure reorganisation upon complex formation (Fig. 1D). The subtraction of both spectra shows some substantial differences, witnessing secondary structure reorganisation upon complex formation (Fig. 1D).

3.2. The FhuA–pb5 complex is extremely stable

Real-time Surface Plasmon Resonance (SPR) experiments show that the complex between FhuA and pb5 has a very high affinity. The binding signal was not due to aggregation of FhuA on the surface, as the signal could be saturated (Fig. S2A), and no dissociation of the complex was detectable after either a night’s wash, or treatments with classical dissociating agents (4 M NaCl, 3 M MgCl₂, 20 mM EDTA, 1 M Na₂SO₄, 4 M Guanidinium-chloride, 1 M diethylenetriamine, 1% acetic acid, 10 mM Glycine pH 2.0, 25 mM HCl, or 100 mM NaOH) (Fig. 2). Dissociation of the complex was observed after injection of 6 M Guanidinium-chloride (Fig. 2), or 50 mM HCl, and was most probably the result of protein denaturation. Indeed, addition of FhuA on the regenerated surface, corresponding to dissociated pb5, did not result in new complex formation, suggesting that pb5 was no longer correctly folded. In order to measure the dissociation constant of the complex, Single Cycle Kinetics were performed [29]. This method involves sequentially injecting an analyte concentration series without any regeneration steps. However, at the high or low level of pb5 immobilisation and at the low FhuA concentrations tested, and with the presence of detergent in the running buffer, the detection limits of the Biacore 3000 were reached without the possibility of measuring either a kₐ (i.e. dissociation of the complex following FhuA injection) or reaching an equilibrium during the injection time (i.e. reaching a plateau during the injection of FhuA) (Fig. S2B). Thus, the data could not be analysed either within a kinetic or in a thermodynamic frame, and the kₐ could not be calculated. Tentative determination of the kₐ by fitting curves shown in Fig. S2A resulted in an upper limit of the kₐ value of the order of a hundred of pM.

To further characterise the interaction between the two proteins, thermal denaturation of the proteins and the complex was probed by SRCD (Fig. 3). Analysis of the spectra of FhuA with Convex Constraint Algorithm [22] shows that three basis spectra are needed to reconstitute the whole dataset (Fig. S3A,D), indicating a three-state unfolding process, that of the complex for the unfolding of a dimer to unfolded monomers with two-state model. Squares represent the residuals from the model.

Fig. 2. SPR analysis of the FhuA-pb5 complex. A. Pb5 was immobilised on the chip onto which FhuA was injected, forming the complex (see Materials and Methods). Sensorgram of the raw signal after injection of classical denaturing reagents (20 µl at 20 µl/min). 1. 4 M NaCl, 2. 3 M MgCl₂, 3. 20 mM EDTA, 4. 4 M Guanidinium-HCl and 5. 6 M Guanidinium-HCl. All solutions contained 0.05% LDAO. R.U.: resonance unit.

Fig. 3. SRCD thermal denaturation. A, B and C: Spectra of FhuA, pb5 and the complex, respectively, collected as a function of temperature. Data were collected at 5-deg increments with 3 min equilibration at each temperature. Black arrows show the evolution of the spectrum as the temperature increases. D, E and F: SRCD signal (circles) and fitted curves (solid line) for FhuA at 196 nm, pb5 at 197 nm and the complex at 218 nm, respectively. The raw data of FhuA were fitted with equations for the unfolding of a monomer with three-state model, that of pb5, with equations for the unfolding of a dimer to unfolded monomers with two-state model. Squares represent the residuals from the model.
mechanism. Analysis of the basis spectra indicates that at around 65 °C, FhuA unfolds into a stable intermediate species characterised by the drop in the CD signal and the shift from 196 to 193 nm of the positive peak (27% β-sheet and 34% α-helix). Above 75 °C the unfolded state is characterised by a negative peak at 208 nm (12% β-sheet and 35% α-helix) (Fig. 3A). In agreement with a three-state unfolding mechanism, the CD signal of FhuA at 196 nm as a function of temperature is best fitted using a three-state model and shows two inflections points at 60 and 74 °C (Fig. 3D). For pb5 and the complex, only two basis spectra were needed to fit the SRCD thermal denaturation data (Fig. 3B,E and F), indicating that both pb5 and the complex follow a two-state unfolding mechanism. Analysis of the basis spectra indicates that denatured pb5 retains an overall β-strand (40%) conformation during thermal denaturation (Fig. 3B). Interestingly, within the complex, both proteins unfold cooperatively and lose almost all their secondary structures, as witnessed by the almost flat CD signal for the denatured complex (Fig. 3C). The unfolding not being reversible, unfolding enthalpies calculated from the fitting may not be relevant. However, calculation of unfolding Tm is independent of the reversibility of the reaction. The evolution of the CD signal at 197 nm for pb5 and 218 nm for the complex, as a function of temperature, are best fitted by equations describing a two-state unfolding mechanism, and provide unfolding Tm of 43 °C for pb5 and 89 °C for the complex (Fig. 3E,F). These results are in agreement with previous differential scanning calorimetry results [15,16], where the two transitions displayed by FhuA were attributed to the unfolding of the loops and cork at 65 °C and of the barrel at 74 °C. The large difference in the transition temperatures between the complex and the two isolated proteins indicates that the complex has a strong stabilising effect on both proteins. The fact that the first transition of FhuA, corresponding to the unfolding of the cork is not present in the complex suggests that pb5 also binds to the cork, or alternatively that pb5 binding to extracellular loops indirectly locks the cork in the barrel.

3.3. Aromatic residues environment changes upon formation of the FhuA–pb5 complex

Near-UV (250–320 nm) CD probes the overall tertiary structure of proteins, being sensitive to the environment and flexibility of aromatic side chains within the proteins: an intense signal witnesses a rigid structure and a well-folded protein. Fig. 4 shows the normalised spectra of FhuA (36F, 41Y, 9W), pb5 (26F, 31Y, 10W) and of the complex. Pb5 shows a rather broad spectrum with an indiscrete peak at 292 nm, and an important shoulder at 250–270 nm, suggesting a stronger contribution of tryptophans and phenylalanines, respectively. FhuA’s spectrum is dominated by the contribution of tyrosines, with a peak centred around 280 nm. The comparison of the measured and calculated signal of the complex (Fig. 4B) shows a huge increase in the overall CD signal. The measured spectrum preserves the same shape as that of FhuA, dominated by the tyrosine contribution. These results strongly suggest that upon complex formation, the tertiary structure of the proteins becomes more rigid, and that the environment of aromatic residues are better defined, with a possible stacking of the latter at the interface between the two proteins. Indeed, the loops of FhuA count a rather large number of aromatics, especially tyrosines, which could be involved in interaction with pb5.

3.4. Proteolysis

Limited proteolysis revealed further evidence for the conformational changes occurring upon formation of the complex. Pb5 and FhuA were separately digested by subtilisin and then assayed for their ability to form a stable complex with their unproteolysed partner. A convenient means of assessing the FhuA–pb5 complex formation is SDS gel electrophoresis as the FhuA–pb5 complex is not dissociated by SDS, and migrates as a unique band unless heated [15]. When incubated with subtilisin, pb5 undergoes extensive proteolysis, as attested by the absence of discrete bands on denaturing electrophoresis (Fig. 5, lane 1). Subtilisin being an a specific protease, proteolysis occurs randomly, leading to
a mixture of peptides of different length resulting in an ‘invisible smear’ on the gel after denaturation of the protein by SDS. Strikingly, digested pb5 forms an SDS stable complex when mixed to FhuA (Fig. 5, lane 2). This is shown by the presence of a sharp band migrating at the same position as the complex (compare with lane 7). Only a very small amount of free FhuA is detected as a fuzzy band (compare with lane 5). This indicates that cleavage of pb5 in multiple sites does not alter the ability of the protein to form a stable complex with FhuA, suggesting that the domain that interacts with FhuA has conserved its three-dimensional structure. This hypothesis is confirmed by ATR-FTIR that shows that both proteolyzed and untreated pb5 have identical spectra (Fig. S4B).

Size exclusion chromatography further suggests that pb5 folds as a unique domain, as both untreated and digested pb5 elute at the same volume when loaded onto an SD200 column (Fig. S4C). We thus assume that pb5 adopts a very tight (but SDS sensitive) core structure, sensitive to proteolysis in external loops that do not or little affect the interaction with FhuA. FhuA is also rather sensitive to subtilisin, its tertiary structure however remaining unaffected (not shown). Proteolysis likely mainly occurs in the external loops, to subtilisin, its tertiary structure remaining unaffected structure, sensitive to proteolysis in external loops that do not or thus assume that pb5 adopts a very tight (but SDS sensitive) core structure, sensitive to proteolysis in external loops that do not or little affect the interaction with FhuA. FhuA is also rather sensitive to subtilisin, its tertiary structure however remaining unaffected (not shown).

The FhuA protein, as well as the conformational changes occurring within the complex likely occurs as a result of a loss of the secondary structure stabilising each of the two proteins, and destabilisation of the complex likely occurs as a result of a loss of the secondary structure of both proteins.

Previous studies suggested that the external loops 4 [30] and 8 [13] of FhuA are involved in the binding of phage T5. The first transition of FhuA, at 60 °C, corresponding to unfolding of the cork, is absent in the complex. This suggests that in the complex, the cork domain of FhuA is stabilised and dependent on the rest of the complex, and thus that pb5 interacts with this domain as well as with the loops of the barrel. This is in agreement with the fact that even though T5 infects cells baring a FhuA mutant in which the cork domain has been deleted (FhuAA51–128) [31], the affinity of binding of T5 to FhuAA is reduced with respect to binding to WT FhuA [16]. Thus, the interaction between FhuA and pb5 seems to involve a large area, including loops from the cork domain as well as loops of the barrel. Near-UV results also suggest an important contribution of aromatic residues stacking in the interaction. These observations can explain the high affinity between the two proteins and the large biochemical and thermal stability.

4.4. Conformational changes within the complex

Upon FhuA–pb5 interaction, significant conformational changes occur within the complex. More specifically, i) β structures are formed at the expense of all other secondary structures, as shown by SRCD and ATR-FTIR; ii) the tertiary structure of the complex appears more rigid, as shown by near-UV CD, and iii) proteolysis experiments clearly indicate conformational changes and/or structurization/ rigidification throughout pb5 upon binding to FhuA. In addition, we note that whereas purified pb5 is poorly soluble (it precipitates at a concentration above 0.5 mg/ml, or in the presence of imidazol, of detergent, or in a dialysis tube), the FhuA–pb5 complex is soluble to at least 20 mg/ml. The interaction with FhuA could mask hydrophobic patches of pb5 exposed to the solvent, either at the interface between the two proteins, or within the protein through conformational changes/rigidification. Furthermore, from SRCD (low signal with respect to FhuA’s signal) and proteolysis data, pb5 appears more structured when in complex than isolated.

Whereas no structural information is available on pb5, several FhuA structures have been determined either unliganded or with a variety of bound ligands on its extracellular side (fericrocin, ferrichrome, phenylferricin, alibomycin, and rifamycin) [32]. In vivo, these ligands are actively transported across the outer membrane in a TonB-dependent manner. Interestingly, none of the structures show secondary structure conformational changes upon ligand binding, apart from the unwinding of the small switch helix belonging to the cork domain, on the periplasmic side of the protein, allowing the binding information to reach TonB. Structure
comparison between the unliganded and the liganded protein only show rigid body movements of loops and of the core. CD and FTIR data obtained on FhuA either free or bound to ferrichrome show very little spectral changes [26]. Furthermore, molecular dynamics performed on both FhuA and FhuA-ferrichrome show movements of the loops as rigid entities [33]. The FhuA structure thus appears very stable, and one can reasonably postulate that no secondary structure conformational changes will occur upon binding of pb5. Thus, it is reasonable to attribute the conformational changes observed by SCDR and ATR-FITR solely to pb5. From the ATR-FITR peak area, and from the decomposition of the SCDR spectra, this would correspond to ~10–12% conversion of random-α-other structures to β-sheets within pb5.

4.3. Pb5 shares no common feature with other known RBPs

Common features of RBPs of known atomic structure include that 1) they are present in moderate to high copy number in the phage particle (18–54), 2) they recognize oligosaccharide receptors, and 3) they fold into multiple domains. This is the case for the RBP of the lactococcal Phosphoviridae phages p2 [34] and TP901-1 [35], which consist of a receptor-binding head domain, a N-terminal domain that anchors the protein to the phage, and a short and rigid neck domain that links both domains. Importantly, these domains appear to behave as independent and stable entities amenable to structural studies as shown for the RBP of the lactococcal phage bl170 [36]. A multi-domain organisation is also a landmark of the RBP of the short tail fibres of the Myoviridae coliphage T4, the RBP of Podoviridae P22, Sf6 and HK620 as well as the RBP of the Tectiviridae phage PRD1 (see [37] for a review).

The characteristics of phage T5 RBP appears however to differ from those of the above-mentioned phages. Pb5 binds a proteic receptor and is present at a low copy number in the phage (less than 3) [38]. Furthermore, our results indicate that pb5 probably folds as a unique domain. Indeed, although cleaved in multiple sites by proteolysis, isolated pb5 migrates at the same retention volume as the uncleaved protein on gel filtration, and forms a complex with FhuA that is not dissociated in SDS-PAGE. FhuA is also the receptor for the lambdoid phages T1, φ80, N15 and HK022 [32]. Yet search for sequence homologies between pb5 and the putative RBP of these phages failed. On the other hand, alignment of pb5 with the RBP of the T5-related phages BF23 [39], H8 [40], EPS7 [41] and SPC35 [42] highlights strong sequence identity at the N-terminus and in patches scattered along the sequence (Fig. S5). Since these phages bind to different receptors, it is likely that the conserved sequences correspond to regions of the protein interacting with the rest of the phage, whereas regions with less homology would be specific for binding to the receptor. No clear domain could be delineated in these sequences, confirming the idea that the RBP of the T5-related phages would fold as a unique domain. Earlier studies suggested that pb5 is located at the top of the straight T5 fibre nearby the baseplate [43]. Yet, recent electron microscopy observations indicate that pb5 is most likely located at the very tip of the straight fibre (Zivanovic et al., in preparation). This raises the question of which of the other proteins of the straight fibre interact with pb5 and how they contribute in transmitting the conformational changes through the phage tail and potentially in DNA transport. This information would not only be of importance for understanding the atypical mechanism of transport of phage T5 DNA, but could also be relevant for phages belonging to the same family.

Acknowledgements

We thank L. Letellier, J.-L. Popot, B. Miroux and C. Ebel for support during the course of this work, D. Picot for stimulating discussions, N. Thielens for help with the SPR measurements and D. Housse for help with the Fitx fitting program. L. Letellier and C. Ebel are acknowledged for a critical reading of the manuscript. We acknowledge access to the Soleil Synchrotron and to the Biacore Platform of the Partnership for Structural Biology (PSB, Grenoble). The research leading to these results has received funding from the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement n°211800.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biochi.2012.05.021.

References


