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The C-terminus of the P22 tailspike protein acts as an independent oligomerization domain for monomeric proteins

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Abstract

TSP (P22 tailspike protein) is a well-established model system for studying the folding and assembly of oligomeric proteins, and previous studies have documented both *in vivo* and *in vitro* folding intermediates using this protein. Especially important is the C-terminus of TSP, which plays a critical role in the assembly and maturation of the protrimer intermediate to its final trimeric form. In the present study, we show that by grafting the C-terminus of TSP on to the monomeric MBP (maltose-binding protein), the resulting chimaera (MBP-537) is a trimeric protein. Moreover, Western blot studies (using an anti-TSP antibody) indicate that the TSP C-terminus in the MBP-537 chimaera has the same conformation as the native TSP. The oligomerization of the MBP-537 chimaera appears to involve hydrophobic interactions and a refolding sequence, both of which are analogous to the native TSP. These results underscore the importance of the TSP C-terminus in the assembly of the mature trimer and demonstrate its potential utility as a model to study the folding and assembly of the TSP C-terminus in isolation.

Keywords

P22 tailspike; protein assembly; protein folding; viral adhesion protein

INTRODUCTION

TSP (P22 tailspike protein) is the adhesion protein for the P22 bacteriophage. Like most viral fibre adhesion proteins, TSP contains three structural elements: an N-terminal head-binding domain, a central β -helix and a C-terminus (Figure 1A) [1]. The C-terminus, which is formed by association of β -sheets from the three monomers in a prism-like structure [1] (Figure 1B), is required for TSP stability and assembly [2]. The interior of this β -prism is dominated by hydrophobic residues, which are critical to the formation of the C-terminus [3]. The *in vitro* folding and assembly pathways of the TSP trimer have been well-characterized [4] (Figure 1C). The initial folding step involves the formation of the β -helix domain and results in a stable monomeric intermediate [5]. Hydrophobic interactions in the

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C-terminus promote monomeric assembly into a dimeric and subsequently an immature trimeric protein known as the protrimer [3]. The protrimer undergoes a structural rearrangement, involving specific ionic interactions, to form the final mature trimer [6,7].

The TSP C-terminus performs two critical functions in the assembly of the mature TSP trimer. First, truncation of the C-terminus inhibits trimer formation [3,5], providing evidence for its involvement in the trimerization process. Alternatively, truncation of the N-terminus does not affect trimerization or protein stability [8]. Second, the three polypeptide chains intertwine between amino acids 541 and 567 in the C-terminus to form a ‘molecular clamp’. This clamp is critical for trimer maturation and significantly increases the stability of the mature protein over folding intermediates [7]. The only known mutations that destabilize the protein while allowing trimer formation are located in this region [6]. These results suggest that the C-terminus acts as an independent oligomerization domain for TSP. It follows that attachment of this domain to a naturally monomeric protein should also lead to oligomerization and the chimaeric protein should follow a similar assembly pathway to TSP.

In the present study, we tested this hypothesis by fusing the C-terminus of TSP to MBP (maltose-binding protein). MBP is a monomeric 370 residue protein involved in the uptake and catabolism of maltodextrins in *Escherichia coli* [9]. MBP was chosen as the TSP C-terminus fusion partner because it is well-characterized, can be conveniently and robustly expressed, and has clearly defined folding kinetics and stability [10]. When the TSP C-terminus was attached to the monomeric MBP, the resulting chimaera (MBP-537) formed a trimer analogous to TSP. Results from Western blots further revealed that the TSP C-terminus expressed in the chimaera had the same conformation as in the native TSP. Refolding experiments suggested that the MBP-537 chimaera followed a similar assembly sequence to the native TSP. Collectively, these results underscore the importance of the C-terminus in TSP assembly and they demonstrate the utility of this chimaera for studying the role of the TSP C-terminus in formation of the TSP trimer.

MATERIALS AND METHODS

Materials

MBP vector and restriction enzymes were obtained from New England Biolabs. Primers used for cloning and mutagenesis reactions were purchased from Integrated DNA Technologies. *PfuUltra* DNA polymerase and nucleotides were obtained from Stratagene. All other chemicals were obtained from Sigma unless otherwise indicated.

MBP-537 cloning

The *TSP* gene sequence encoding amino acids 537–666 was amplified by PCR using *PfuUltra* polymerase. The forward primer sequence was ATTAAGAATTCATGTTGCTAATTT-GGCAGAAGAAGGG and contained an EcoRI restriction site. The reverse primer sequence was ATGGACAAGCTTGCTCAA-AGTGTGCGCAAGGATAATC and contained a HindIII restriction site. The resulting PCR product and the pMal-c2g vector were both digested with HindIII and EcoRI, and the PCR product was ligated into the vector using T4 ligase. The ligated product was transformed

into DH5 α cells, plated on LB [11]+ Amp (Luria– Bertani broth plus 100 μ g/ml ampicillin) plates and grown overnight. Colonies were isolated from the plated transformation mixture and grown overnight in LB + Amp. Plasmids were isolated from each colony. The purified plasmids were sequenced to verify that the native sequence had been cloned.

Generation of MBP-537 mutants

Mutant MBP-537 genes were generated by PCR amplification as described above using mutant TSP genes from a previous study [3] as templates.

Protein expression

Chemically competent *E. coli* BL21(DE3) cells (Novagen) were transformed with the pMal-c2g plasmid containing the appropriate gene and selected for on LB + Amp plates.

Individual colonies were grown overnight in LB + Amp medium. The overnight starter culture was used to inoculate a 1.5 litre culture that was grown to a $D_{600} \sim 0.6$ at 37 °C and induced using 1 mM IPTG (isopropyl β -D-thiogalactoside) at 30 °C for 24 h. Cells were harvested by centrifugation (4200 g for 30 min at 4 °C) and resuspended in lysis buffer (50 mM Tris, 5 mM MgCl₂, 0.1 % Triton X-100, 0.1 mg/ml lysozyme and 0.1 mg/ml DNase, pH 7.4). After two freeze/ thaw cycles (–80 °C/20 °C), the cell debris was removed by centrifugation at 12000 g for 20 min. Both the pellet and the supernatant from samples for SDS/PAGE and non-denaturing PAGE analyses were collected and the pellet was resuspended in a volume of lysis buffer equivalent to the supernatant prior to analysis. Only the supernatant was collected from large-scale expressions for purification.

Protein purification

The chimaeric MBP-537 protein was purified using amylose resin (New England Biolabs). Cell lysate was loaded using binding buffer (20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol) and the column was washed to remove unbound protein. Chimaeric protein was eluted using binding buffer containing 10 mM maltose. Eluted protein was > 90% pure as judged by Coomassie Blue staining. Eluted protein was concentrated and further purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare). The final protein was > 98% pure by both silver and Coomassie Blue staining. The final protein concentration was determined by measuring the D_{280} ($D_{280} = 0.736$ mg/ml MBP-537).

Genenase™ I digestion

Purified MBP-537 was diluted to 0.5 mg/ml in binding buffer and incubated overnight at 20°C with 1 μ g of Genenase™ I. Cleavage efficiency was determined by SDS/PAGE.

Electrophoresis, staining and Western blotting

Non-denaturing PAGE was performed at 4°C, and SDS/PAGE was performed at room temperature (25°C). For initial characterization of the MBP-537 chimaera, 10% acrylamide resolving gels were used for both non-denaturing PAGE and SDS/PAGE. Non-denaturing 7% acrylamide resolving gels were used for refolding experiments, with the exception of the gels used for the Ferguson plot analysis. In all cases, the stacking portion of the gel was 4.3

% acrylamide. Silver staining was performed as described by Sather and King [12]. Western blotting was performed by transferring samples from non-denaturing and SDS polyacrylamide gels on to nitrocellulose overnight at 4°C. The nitrocellulose was blocked using a 5% (w/v) non-fat skimmed milk solution and then incubated with either anti-TSP or anti-MBP antibody (NEB) for 1 h. The blot was washed and then treated with an anti-mouse secondary antibody for an additional 1 h. The blot was then washed and treated with ECL[®] (enhanced chemiluminescence) plus (GE Healthcare) developing reagent and then imaged using an Alpha-Innotech gel imaging system.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed on a Beckman-Coulter Proteome Lab XL-A analytical ultracentrifuge. Sedimentation equilibrium experiments were performed on a Beckman-Coulter Model XL-I analytical ultracentrifuge. All experiments were performed at 20°C. Sedimentation velocity experiments were performed with a rotor speed of 45000 rev./min over a protein concentration range of 4–16 µM. Typically, 35–40 scans were collected for each sample per experiment and analysed with SEDFIT using a partial specific volume of 0.73 cm³/g based on the predicted prolate ellipsoid geometry. The solvent density and viscosity values used for the fits were 1.004 g/ml and 0.0102 P respectively as determined experimentally. Sedimentation equilibrium experiments were conducted at 8000 and 10000 rev./min for protein concentrations of 4, 8 and 16 µM. Samples were equilibrated at each speed for 18 h with scans collected every 6 h to ensure that equilibrium was reached at each speed. The 18 h scans were analysed with SEDPHAT using a partial specific volume of 0.73 cm³/g.

Protein refolding

Chimaeric MBP-537 or wild-type tailspike protein was denatured on ice in 8 M urea and 50 mM Tris, pH 3.0, for 1 h at a concentration of 1 mg/ml. Refolding was initiated by dilution of the denatured protein into refolding buffer (50 mM Tris, pH 7.6 and 1 mM EDTA) to the final experimental concentration. Samples were removed at various time points and quenched on ice in 3× non-denaturing sample buffer (15 mM Tris, pH 7.4, 120 mM glycine, 30% glycerol and Bromophenol Blue). It has been previously demonstrated that intermediates in the tailspike refolding pathway can be trapped and resolved by quenching the samples on ice and subjecting them to non-denaturing gel electrophoresis at 4°C [13]. Samples were separated on a 7% non-denaturing PAGE gel and silver-stained.

Ferguson plot analysis

Purified MBP-537 was denatured and refolded as described above. Refolding samples were separated on 7, 8, 9 and 11% non-denaturing polyacrylamide gels. Gels were run overnight at 4°C, silver stained and imaged using an Alpha-Innotech gel imaging system. The mobility of each species was calculated by dividing the distance from the top of the gel to the top of each band by the distance travelled by the dye front. The log of this value was plotted against acrylamide percentage and fit by linear extrapolation to determine the slope and y-intercept for each species (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/419/bj4190595add.htm>).

RESULTS AND DISCUSSION

Fusion of TSP C-terminus to MBP results in the formation of an oligomeric species

The TSP C-terminus is critical for oligomerization and stability of the TSP trimer [3,7], suggesting that this region acts as the oligomerization domain for TSP. To test this hypothesis, a chimaeric protein (MBP-537) was created by fusing the TSP C-terminus (amino acids 537–666) on to the C-terminus of the MBP gene. The chimaeric protein migrated to a position consistent with its predicted molecular mass via SDS/PAGE (Figure 2A, lane 2). Furthermore, digestion of the MBP-537 chimaera with Genenase™ I resulted in two faster migrating proteins, MBP and the cleaved 537–666 TSP fragment (Figure 2A, lane 3). The 537–666 fragment reproducibly exhibits a weaker intensity than the MBP fragment. The lower intensity of the 537–666 fragment may result from loss of protein due to aggregation of this very hydrophobic region. As Genenase™ I is very specific, it is less likely that this is a non-specific proteolysis event.

The MBP-537 chimaera was next analysed by non-denaturing (native) PAGE to determine if fusion of the TSP C-terminus to MBP resulted in oligomerization of the MBP. The MBP-537 chimaera migrated to a similar position as TSP under non-denaturing conditions, consistent with the chimaera being in an oligomeric state. Upon Genenase™ I digestion of the MBP-537 chimaera, a band is observed at a position comparable to monomeric MBP (Figure 2B). The 537–666 fragment of TSP was not observed in the digested sample by native PAGE. There are two potential explanations for this observation. First, the protein we observe as a lower-molecular-mass species on SDS/PAGE may be highly aggregate-prone, particularly under non-denaturing conditions. Second, the 537–666 region of TSP is highly negatively charged and this charge could alter the migration of the fragment in the native gel. It is currently not possible to distinguish between these two alternatives.

A useful feature of TSP in characterizing folding intermediates is its resistance to SDS denaturation in the absence of heat. As shown in Figure 2(A), TSP migrated as a trimer even in the presence of SDS (lane 1). However, resistance to SDS denaturation was not conferred on to the MBP-537 chimaera by the presence of the C-terminus. The MBP-537 chimaera migrated in the SDS/PAGE gels as a monomer, even when samples were not heated prior to gel loading (Figure 2A). This suggests that other regions of the protein in addition to the C-terminus are critical to SDS-resistance: the TSP C-terminus alone is not sufficient to confer SDS-resistance to a protein.

MBP-537 chimaera forms a trimer

To confirm the identity of the higher-molecular-mass band on SDS/PAGE, size-exclusion chromatography and analytical centrifugation were utilized. Size-exclusion chromatography demonstrated that the MBP-537 chimaera is oligomeric (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/419/bj4190595add.htm>), but was not able to provide an accurate molecular mass. Analytical ultracentrifugation is a very reliable technique for estimating molecular mass, and the results were consistent using both sedimentation velocity and sedimentation equilibrium. Raw sedimentation velocity data (Figure 3A) suggested a monodisperse species with a sedimentation coefficient of 7.25 ± 0.003 S. Fitting this data to

a model of a prolate ellipsoid geometry, modelled after the TSP shape, yielded a predicted molecular mass of 165.7 kDa. This molecular mass was consistent with the model of a homotrimer where each monomer had a molecular mass of 55.2 kDa. The experimentally determined monomeric mass was in close agreement to the predicted molecular mass of the monomeric chimaera (56.7 kDa).

Sedimentation equilibria were used to confirm the molecular mass as well to examine the potential for a monomer–trimer equilibrium suggested by a small peak on the velocity experiments at 2.75 S (Figure 3A). The raw data from the sedimentation equilibrium experiments were fitted to a non-associated single species model (Figure 3B). Analysis yielded a molecular mass for the species of 170 kDa, which is in complete agreement with the predicted molecular mass of the chimaeric trimer. Fitting the data to a monomer–trimer equilibrium, however, did not improve the fit of the data (results not shown), indicating that if such an equilibrium exists, it heavily favours the trimeric form of the protein.

TSP C-terminus in MBP-537 chimaera assumes a similar structure as native TSP

The above experiment indicated that the MBP-537 chimaera is trimeric, as in the native TSP, but it does not follow that the chimaera C-terminus has the same conformation. A variety of antibodies exist for various refolding states of TSP. One anti-TSP antibody, mAB155, has been shown to react with later-stage intermediates in the folding pathway and is considered to be a reliable indicator of the native tailspike trimer structure [14]. The mAB155 antibody will only bind to the MBP-537 chimaera if it has a structure similar to the native TSP.

Purified MBP-537 chimaera and MBP-537 chimaera digested with Genenase™ I were separated on both SDS/PAGE and non-denaturing PAGE gels and transferred on to nitrocellulose for Western blotting with both anti-MBP and anti-TSP antibodies. Binding of the anti-MBP antibody was observed on both the digested and undigested MBP-537 chimaera in both the SDS and the non-denaturing PAGE gels (Figure 4). The anti-TSP antibody did not bind to the MBP-537 chimaera in proteins separated by SDS/PAGE (Figure 4A). This was expected because the MBP-537 chimaera was monomeric under SDS/PAGE conditions.

In the non-denaturing PAGE gel, the anti-TSP antibody bound to the undigested MBP-537 chimaera (Figure 4B). The epitope formed by the MBP-537 chimaera must be in a conformation that is nearly identical to the epitope in the native TSP in order for the anti-TSP antibody to bind. Although the specific epitope for the anti-TSP antibody is unknown, it probably includes contributions from more than one chain because this antibody binds to the native TSP but not to nascent TSP chains that are still attached to the ribosome, even though the C-terminus is exposed [14a]. Hence, not only does the C-terminus induce an oligomeric form of MBP, it also appears to form a structure similar to the native TSP, making it a potentially ideal system for studying the assembly of the C-terminus in isolation from the rest of the tailspike protein.

Mutation of the hydrophobic domain of TSP inhibits oligomerization

Insertion of either polar or charged residues in the C-terminus has been shown to interfere with hydrophobic core interactions and thereby disrupt oligomerization of TSP [3]. Therefore, a similar disruption should occur in chimaeric MBP-537 if oligomerization occurs by the same mechanism. To test this hypothesis, a series of mutations were generated in the MBP-537 protein at Leu⁶⁰⁶ (TSP numbering system), which is in the centre of the hydrophobic core, to test this hypothesis. Residue 606 has been shown previously to be sensitive to mutation to either polar or charged residues [3]. A representative series of the mutations from a previous study [3], including non-polar, polar and charged substitutions, were investigated.

Our findings were consistent with previously published results [3] (Figure 5). The leucine to valine substitution in the chimaera was the only modified protein that exhibited wild-type expression levels on SDS/PAGE gels (Figure 5A). MBP- β Gal (β -galactosidase) was obtained from expression of the empty pMal-c2g plasmid. This plasmid contains a β Gal gene fused C-terminally to the MBP gene for screening purposes, resulting in an MBP- β Gal fusion protein when expressed.

Western blot results with the anti-MBP antibody revealed weak expression levels for all the clones, which were not as apparent from the Coomassie Blue-stained gel (Figure 5B). Substitution of Leu⁶⁰⁶ with either polar or charged residues resulted in decreased protein expression. We hypothesize that by disrupting the hydrophobic core, the chimaeric protein is more sensitive to proteolysis, resulting in the lower protein yields. We observed an increased number of lower-molecular-mass species that were reactive with the anti-MBP antibody in the mutant chimaera variant expressions, consistent with this hypothesis.

The L606V variant was the only variant visible by Coomassie Blue staining on native PAGE gels (Figure 5C), consistent with previous work [3]. Finally, the L606V variant was the only modified protein that reacted with either the anti-TSP (Figure 5D) or anti-MBP (Figure 5E) antibodies. In summary, the conserved leucine to valine mutation was the only modification to Leu⁶⁰⁶ that did not affect either protein expression levels or the ability of the MBP-537 chimaera to oligomerize. These results further demonstrate that the MBP-537 chimaera assembles in a similar manner to TSP and that assembly is driven by formation of a hydrophobic core.

MBP-537 chimaera refolds following a similar pathway as TSP

If the C-terminus of TSP acts as an independent oligomerization domain, the MBP-537 chimaera should exhibit a refolding behaviour similar to the native TSP. The chimaera would first form a monomer, then a dimer and finally a trimer during refolding. To test this theory, purified MBP-537 protein was refolded using a standard TSP refolding protocol (Figure 6A). The initial refolding sample (zero time) comprises three species, two lower in molecular mass than the native MBP-537 chimaera (presumably monomeric and dimeric MBP-537) and one higher. The higher-molecular-mass species was similar to a high-molecular-mass species detected during native TSP refolding, shown to be an immature (protimer) form of the TSP. The concentration of the higher-molecular-mass species

decreased with the concurrent appearance of a new species after 8 min, which has been identified as the folded MBP-537 chimaera due to its position on the gel relative to the purified MBP-537 chimaera. The concentration of the second species continued to increase throughout the experiment. The progression of intermediates was nearly identical to that observed during refolding of the native TSP, suggesting that the MBP-537 trimer follows essentially the same assembly pathway as the native TSP.

Interestingly, the behaviour of the higher-molecular-mass species was similar to the protimer seen in native tailspike refolding experiments. To gain insight into this slowly migrating species, refolding conditions were altered to populate intermediates along the refolding pathway. Initial identification of the TSP protimer was made by trapping the protimer species at 4°C and then warming the sample to 30°C, which matured this species to the final trimer [15]. Thus, if the high-molecular-mass species represents a protimer-like intermediate in the folding pathway, it should accumulate without any formation of native MBP-537 when refolding is initiated on ice.

Refolding of the MBP-537 chimaera was initiated on ice for 4 h then examined using non-denaturing electrophoresis. The predominant MBP-537 folding intermediate was the monomer, in contrast to TSP, which predominately populates the dimer and protimer species (Figure 6B). This is consistent with our other refolding results that showed monomer and dimer species that are much longer lived than in native TSP refolding, suggesting that the refolding kinetics are slower for the chimaera. There are several possible explanations for this observation but insufficient data to explain the difference. Regardless of the difference in kinetics, there is clearly an accumulation of the slowly migrating species and no formation of mature MBP-537 trimer, similar to that observed for TSP. Thus, the higher-molecular-mass species may represent a protimer-like intermediate along the folding pathway. The formation of the native MBP-537 may require crossing an energy barrier, similar to the barrier required for formation of the native tailspike.

A Ferguson plot analysis was conducted on refolding samples to further characterize the species formed during refolding [16]. Refolding samples were prepared and separated on 7, 8, 9 and 11% non-denaturing acrylamide gels. The log of the retention factors for each species was plotted against the percentage of acrylamide (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/419/bj4190595add.htm>). The data for each species were fitted by linear extrapolation; the slopes, intercepts and correlation coefficients are shown in Table 1. For all four species, the linear fit had an $R^2 > 0.99$, indicating that the data could accurately be extrapolated to 0% acrylamide.

As shown in Table 1, the slope decreased and the y-intercept increased for each species from the bottom to the top of the gel. These changes are consistent with an increasing hydrodynamic radius and an increasing negative charge on the protein. Exceptions are the presumed trimer and protimer species; the protimer has a larger slope, but the two proteins have identical y-intercepts, consistent with two species that have identical charges but different hydrodynamic radii. Ferguson plot analysis on TSP refolding intermediates exhibits a similar pattern of intercepts and slopes [17]. Collectively, these data provide

strong evidence that the higher-molecular-mass species in chimaera refolding is a protimer-like intermediate.

Perhaps the most surprising result of this study is that the MBP-537 chimaera formed an immature trimer species that underwent a similar maturation as TSP during refolding. The MBP-537 chimaera contains the amino acids (540–580) that have been identified as important to TSP refolding [6,7]. The maturation process leading to the formation of the final, mature trimer involves a structural rearrangement that results in a change in exposed amino acids on the surface of the protein [17]. Work to date has shown that proper association and orientation of the three subunits in the protimer is critical, allowing the final conformational changes associated with maturation to occur. That similar conformational changes were observed in the MBP-537 chimaera indicates that all the elements needed for maturation are also present in the C-terminus of TSP. It also further supports the conclusion that the C-terminal region attached to the MBP is in a similar structure as in TSP. The maturation of the MBP-537 trimer must have a similar energy barrier associated with the maturation reaction as TSP. Like tailspike, it is possible to assemble the protein through the protimer stage but not to generate mature MBP-537 at 0°C. This indicates that a certain amount of energy is required to complete the maturation process. As mentioned previously, the transition in tailspike is thought to involve breaking of bonds in conjunction with the formation of new bonds, giving the process a high energy of activation [18,19]. The maturation barrier in MBP-537 suggests that the same process occurs during maturation of the chimaeric trimer.

Concluding remarks

The present study demonstrates that the C-terminus of the P22 tailspike protein is an independent oligomerization domain that can induce oligomerization in monomeric proteins. The present results show that this domain has the same conformation as in the native TSP, as determined by Western blotting and analytical ultracentrifugation. MBP-537 also probably undergoes a similar conformational maturation as the native TSP, as shown by the refolding experiments.

Based on these findings, there is potential for future studies on the TSP C-terminus using this chimaera. Because the C-terminus of other viral adhesion proteins, such as the T4 fibrin C-terminus [20,21], have also been shown to act as independent oligomerization domains, we speculate that the C-terminus of viral adhesion proteins may be exchanged between different adhesion proteins without losing functionality. If this hypothesis is true, all adhesion proteins may share a common evolutionary ancestor. Accordingly, the C-termini of the HK620 bacteriophage and *Shigella* phage Sf6 spike proteins contain sequence motifs that are conserved with TSP [22], further supporting this hypothesis. The C-terminus in the Sf6 tailspike protein is known to result in increased stability of the Sf6 tailspike protein [23]. The structure of the Sf6 tailspike protein was recently published [24], providing a second tailspike protein structure to compare. Future studies will include exchanging the C-termini of TSP and Sf6 in order to determine the effects on functionality and stability. Furthermore, this could be expanded as additional structures of viral adhesion proteins

become available. With this additional information, it will be possible to elucidate the evolutionary relationship between adhesion proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

βGal	β-galactosidase
MBP	maltose-binding protein
TSP	P22 tailspike protein

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Figure 1. Structure and folding pathway of TSP

(A) Ribbon diagram of the structure of TSP. The four main structural features are indicated on the structure. (B) Ribbon diagram of the C-terminus, orientated to view the structure down the long axis of the protein from the β -helix domain to the end of the protein. Sheets D and E are indicated with arrows. (C) *In vitro* folding and aggregation pathway of the TSP. Unfolded monomer folds and forms either aggregate-prone monomer (bottom) or folding competent monomer (top). Adapted from [3] with permission © (2003) Wiley.

Figure 2. Comparison of the electrophoretic mobility of MBP-537 chimaera compared to native TSP

Purified protein (0.5 $\mu\text{g/ml}$) was separated by SDS/PAGE (A) or non-denaturing PAGE (B) and stained with Coomassie Blue. Lane 1, TSP; lane 2, purified MBP-537; lane 3, MBP-537 digested overnight with GenenaseTM I. Lane 4 in (A) is the molecular mass markers, with values in kDa to the right. All lanes were from the same gels and were grouped together for publication purposes. Dividing lines indicate where each gel was merged.

Figure 3. Sedimentation velocity and sedimentation equilibrium analysis of the MBP-537 chimaera

(A) Raw sedimentation velocity data (grey line) fitted to a monodisperse species (black line). The calculated (SEDFIT) sedimentation coefficient is 7.25, which is consistent with a species having a molecular mass of 165.7 kDa. (B) Equilibrium sedimentation data (circles, 10 000 rev./min; diamonds, 8000 rev./min) were analysed with SEDPHAT using a model for monodisperse protein, which gives a calculated species molecular mass of 170 kDa. The

fit of the data is shown as the lines through the points, with residuals plotted below the graphs.

Figure 4. Comparison of antibody reactivity of the chimaeric MBP-537 with antibody reactivity of the TSP and monomeric MBP

Purified protein (0.1 $\mu\text{g/ml}$) was separated by SDS/PAGE (**A**) or non-denaturing PAGE (**B**) and transferred on to nitrocellulose for Western blotting. Lane 1, TSP; lane 2, purified MBP-537; lane 3, MBP-537 chimaera that has been digested overnight with GenenaseTM I to separate the MBP from the TSP C-terminus. The blots on the left were incubated with anti-MBP antibody (NEB). The blots on the right were incubated with the mAB155 (anti-TSP)

antibody. All lanes were from the same blots and were grouped together for publication purposes. Dividing lines indicate where each blot was merged.

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Figure 5. SDS/PAGE and Western blot analysis of mutated MBP-537 chimaeras

Whole-cell lysates from expressions of MBP-537 chimaeras containing various mutations at Leu⁶⁰⁶ (TSP numbering) were analysed using both SDS/PAGE and native PAGE. **(A)** Coomassie Blue-stained SDS/PAGE gel of whole-cell lysates. **(B)** Western blot of SDS/PAGE-separated lysates treated with an anti-MBP antibody. **(C)** Coomassie Blue-stained native gel of whole-cell lysates. **(D)** Western blot of native PAGE-separated lysates treated with an anti-TSP antibody. **(E)** Western blot of native PAGE-separated lysates treated with an anti-MBP antibody. Lane 1 of each gel contains purified TSP and lane 2 contains purified

monomeric MBP. The remaining lanes contain the soluble portion (S) and pellet (P) from each lysate. Lanes 3 and 4 contain the soluble and pellet fractions from an expression of the empty pMal plasmid, which expresses a MBP- β Gal fusion protein. Lanes 5 and 6 contain the soluble and pellet fractions from expression of the native MBP-537 chimaera. The soluble and pellet fractions from expressions of individual mutant MBP-537 chimaeras were separated in lanes 7–18, with the individual mutation indicated over each pair of lanes.

Figure 6. Analysis of MBP-537 refolding

(A) Denatured MBP-537 and TSP were refolded at 100 $\mu\text{g/ml}$ and 20°C. Samples were collected, quenched on ice and analysed by non-denaturing PAGE. The time each sample was collected is shown between the two gels. The various refolding species are indicated on the right side of each gel. A similar progression from monomer to dimer to the final form is seen with both proteins. Interestingly, an early intermediate similar to the TSP protrimer is seen in the MBP-537 sample. (B) TSP and MBP-537 protein were refolded at 100 $\mu\text{g/ml}$ and 0°C for 4 h. The samples were separated on a native gel and the gel was silver-stained. Lane 1, refolded TSP; lane 2, refolded MBP-537; lane 3, blank; lane 4, purified MBP-537; lane 5, purified TSP. TSP primarily forms a dimer and protrimer, whereas the MBP-537 chimaera forms primarily monomer. TSP dimer and mature trimer migrate very similarly on 7 % native gels due to similar Stokes radii [25].

Table 1

Linear extrapolation values for Ferguson plot analysis

Species	Slope	y-intercept	R^2
Monomer	-0.0723	0.1676	0.9964
Dimer	-0.0956	0.2083	0.9981
Trimer	-0.1279	0.346	0.997
Protrimer (?)	-0.1356	0.339	0.9998