Characterisation of the meningococcal transferrin binding protein complex by photon correlation spectroscopy

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Abstract Photon correlation spectroscopy demonstrated for the first time that co-purified meningococcal TbpA+B form a complex in solution. This structure bound hTf and the resultant species underwent partial dissociation after exposure to additional hTf or following prolonged incubation. Purified TbpA and TbpB had similar apparent sizes but showed distinctive size profiles suggesting that TbpA forms a largely homogeneous population while TbpB may produce more variable particle sizes under these conditions.

Key words: Transferrin; *Neisseria meningitidis*; Photon correlation spectroscopy

1. Introduction

All species of pathogenic *Neisseria* express two structurally distinct transferrin binding proteins now known as TbpA (100 kDa) and TbpB (65–85 kDa dependant on strain) [1]. Both proteins specifically bind human serum transferrin (hTf, 80 kDa) and while TbpA and TbpB bind hTf independently both are essential for optimal iron uptake from this source [2–5]. The suggested structural distinctiveness of TbpA and TbpB, coupled with their co-expression, shared ligand specificity and mutual contribution to bacterial virulence suggests that these proteins act in tandem, together forming the functional hTf receptor [6–8]. Despite the convincing and growing body of indirect evidence suggesting this association, as yet there has been no direct demonstration of a TbpA+B complex. We have used photon correlation spectroscopy (PCS) to characterise TbpA+B, co-purified from *Neisseria meningitidis* and consequently offer novel direct demonstration that these proteins interact in vitro.

Photon correlation spectroscopy is a particle sizing technique in which light from a continuous, visible laser beam is directed through an ensemble of macromolecules or particles in suspension and moving under Brownian motion. Some of the coherent laser light is scattered by these particles and is collected by a lens, typically set at 90° to the incident beam. This reflected light is then detected by a photon counting photomultiplier (PMT) which generates a digitised signal proportional to the intensity of the detected light. As the particles move relative to each other, the light they collectively scatter changes in phase, and therefore in intensity at a rate proportional to the speed at which the particles are moving, specifically their diffusion coefficient (D_t). This, in turn, can be inversely related to their size or hydrodynamic sphere equivalent radius (r_h) via the Stokes-Einstein equation:

$$D_t = K_B T/6\pi\eta r_h.$$

K_B is the Boltzmann constant, T the temperature and η the solvent viscosity.

The characteristic time-scale of intensity fluctuation is extracted from the detector signal by autocorrelation, which generates an exponential curve, the rate of decay of which allows the determination of mean D_t. Through detailed analysis of its multi-exponentiality, a particle size distribution can be constructed representing the species in solution [9,10].

2. Materials and methods

2.1. Bacterial strains and growth conditions

Group B *N. meningitidis* strains SD [11] and B16B6 were inoculated onto agar plates containing 5% (v/v) horse blood and grown overnight at 37°C in an atmosphere of 5% CO_2. Colonies from these plates were used to inoculate 100 ml of Mueller Hinton broth (MHB) in a 250 ml conical flask which was incubated with shaking at 37°C for 6 h. 5 ml of this culture was introduced into 750 ml MHB in 2 l conical flasks, made iron-limited by the addition of ethylendiamine-*N,N*-diacetic acid. (EDDA, 28 μM), and incubated with shaking at 37°C for 18 h.

2.2. Co-purification of TbpA+B

These proteins were solubilised from iron-limited *N. meningitidis* whole cells using Elugent detergent (Calbiochem) and hTf Sepharose affinity chromatography [12]. Extracts were prepared from strains SD and B16B6 which express high and low molecular mass forms of TbpB (85 kDa and 65 kDa respectively). Separate, functionally active TbpA and TbpB were prepared by chromatofocusing according to the published method [13]. The purity of TbpA+B in each preparation was assessed by SDS-PAGE [14] and hTf binding capacity was confirmed by dot blotting, probed using hTf horse-radish peroxidase conjugate (hTf-HRP).

2.3. hTf extraction

A crude protein extract was prepared by ammonium sulphate precipitation of human serum. hTf was purified by gel filtration using a Superose 12 HR column (Pharmacia Biotechnology) and subsequent ion-exchange chromatography involving a Q Sepharose FF HiLoad 16/10 column (Pharmacia Biotechnology) on FPLC. The purity and iron binding capacity of the final protein extract were determined by SDS-PAGE and electrophoresis in the presence of 6 M urea [15]. hTf treated in this way was used in the affinity purification of TbpA+B described earlier.

2.4. Photon correlation spectroscopy

300 μl samples of co-purified TbpA+B, individual TbpA, TbpB and iron free (apo) hTf were prepared in phosphate buffered saline (PBS) pH 7.4+2% (v/v) Elugent. A solution of apo hTf was also prepared in PBS. All solutions contained approx. 500 μg/ml protein as determined

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by spectrophotometric analysis using a GENEQUANT micro-spectrophotometer (Pharmacia Biotechnology). Samples were passed through a 0.2 μm filter prior to analysis in order to remove any dust, bacteria or other particulate matter from the solution. Samples were then placed in 1 cm light path cuvettes which were allowed to equilibrate at 25°C for 10 min in the index matching water bath within the PCS instrument (Malvern Instruments 4700_{CE} PCS system incorporating a variable angle light scattering spectrometer/goniometer). Each sample was then analysed using a monochromatic, vertically polarised laser beam at 488 nm (Spectra-Physics 3 W Argon Ion Laser, Model 2020 operated under constant power mode). The PMT detector was set at 90° to the incident beam and light collected through a 100 μm aperture to define a suitable coherence area. The laser intensity (typically 300 mW) was adjusted for each sample type such that the mean count rate detected was close to the optimum of 150 kcounts/s. Sample times were selected automatically by the PCS instrument manufacturer's proprietary software (Automeasure Programme Ver 4.0, Malvern Instruments Ltd.). PCS measurements were automatically averaged from 10 separate data accumulation periods, each with a duration of 60 s. Data sets whose figures indicated the transitory presence of dust of other contaminating material (identified by excessively long time-scale correlation functions under the instrument setting described) were automatically rejected from the averaging process. From the data thus collected (a multi-exponential correlation function) a particle size distribution could be estimated using the Malvern Instruments Ltd. Proprietary deconvolution programme based on the method of cumulants (for full details of cumulants analysis see [10]). Because light scattering data is biased towards larger particles (intensity of light scattering varying with particle volume^2), results are expressed as intensity weighted particle size distributions.

To ensure consistency of results the above analyses were carried out in triplicate. Standard deviations and mean values were calculated using Microsoft Excel spreadsheet package (Version 5.0a Microsoft Corporation).

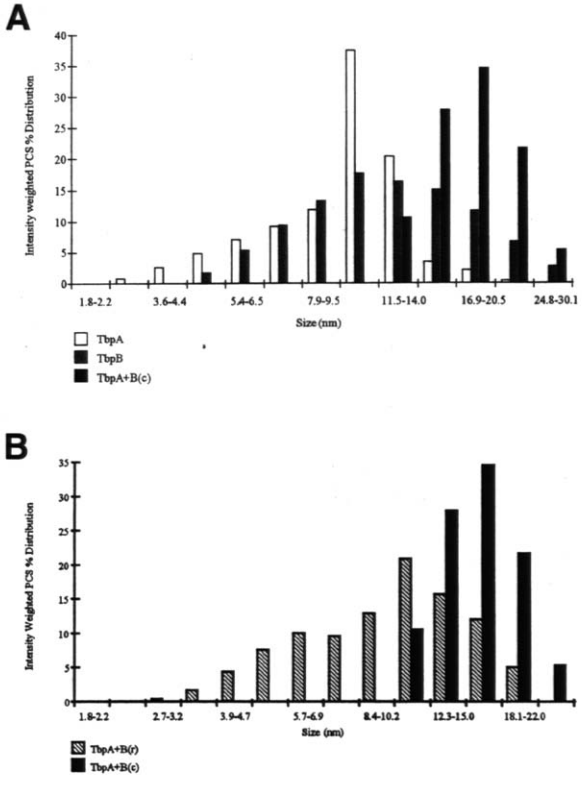

Fig. 1. A: Analysis of purified TbpA, TbpB and co-purified (c) TbpA+B by PCS. B: PCS analysis of co-purified (c) TbpA+B and recombined (r) TbpA and TbpB (1:1 molecular ratio).

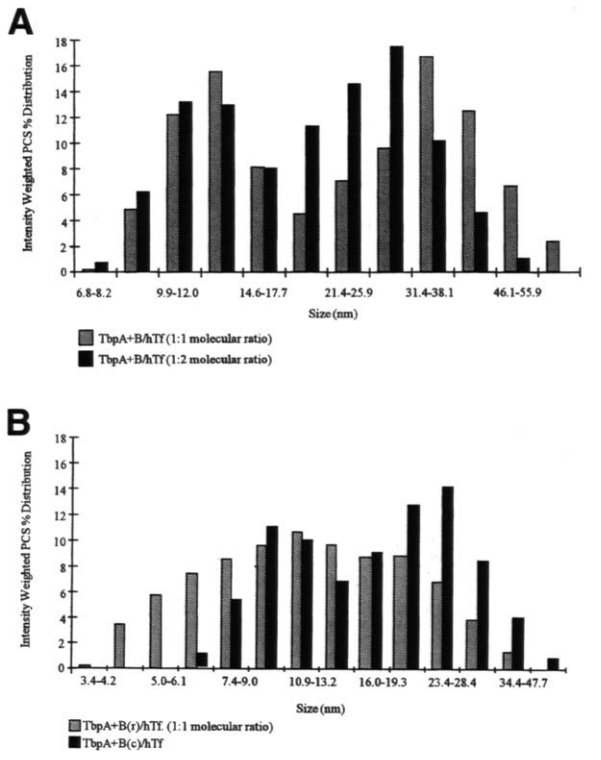

Fig. 2. A: PCS analysis of TbpA+B/hTf complex sizes at variable concentrations of hTf. B: Comparison of TbpA+B/hTf complex formation using co-purified (c) and recombined (r) TbpA+B.

3. Results

Results obtained from blank solutions containing only filtered PBS+2% (v/v) Elugent showed no characteristic particle size peaks even under laser intensities significantly higher than those used in the analysis of samples containing added protein (data not shown). While PCS does not require calibration, correct operation of the instrument was confirmed using 500 nm polystyrene microsphere standards (data not shown).

All protein samples were analysed under the same solvent conditions and because the experiment was designed only to determine comparative changes in molecular size and size distributions of the added protein, values for solvent R_i and viscosity (η) were assumed to be those of water. Given this internal consistency adjustments for the presence of solvent constituents such as detergent were unnecessary. In addition, previous analyses (these authors unpublished observations) have established that Elugent, used at the concentration described above does not prevent hTf binding by TbpA, TbpB or TbpA+B.

Particle size distributions were produced for individual TbpA and TbpB solutions, co-purified TbpA+B and separate Tbps recombined in an approx. 1:1 molecular ratio. Each sample was prepared in PBS+2% (v/v) Elugent in order to prevent protein precipitation. hTf was introduced to samples of co-purified TbpA+B, individual TbpA and TbpB and recombined Tbps, initially in an approx. 1:1 molecular ratio. On those occasions where complex formation could be demonstrated, the hTf/Tbp molecular ratio was increased to 2:1 in order to determine whether this effected the ultimate complex size. The hTf-Tbp binding event was also monitored over

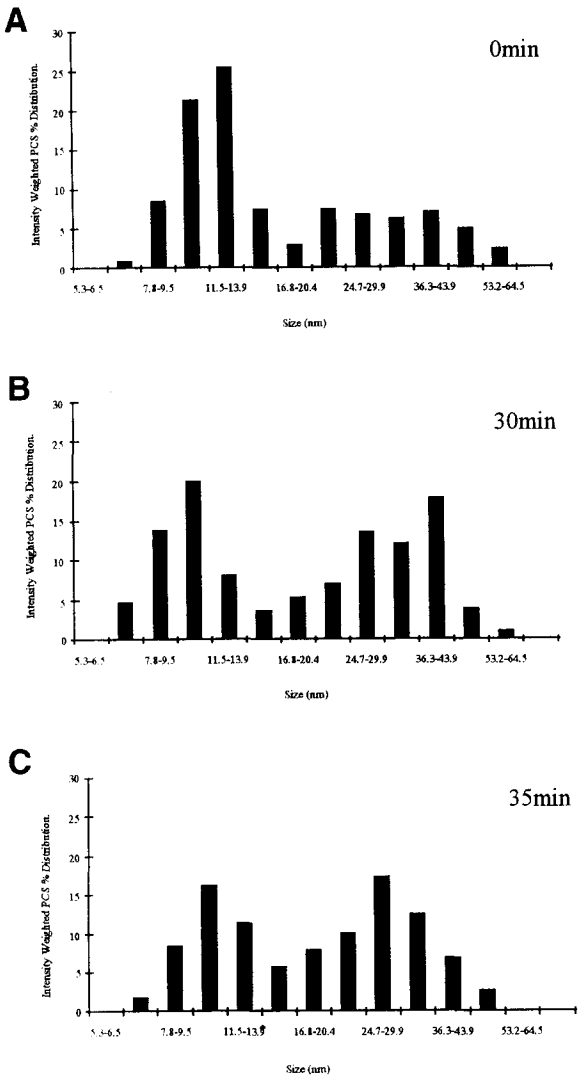

Fig. 3. Time course analysis of TbpA+B/hTf complex formation.

time, by analysing a sample of hTf and co-purified TbpA+B mixed with an approx. 1:1 molecular ratio. Data were collected at 5 min intervals over a period of 35 min.

Preliminary analyses were carried out using hTf in the iron free (apo) and iron saturated (holo) form. Under these conditions variable iron saturation was found to have no detectable effect on apparent particle size or on the formation of Tbp-hTf complexes. Furthermore, PCS failed to demonstrate heterogeneity in the molecular weight of TbpB expressed by meningococcal strains SD and B16B6. Consequently, the remaining analyses were carried out using apo hTf and Tbps purified from *N. meningitidis* strain B16B6.

Purified TbpA and TbpB each had an apparent particle size of 9.5–11.5 nm under these conditions (Fig. 1A). The trace produced by TbpA had a pronounced peak while TbpB produced a much broader profile of particle sizes. Co-purified TbpA+B exhibited an apparent size range of 11.5–30.1 nm reaching a maximum value at 16.9–20.5 nm. This indicated the presence of a species absent from, and significantly larger than those found in either sample of purified TbpA or TbpB. Recombination of TbpA and TbpB in a 1:1 molecular ratio produced a trace with a broad range of particle sizes similar to that shown by TbpB. The large peak, present in co-purified TbpA+B could not be demonstrated in this sample (Fig. 1B).

The addition of hTf to solutions of purified TbpA and TbpB produced no evidence of complex formation under these conditions, although both proteins bound hTf on dot blotting, in ELISA based studies (these authors, unpublished data) and in analyses using surface plasmon resonance biosensors (Bouton et al., in preparation). The addition of hTf to co-purified TbpA+B in a 1:1 molecular ratio produced a trace with one peak at 12.0–14.6 nm, and a second, larger peak indicating a predominant particle size of 31.4–38.1 nm (Fig. 2A). The minor peak spanned a range of values typical of hTf while the second was significantly larger than either TbpA, TbpB, hTf or co-purified TbpA+B. Increasing the concentration of hTf to a 2:1 molecular ratio consistently reduced the apparent size of this complex by approx. 10 nm but had no significant effect on the position or magnitude of the minor peak. Further increasing the level of hTf in the reaction mixture had no additional effect on the larger peak (data not shown) and the introduction of hTf to TbpA and TbpB, recombined in a 1:1 molecular ratio produced no evidence of complex formation (Fig. 2B).

Examining the Tbp-hTf binding event over time indicated the initial presence of a reflecting species of 9.5–11.5 nm (Fig. 3A). Subsequent analysis showed the progressive development of a large peak at 36.3–43.9 nm accompanied by a reduction in the original peak size (Fig. 3B). As the reaction proceeded there was a pronounced and consistent down-shift in the newly formed, large peak producing a final apparent complex size of 24.7–29.8 nm (Fig. 3C). No further changes in peak profile were noted after the 35 min experimental period. Throughout these analyses standard deviations from the mean value represented in each particle size category were consistently below 10%. As a consequence of this minimal variation and the large number of experimental replicates (30) these data have been omitted from all figures.

4. Discussion

The results of these analyses provide the first direct demonstration that co-purified meningococcal TbpA and TbpB associate in solution, forming a complex larger than either individual component. This complex bound hTf under these conditions, again producing a structure larger than purified TbpA, TbpB, co-purified TbpA+B or hTf. The addition of further hTf resulted in a consistent diminution in the size of this complex although the resultant species remained larger than any of the individual reactant proteins. This pronounced down-shift in apparent size inferred that the initial TbpA+B complex underwent partial dissociation in solution and the consistency of this change suggests the presence of a specific dissociation event. Consequently, we suggest that co-purified TbpA+B binds up to two molecules of hTf, consistent with the meningococcal hTf receptor being formed from two proteins each capable of independent ligand interaction. At higher concentrations of hTf, both accessible binding sites may be occupied, possibly resulting in stearic interference between the bound ligands causing dissociation of the receptor complex. Time course analysis of TbpA+B/hTf binding clearly demonstrated the progressive formation of a single large complex, accompanied by a reduction of intensity in the initial smaller species. The larger, putative TbpA+B/hTf complex also

underwent a similar down-shift in size following prolonged incubation with hTf, again inferring partial dissociation and consistent with previous observations.

Individual purified TbpA and TbpB had very similar predominant particle sizes but showed distinctive analytical profiles, possibly resulting from the suggested structural differences between these proteins [16,17]. The broad spectrum of sizes apparent in TbpB analysis, coupled with the absence of any significant peak may suggest that this protein forms a heterogeneous population in solution. It is implied that in vivo TbpB is anchored in the meningococcal outer membrane by lipid moieties and these may contribute to the inherent adhesiveness of this protein, possibly favouring non-specific aggregation [5]. The size profile produced by purified TbpA was dominated by a single peak, indicating that this protein forms a largely homogeneous light scattering population in solution. The predicted structure of TbpA suggests that it may be an integral membrane, porin-like molecule composed largely of anti-parallel β strands joined by loops exposed at the cell surface and in the periplasm [18]. Although PCS data are subject to the limitations discussed below, analysis of TbpA by this method confirms that this protein has sufficient size to span the lipid bilayer consistent with the proposed model [19].

Purified TbpA and TbpB, recombined in an approx. 1:1 molecular ratio did not demonstrate complex formation, either in the presence or absence of hTf. However, the functional activity of these proteins is not in question, having been confirmed using a variety of techniques in both the solid and fluid phase and in the presence of Elugent (Boulton et al., unpublished observations). In contrast with PCS, one interactant, either hTf, TbpA or TbpB was immobilised in each of these previous analyses. In this study the failure of TbpA or TbpB to reform the TbpA+B complex or to bind hTf (irrespective of iron saturation) suggests that the presence of both reactant species entirely free in solution and in the defined physical conditions may prevent the association hTf and individual Tbps. However the similarity between traces produced by purified TbpB and recombined TbpA+B may equally indicate that the 1:1 ratio of TbpA to TbpB used in this study contained an excess of TbpB in comparison with co-purified TbpA+B. Consequently, we speculate that co-purified TbpA+B may contain more TbpA than TbpB.

While in the presence of detergent, essential in these analyses, proteins in solution may be incorporated into micelles. It is possible that the presence of TbpA and TbpB within such inclusions could influence their capacity to bind hTf and to reform the TbpA+B complex. In addition, this effect could also influence the hydrodynamic radius of molecules in solution with clear implications for the apparent particle size as determined by PCS. However, as Elugent was included at the same concentration in each solution and failed to produce any reflective signal when analysed independently we propose that possible micelle formation had a minimal impact on our data. Altering the concentration or nature of this detergent, or indeed changing other physical conditions would undoubtedly influence apparent particle size and possibly effect the formation of protein complexes. However, the consistency of detergent concentration, pH, osmolarity and temperature within this experimental system validates our comparative analyses and we make no claims concerning the absolute particle sizes indicated by our data.

PCS is a favourable method for the analysis of proteinaceous solutions, as samples remain in the fluid phase with no requirement for either chemical or radio labelling. Data may also be collected in real time and sample consumption is relatively low [20]. However, while PCS is an accurate technique for determining the diffusion coefficient of such samples and is useful in establishing comparative changes in samples following protein aggregation or disassociation, several assumptions must be employed. In extrapolating D_t data through to protein size (hydrodynamic radius) or, in particular, to molecular weight the Stokes-Einstein approximation assumes a hard sphere model and while proteins are neither necessarily 'hard' (insensitive to nearest neighbour proteins), or true spheres, many proteins can be considered sufficiently globular (exhibiting an aspect ratio significantly smaller than 3) and freely diffusing for the Stokes-Einstein relationship to be valid. Furthermore, protein conformation and structure is so variable that estimations of molecular weight from D_t or protein size data, can be difficult to accurately predict without substantial a priori knowledge of the protein [21]. Accordingly, data in this paper restrict themselves to comparative measurements of estimated protein size and we avoid speculation on the molecular weight of the TbpA+B complex. Despite these limitations, PCS is an ideal technique for the detection of complex formation in this size region as the intensity of light scattering (I_s) varies directly with particle size (r_h^6) [22]. Clearly then, PCS is acutely sensitive to complex formation in solution and, subject to appropriate interpretation of data, constitutes a valuable tool in analyses of this type.

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