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Short communication

An asymmetric and slightly dimerized structure for the tetanus toxoid protein used in glycoconjugate vaccines

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ABSTRACT

Tetanus toxoid protein has been characterized with regard oligomeric state and hydrodynamic (low-resolution) shape, important parameters with regard its use in glycoconjugate vaccines. From sedimentation velocity and sedimentation equilibrium analysis in the analytical ultracentrifuge tetanus toxoid protein is shown to be mostly monomeric in solution (~86%) with approximately 14% dimer. The relative proportions do not appear to change significantly with concentration, suggesting the two components are not in reversible equilibrium. Hydrodynamic solution conformation studies based on high precision viscometry, combined with sedimentation data show the protein to be slightly extended conformation in solution with an aspect ratio ~3. The asymmetric structure presents a greater surface area for conjugation with polysaccharide than a more globular structure, underpinning its popular choice as a conjugation protein for glycoconjugate vaccines.

1. Introduction

Tetanus toxoid is a chemically (formaldehyde) detoxified form of the tetanus toxin produced from *Clostridium tetani*. The tetanus neurotoxin is a 1292 amino acid protein consisting of two chains (N-terminal light chain of 52 kDa and C-terminal heavy chain of 98 kDa) linked by a single disulfide bridge (Eisel et al., 1986; Halpern & Ofthus, 1993). In addition to being a potent antigen, tetanus toxoid has been frequently used as the carrier protein in conjugate vaccines (see for example Astronomo & Burton, 2010; Kaplan et al., 1994; Robbins & Schneerson, 1990; Sigurdardottir et al., 1997), adding much greater efficacy to carbohydrate vaccines such as from *Streptococcus pneumoniae* (Harding et al., 2012). Some *C. tetani* strains are non-toxigenic and are indistinguishable from the toxigenic strains by their phenotypic characteristics and DNA homology, except for the production of toxins (Song, 2003).

The toxoid form has been shown to maintain a high degree of secondary and tertiary structure when compared to native toxin (Robinson, Picklesimer, & Puett, 1975). In addition it appears to have good thermal stability, retaining its structure and polyclonal antibody binding properties after storage at 37.0 °C for at least 2 months (Xing et al., 1996). Although it has thus far resisted attempts to crystallize and give diffraction quality crystals, it has been possible to get a fairly detailed low-resolution picture of its solution structure and oligomeric state on the basis of molecular hydrodynamic measurements.

2. Materials and methods

2.1. Materials

Tetanus toxoid protein was prepared as follows: the reconstituted *Clostridium tetani* strain was grown in a meat free medium (NTM), in a fermentor under regulated conditions. Following the growth of the bacteria, the toxin was released into the culture through natural lysis. After separation from cells (depth filtration) and concentration (ultrafiltration), the toxin was subjected to a detoxification procedure using formalin. Following ultrafiltration, the tetanus toxoid was then purified in two steps by ammonium sulphate precipitation. Finally, after elimination of the residual ammonium sulphate and pH and NaCl content adjustment, the purified tetanus toxoid was sterile filtered and stored at +2/+8 °C.

For analysis, samples were dissolved in phosphate chloride buffer pH ~ 6.8 adjusted to an ionic strength $I = 0.1$ mol l^{-1} according to Green (1933).

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Table 1
Relative proportions of monomer and dimer from sedimentation velocity.

Loading conc. c (g/ml)	s (S) (1st peak)	M (kDa) (1st peak)	Proportion (%) (1st peak)	s (S) (2nd peak)	M (kDa) (2nd peak)	Proportion (%) (2nd peak)
0.1	7.70	145	89	12.03	280	11
0.3	7.60	150	87	11.29	275	13
0.5	7.50	145	84	11.35	280	16
0.7	7.54	150	88	11.45	275	12
1.0	7.50	150	84	11.40	275	16
1.5	7.50	145	83	11.35	250	17
2.0	7.47	150	84	11.50	260	16

2.2. Sedimentation velocity analytical ultracentrifugation

Sedimentation coefficients and sedimentation coefficient distributions were evaluated using a Beckman Optimal XL-I analytical ultracentrifuge (Beckman Instruments, Palo Alto, USA). 400 μl of solution or reference solvent/dialysate were injected into appropriate channels of 12 mm double sector aluminium epoxy cells with sapphire windows. Solutions were centrifuged at 45,000 rpm at a temperature of 20.0 °C. Solute distributions were captured using the Rayleigh interference optical system and data were analysed using the $c(s)$ and $c(M)$ methods of the SEDFIT suite of algorithms from numerical solutions to the Lamm equation (Schuck, 1998, 2000). The weight average s for a particular component was then corrected to standard solvent conditions to yield $s_{20,w}$ (S) using the SEDNTERP algorithm (Laue, Shah, Ridgeway, & Pelletier, 1992). Loading concentrations from 0.1 to 2.0 mg/ml were employed.

2.3. Sedimentation equilibrium analytical ultracentrifugation

Absolute weight average and z-average molecular weights were estimated using low speed sedimentation equilibrium in the analytical ultracentrifuge. Long (20.0 mm) optical path length double-sector titanium cells with sapphire windows were loaded with 70 μl of dialysed protein solution and a matching amount of reference buffer dialysate in appropriate channels. Solutions were centrifuged at ~9000 rpm. Solute distributions at sedimentation equilibrium were analysed to give the weight average apparent molecular weight $M_{w,app}$ using the *MSTARI* algorithm (Cölfen & Harding, 1997) implemented by K. Schilling (Nanolytics Ltd., Germany) for ORIGIN (version 6.0) (Originlab Corporation, Northampton, USA). The z-average molecular weight M_z was also calculated using the algorithm *MFIT* (Ang & Rowe, 2010). The long path length cells meant that a low loading concentration could be used (~0.3 mg/ml) to give a sufficient signal – at such a low concentration, non-ideality effects will be small and hence the apparent weight and z-average molecular weights will be approximately equal to the true weight average M_w and z-average M_z molecular weights, respectively.

2.4. Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS)

Absolute molecular weight analysis (M_w and M_z) was also performed using SEC-MALS. The apparatus comprised an X-Act 4 channel degassing unit (Jour Research, Onsala, Sweden), Jasco Intelligent HPLC Pump – PU-1580 (Jasco Corporation, Great Dunmow, U.K.), fitted with a Spark-Holland Marathon Basic autosampler (Emmen, the Netherlands) combined with a guard column and TSK Gel G6000, 5000, and 4000 columns connected in series (Tosoh Biosciences, Tokyo, Japan), together with a column temperature regulator (Anachem, Luton, U.K.). Light scattering intensity was detected using a DAWN® HELEOS™ II, an 18-angle light scattering detector plots (Wyatt Technology Corporation, California, USA) connected in series to an Optilab® rEX refractive index detector (Wyatt Technology Corporation, California, USA) to measure the concentration. Molecular weights were obtained from standard Debye extrapolation to zero angle (Wyatt, 1992). A solution of tetanus toxoid protein prepared at a concentration of 1.5 mg/ml was filtered through a 0.45 μm syringe filter (Whatman, Maidstone, England) to remove any insoluble material or dust prior to injection. 100 μl of each solution was injected onto the columns at ambient temperature. The pH ~ 6.8; I = 0.1 solvent was pumped at a flow rate of 0.8 ml/min. Data was analysed using the ASTRA™ (Version 5.1.9.1) software (Wyatt Technology Corporation, Santa Barbara, USA). A refractive increment (dn/dc) = 0.17 ml/g was employed.

2.5. Anton Paar™ conventional rolling ball viscometer

Measurement of intrinsic viscosity [η] was carried out using a capillary viscometer AMVn (Anton Parr, Graz, Austria) at a concentration series of (0.1–2.0 mg/ml) based on the rolling ball viscosity method employing a silanized steel ball moving in a1.6 mm diameter glass capillary. The experiment was performed at different reclining angles of 70° (at an average of 4 times), 60° (at an average of 4 times) and 50° (at an average of 6 times) under precise temperature control (20.00 ± 0.01 °C). Huggins (1942) – Eq. (1) and Kraemer (1938) – Eq. (2) extrapolations were used:

$$\frac{\eta_{sp}}{c} = [\eta](1 + K_H[\eta]c) \quad (1)$$

$$\frac{\ln(\eta_{rel})}{c} = [\eta](1 - K_K[\eta]c) \quad (2)$$

where K_H and K_K are the Huggins and Kraemer coefficients, respectively.

3. Results

3.1. Sedimentation velocity in the analytical ultracentrifuge

Sedimentation coefficient $c(s)$ vs s profiles (Fig. 1A) reveal the presence of two clear components with approximately 86% monomer sedimenting at ~7 S and a 2nd component at ~11 S. The proportions of each do not seem to alter significantly with increasing total loading concentration (Fig. 1a and b and Table 1): this implies that these two components are not in reversible equilibrium. Extrapolation of $s_{20,w}$ values of both components to c = 0 (Fig. 2) using the relation of Gralén (1944)

$$\frac{1}{s_{20,w}} = \frac{1}{s^0_{20,w}}(1 + k_s c) \quad (3)$$

Table 2
Sedimentation data for the oligomeric components of tetanus toxoid protein.

Component	Proportion (%)	$s^0_{20,w}$ (S)	M_w (kDa)
Monomer	86 ± 2	7.6 ± 0.1	150 ± 5
Dimer	14 ± 2	11.6 ± 0.2	270 ± 15

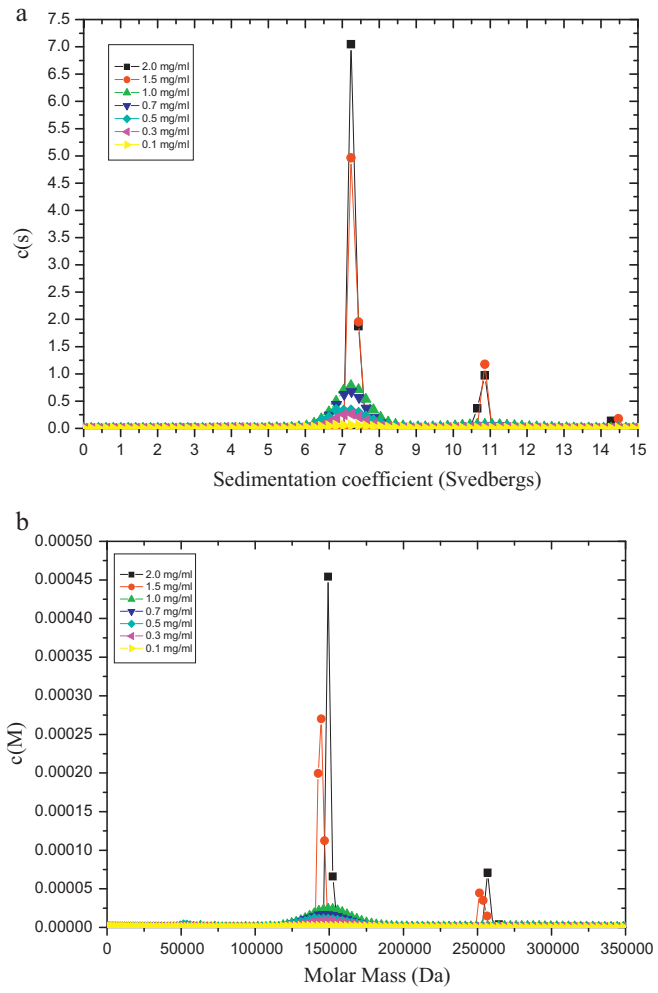

Fig. 1. Sedimentation coefficient distributions (a) and molar mass distributions (b) of tetanus toxoid protein for 7 loading concentrations showing ~14% of dimer.

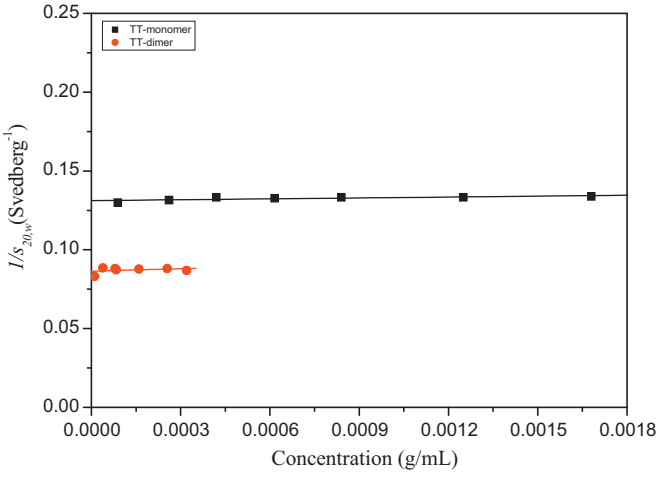

Fig. 2. Concentration dependence of the reciprocal of the sedimentation coefficient for the monomer and dimer species. Linear regression (and subsequent normalization to standard solvent conditions of the density and viscosity of water at 20.0 °C) yields $s^0_{20,w}$ values of 7.6 ± 0.1 S and 11.6 ± 0.2 S for the monomer and dimer forms, respectively.

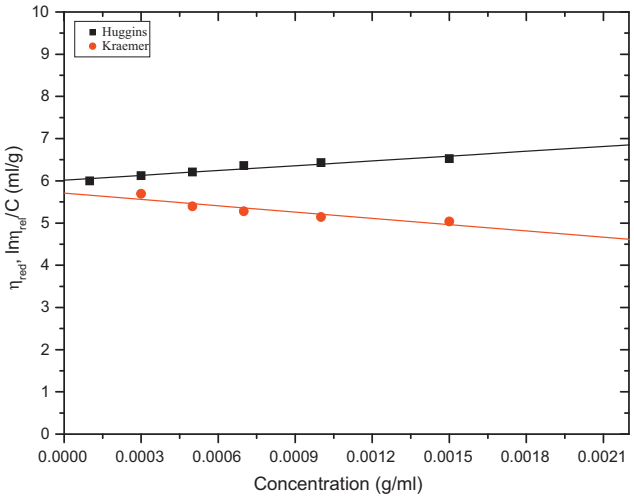

Fig. 3. Plots of reduced viscosity (black data points) and inherent viscosity (red data points) from the rolling ball viscometer at 20.0 °C. Reduced viscosities fitted to the Huggins equation and inherent viscosities to the Kraemer equation, yielding an intrinsic viscosity [η] of 5.7 ± 0.1 ml/g. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with k_s the Gralén coefficient yields "infinite dilution" (i.e. free of the effects of non-ideality) $s^0_{20,w}$ values of 7.6 ± 0.1 S and 11.6 ± 0.2 S for the two components (Table 2).

Although the sedimentation coefficient is a manifestation of molecular shape as well as molecular weight it is possible to estimate the molecular weight distribution by estimating the diffusion coefficient (or equivalently the friction coefficient) as well as the sedimentation coefficient from solutions of the Lamm equation for a discrete number of components (Schuck, 1998, 2000). This allows the conversion of the sedimentation coefficient distribution plot ($c(s)$ vs s) to a molecular weight distribution plot (Fig. 1b). From these data the molecular weight (in Da or equivalently the molar mass g/mol) of the 1st peak $M_1 \sim 150{,}000 \pm 5000$ Da and the 2nd peak $M_2 \sim 270{,}000 \pm 15{,}000$ Da, are consistent with a monomer–dimer system (Tables 1 and 2).

3.2. Absolute molecular weight analysis

Absolute weight average molecular weight M_w and z-average molecular weight M_z values from sedimentation equilibrium analysis are displayed in Table 3 and are consistent with the sedimentation velocity results if the 1st component observed in the latter is a monomer and the 2nd component a dimer. The existence of dimers appears to be confirmed by SEC-MALS, and values for the weight average molecular weight, M_w and z-average molecular weight M_z – for tetanus toxoid protein from SEC-MALS are also reported in Table 3. These values represent the values averaged over both components.

3.3. Viscometry

The Huggins and Kraemer extrapolations for the intrinsic viscosity [η] are shown in Fig. 3, and they extrapolate to a common intercept [η] = 5.7 ± 0.1 ml/g.

4. Discussion

It appears that tetanus toxoid protein consists of two components that are not in reversible equilibrium, and comprise approximately 86% of a major "monomeric" component of

Table 3
Average molecular weight estimates from SEC-MALs and sedimentation equilibrium.

M_w (kDa) (SEC-MALS)	M_z (kDa) (SEC-MALS)	M_w (kDa) (sedimentation equilibrium)	M_z (kDa) (sedimentation equilibrium)
190 ± 10	195 ± 25	185 ± 10	195 ± 10

$M \sim 150{,}000$ and 14% of "dimer" component. It is possible to comment on the significance of the dimer component. The appearance of dimers seems to be a consequence of the detoxification process by formalin (see Latham et al., 1965): although the phenomenon is clearly non-reversible the dimers themselves will have no direct effect as the tetanus toxoid itself is behaving as a carrier protein and has no antigenic behaviour. However the existence of dimers can affect the efficiency of the conjugation process – presumably through steric hindrance at the protein surface – leading to a loss of activity, and it is considered desirable that the level of monomers should not drop below 80% – this seems to be the case here.

It is possible also to comment on the approximate conformation of the monomer species. We can estimate the approximate aspect ratio of the monomeric species however from both the sedimentation and viscosity data.

1. For an $s^0_{20,w} \sim 7.6$ S, $M_w = 150{,}000$ kDa, $\bar{\nu} = 0.74$ ml/g: these data give a frictional ratio $(f/f_0) = 1.29$ using the routine UNIVERSAL_PARAM and ELLIPS1 (Harding, 1997; Harding, Cölfen, & Aziz, 2005). For an $(f/f_0) \sim 1.29$, the Perrin (P) universal shape function = 1.13 (for a hydration δ of 0.35 g/g) and = 1.08 (for 0.50 g/g). Running ELLIPS1 these data correspond to an aspect ratio $(a/b) \sim 2.6$–3.2 (for a prolate ellipsoid).
2. From $[\eta] = 5.7$ ml/g, $\bar{\nu} = 0.74$ ml/g, these give a Simha–Saito (Simha, 1940; Saito, 1951; Harding, 1997) universal shape function $\nu \sim 4.5$ (for a hydration δ of ~0.35 g/g) and =4.0 (for $\delta \sim 0.50$ g/g). Running ELLIPS1, $(a/b) \sim 3.3$–4.2 for a prolate ellipsoid.

Both estimations suggested the tetanus toxoid protein has an aspect ratio of $\sim(3 \pm 1)$:1 within the range of plausible hydrations (Fig. 4). The estimation from intrinsic viscosity will be slightly affected by the presence of the small amount of dimer but the value is consistent with the estimation from the sedimentation coefficient. This asymmetric structure – coincidentally similar to the cartoon representation of it by Astronomo and Burton (2010) – presents a greater surface area for conjugation with polysaccharide than a more globular structure, underpinning its popular choice as a conjugation protein for glycoconjugate vaccines.

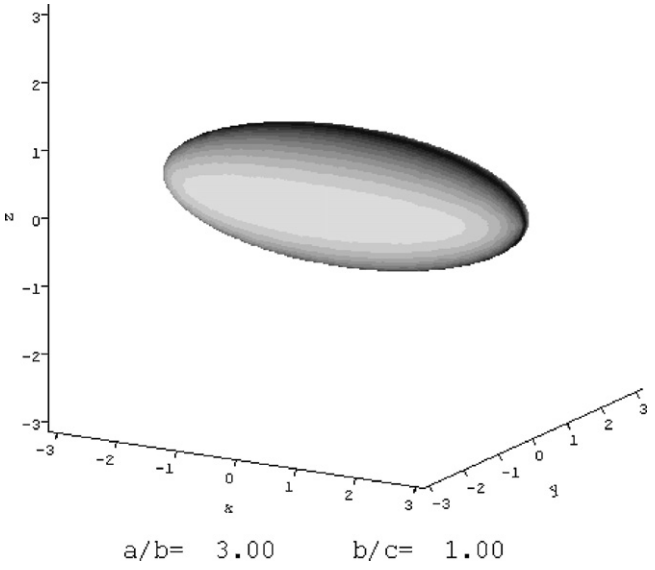

Fig. 4. Prolate ellipsoid representation for monomeric tetanus toxoid protein showing an asymmetric structure of axial ratio ~3.

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