The Angiogenic Inhibitor Long Pentraxin PTX3 Forms an Asymmetric Octamer with Two Binding Sites for FGF2*§

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The inflammation-associated long pentraxin PTX3 plays key roles in innate immunity, female fertility, and vascular biology (e.g. it inhibits FGF2 (fibroblast growth factor 2)-mediated angiogenesis). PTX3 is composed of multiple protomers, each composed of distinct N- and C-terminal domains; however, it is not known how these are organized or contribute to its functional properties. Here, biophysical analyses reveal that PTX3 is composed of eight identical protomers, associated through disulfide bonds, forming an elongated and asymmetric molecule with two differently sized domains interconnected by a stalk. The N-terminal region of the protomer provides the main structural determinant underlying this quaternary organization, supporting formation of a disulfide-linked tetramer and a dimer of dimers (a non-covalent tetramer), giving rise to the asymmetry of the molecule. Furthermore, the PTX3 octamer is shown to contain two FGF2 binding sites, where it is the tetramers that act as the functional units in ligand recognition. Thus, these studies provide a unifying model of the PTX3 oligomer, explaining both its quaternary organization and how this is required for its anti-angiogenic function.

PTX3 (long pentraxin-3) is an acute phase glycoprotein produced at sites of inflammation by both somatic and immune cells (e.g. in response to inflammatory cytokines) (1–3). PTX3 is a soluble pattern recognition receptor providing resistance to selected pathogens, such as the opportunistic fungus Aspergillus fumigatus and Pseudomonas aeruginosa (4–6). Furthermore, it is essential for female fertility in mammals being involved in the stabilization of a mucoelastic extracellular matrix, which forms around the oocyte prior to ovulation and is required for fertilization in vivo (7–9). There is also growing evidence that PTX3 plays additional biological roles in vascular biology (e.g. acting as a modulator of angiogenesis) (10, 11). In this regard, blood vessels produce large amounts of PTX3 during inflammation (12), and the levels of circulating PTX3 increase in several pathological conditions affecting the cardiovascular system (13, 14). Importantly, PTX3 interacts directly with FGF2 (fibroblast growth factor-2), a promoter of neovascularization during wound healing and inflammation (15), thereby inhibiting the proangiogenic activity of FGF2 on endothelial cells (16) and blocking the autocrine and paracrine stimulation exerted by this factor on smooth muscle cells (17).

Thus, current research suggests that PTX3 is a key molecule at the crossroads between innate immunity, female fertility, and vascular biology (18). Consistent with this functional complexity, a number of diverse ligands have been described for PTX3. For example, in addition to FGF2, it recognizes the Outer membrane protein A on the surface of Klebsiella pneumoniae (19, 20) as well as complement components C1q, factor H, and ficolin-2, thus modulating activation of the complement system (21–23). Furthermore, PTX3 is essential for correct assembly of the hyaluronan-rich matrix surrounding the oocyte in the preovulatory follicle, where ptx3−/− female mice are severely subfertile due to defective cumulus organization (8). PTX3 has been implicated in cross-linking individual hyaluronan polymers, probably via its interactions with the heavy chains of inter-α-inhibitor that become covalently attached to hyaluronan (9).

This broad ligand-binding spectrum is probably due to the structural complexity of the PTX3 protein, which is composed of multiple protomer subunits held together by a disulfide bond network, and where these protomers are each composed of distinct N- and C-terminal domains (24–26). Although the N-terminal region (residues 18–178 in the preprotein (1, 2)) is unrelated to any known protein domain, secondary structure predictions indicate that it includes three α-helices (amino acids 78–97, 109–135, and 144–170), which participate in the formation of coiled-coil assemblies (11) (see supplemental Fig. 1A). The C-terminal region (residues 179–381) corresponds to a pentraxin-like domain, which has been modeled on the basis of its similarity to the short pentraxins C-re-
active protein (CRP)\(^3\) and serum amyloid P component (SAP) (24, 26–28). Studies performed with recombinant preparations of the N- and C-terminal domains have indicated that FGF2 and inter-α-inhibitor each bind to the N-terminal region (9, 29); C1q interacts with the pentraxin-like domain (21, 25), whereas both domains have been implicated in the interaction with complement factor H (22). The binding of C1q is affected by the presence of an N-linked glycan at Asn\(^{220}\) in PTX3 (the only glycosylation site in the protein), modulating its activation of the complement system (28).

Previous studies have shown that the PTX3 protomer subunits are assembled into higher order oligomers stabilized by disulfide bonds (25). Our recent investigations using electrophoretic analyses of the recombinant protein indicated that PTX3 is likely to form covalent octamers (26) rather than the decamers that had been suggested previously (25). Moreover, we showed that cysteine residues at positions 47, 49, and 103 in the N-terminal region of PTX3 form three homotypic interchain disulfide bonds holding four protein subunits in a tetrameric arrangement and proposed that two tetramers are linked together to form an octamer by additional interchain bridges involving the C-terminal domain cysteines Cys\(^{317}\) and Cys\(^{318}\) (26) (see supplemental Fig. 1B). In addition, functional analyses of Cys \(\rightarrow\) Ser mutants of PTX3, which affected its oligomerization state, revealed that a tetramer is the minimum size of oligomer that can support cumulus matrix formation \(ex vivo\). These experiments demonstrated that PTX3 oligomerization is necessary for its function, in ovulation at least. However, these data aside, at present, there is a lack of structural information regarding the quaternary organization of PTX3 and how this underlies the various functions of the protein.

Here, biophysical characterization of both recombinant and native human PTX3 demonstrates that the protein is composed of eight identical protomer subunits, where solution methodologies reveal that it is an elongated, asymmetric molecule with two differently sized lobes interconnected by a stalk region. Analysis of recombinant domains of PTX3 shows that the main structural determinant underlying this quaternary organization is located within the N-terminal region of the protomer, which can form disulfide bond-linked tetramers and dimers, where the dimers associate to form non-covalent tetramers. This arrangement gives rise to the asymmetry of the PTX3 molecule and also dictates the number of ligand-binding sites. In this regard, the PTX3 octamer is shown to contain two FGF2 interaction sites, where both types of tetramer can mediate binding. Overall, we provide a unifying model of the PTX3 oligomer explaining both its structural organization and how this arrangement contributes to its recognition of FGF2 and, thus, its antiangiogenic function.

**EXPERIMENTAL PROCEDURES**

**Purified Proteins and Antibodies**—Recombinant human PTX3 (rhPTX3) was purified from a Chinese hamster ovary cell line as described previously (30). Native PTX3 (nPTX3\(^{3837}\)) was isolated from human fibrosarcoma 8387 cells (28). The N-terminal domain (N\(_{\text{PTX3}}\); residues 18–170 of PTX3) and C-terminal domain (C\(_{\text{PTX3}}\); residues 178–381) were purified from HEK-293F and Chinese hamster ovary cell lines, respectively (see supplemental material). N\(_{\text{PTX3}}\)C103S, N\(_{\text{PTX3}}\)C47S/C49S, and N\(_{\text{PTX3}}\)C47S/C49S/C103S mutant constructs were made from the N\(_{\text{PTX3}}\) expression vector by overlapping PCR. The mutants were expressed/purified as described for the wild type N\(_{\text{PTX3}}\) (see supplemental material). Recombinant FGF2 was expressed in *Escherichia coli* and purified as previously reported (31). Biotin-labeled rabbit anti-human PTX3 polyclonal antibody (αPTX3pb) and rat anti-human PTX3 monoclonal antibody (MNB4) were made as described (25, 28).

**Size Exclusion Chromatography-Multiangle Laser Light Scattering (SEC-MALLS)**—Proteins were run on either Superose 6 or Superose 12 HR 300 \(\times\) 10-mm gel filtration columns (GE Healthcare) equilibrated in 20 mM sodium phosphate, 150 mM NaCl, pH 7.40 (PBS) at 0.71 ml/min. Elution was monitored by a Wyatt EOS 18-angle laser photometer (Wyatt Technology, Santa Barbara, CA), an Optilab rEX refractive index detector, and a Jasco UV-2077 Plus UV-visible spectrophotometer (Jasco, Easton, MD); these were coupled to a Wyatt quasielastic light scattering detector for simultaneous measurement of hydrodynamic radius. Molar mass determinations were performed using both Astra 5.3.2.16 software (Wyatt Technology) and the “three-detector method” (see supplemental material). The d\(n\)/d\(c\) value for rhPTX3 was determined experimentally as 0.180 \(\pm\) 0.005 ml/g. Values of mass and hydrodynamic radius are expressed as means \(\pm\) S.E.

In separate experiments, SEC-MALLS analyses were run on 0.60 \(\mu\)M rhPTX3 solutions in PBS containing 2.40 \(\mu\)M FGF2 (i.e. a molar ratio of FGF2/rhPTX3 = 4:1). Molecular mass of the PTX3-FGF2 complex was measured using the three-detector method (see supplemental material).

**Analytical Ultracentrifugation (AUC)**—Velocity and equilibrium sedimentation AUC analyses were done on MALLS-purified proteins in PBS, using an XL-A ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with an An50Ti 8-hole rotor fitted with either two-sector (for sedimentation velocity) or six-sector (for equilibrium sedimentation) Epon-filled centerpieces with quartz glass windows.

Velocity AUC was carried out at 45,000 rpm at 20 °C, with the sedimenting boundary monitored at 230 nm every 90 s. C(s) plots were generated from the experimental data using Sedfit (developed by P. Schuck; available on the World Wide Web), and peaks were integrated to give the sedimentation coefficients. Three independent experiments were carried out for each protein, and s\(_{20,w}\) values are expressed as mean \(\pm\) S.E.

Equilibrium sedimentation was performed at 4 °C, using rotor speeds of either 4,000, 7,000, and 11,000 rpm (for
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—Recombinant and native PTX3 (both at 12.5 μg/ml in PBS) were analyzed by negative staining transmission electron microscopy (TEM) according to conventional protocols (32). Low dose images were obtained on an FEI Tecnai 12 twin electron microscope (FEI, Eindhoven, The Netherlands) operating at 120 keV. Images were converted to Imagic5 format (Image Science Software, Berlin, Germany) for particle picking and image processing (33). Selected particles (5,109 in data set; picked manually) were windowed into 33.6 × 33.6-nm images and band pass–filtered with high and low frequency cutoffs of 20 and 250 Å, respectively. Images were then centered by cross-correlation relative to the total sum of the data set and sorted into classes by multivariate statistical analysis. Characteristic class-sum images were then used as references to align the data set during iterative rounds of multireference alignment.

Small Angle X-ray Scattering (SAXS)—rhPTX3 samples (0.63 mg/ml (1.85 μM) in PBS) were subjected to SAXS analysis on beamline EMBL X33 (34) at the Deutsches Elektronen-Synchrotron (Hamburg, Germany). Data were collected at 10 °C using a 60-s exposure time and 2.4-m sample to detector distance to cover a momentum transfer interval of 0.1 nm⁻¹ < q < 5.0 nm⁻¹ (q = 2πs/λ, 2θ is the angle between incident and scattered radiation, and λ is the X-ray wavelength); bovine serum albumin was used as a mass calibration standard. Buffer scattering intensities were recorded and subtracted from the sample image to remove background scattering. Values for radius of gyration (Rg) and maximum particle size (Dmax) were determined using GNOM (35). Particle shapes were generated ab initio using DAMMIN (36) with imposed P4 symmetry; 20 runs were performed, combined, and filtered to produce an averaged model using DAMAVER (37). HydroPro (38) was used to calculate Rg, sedimentation coefficient (s20,w), and maximum distance (Dmax) from the ab initio DAMAVER model.

4 SAXS experiments performed on 2.04 mg/ml rhPTX3 in PBS (at the Diamond Synchrotron, UK) gave essentially identical scattering curves and demonstrated that the scattering particle mass (I(0)) and Rg values are independent of protein concentration (data not shown).

SDS-PAGE and Western Blotting—Proteins were run on either Tris acetate 3–8% (w/v) gradient or Tris-glycine 10% (w/v) SDS-polyacrylamide gels (with or without reduction using dithiothreitol) and stained using Coomassie Blue. Fractions from SEC-MALLS (i.e. of N_PTX3C103S and N_PTX3C47S/C49S) were analyzed by Western blot using the monoclonal antibody MNB4 (26).

FGF2 Binding—Binding of rhPTX3, N_PTX3, and N_PTX3C47S/C49S/C103S to FGF2 was assessed using 96-well Polysorp plates (Nunc, Roskilde, Denmark) coated with FGF2 (25 pmol/well); all reaction volumes were 100 μl and plates were washed after each step with PBS containing 0.1% (v/v) Tween 20 (PBS-T). After coating with FGF2, wells were blocked with 1% (w/v) bovine serum albumin in PBS-T for 2 h at 37 °C and then incubated with PTX3 samples for 1 h at 37 °C. Bound proteins were detected using αPTX3pb polyclonal antibody (1:5,000 dilution), followed by serial incubation with Extra-Avidin alkaline phosphatase (1:5000; Sigma) and the substrate disodium p-nitrophenyl phosphate (1 mg/ml) in 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, pH 9.3; the absorbance was read at 405 nm, and background from uncoated wells was subtracted. Competition experiments were also performed, where FGF2-coated plates were incubated with biotinylated rhPTX3 (bPTX3) in the presence of rhPTX3, N_PTX3, or N_PTX3C47S/C49S/C103S.

To determine which component(s) of the N_PTX3 preparation bind FGF2, biotinylated FGF2 (bFGF2) and N_PTX3 were preincubated in PBS (at 2.5 and 10 μM, respectively) overnight at 4 °C (100-μl final volume). The bFGF2/N_PTX3 mixture was then incubated with 40 μl of 50% (v/v) streptavidin-agarose resin (Thermo Scientific, Rockford, IL) at room temperature for 1 h in PBS-T. Following extensive washing (five times with 500 μl of PBS-T), bound proteins were released from the resin by the addition of 20 μl of NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) and incubation at 70 °C for 10 min. These were run on a Tris-glycine 10% (w/v) SDS-polyacrylamide gel under non-reducing conditions, and the presence of N_PTX3 was determined by Western blot using the MNB4 antibody as described above. Control experiments were performed on solutions containing only bFGF2 or N_PTX3.

Chemotaxis Assays—Human artery smooth muscle cells at 2 × 10⁶ cells/ml in serum-free medium were seeded in the upper compartment of a Boyden chamber containing an 8-μm polyn vinylpyrrolidone-free polycarbonate filter (Nuclepore, Whatman Inc., Clifton, NJ) coated with 5 mg/liter porcine gelatin. FGF2, with or without rhPTX3, was placed in the lower compartment. Following a 4-h incubation at 37 °C, migrating cells attached to the lower surface of the filter were stained with Diff-Quik (Dade-Behring, Germany) and counted. Data from two independent experiments were obtained in quadruplicate, averaged, and expressed as percentage inhibition of cell migration in the absence of rhPTX3 ± S.E.

RESULTS

PTX3 Is an Octamer Composed of Eight Identical Protomers—Previous reports, mainly based on size exclusion chromatography of recombinant human PTX3 (rhPTX3), indicated that the protein is composed of 10 protomers with potential to form
higher molecular weight aggregates (e.g. 20-mers) (25). In contrast, we have recently proposed that PTX3 is an octamer based on the electrophoretic mobility of the protein on native PAGE (26). To address this inconsistency and unambiguously define the number of protomers forming the functional PTX3 molecule, we analyzed rhPTX3 by SEC-MALLS. As can be seen from Fig. 1A, the chromatogram contains a major species eluting at 10.7 ml (peak 3) along with minor species (peaks 1 and 2). When peak 3 was collected and reanalyzed by SEC-MALLS (Fig. 1B), it had a polydispersity index of 1.001 ± 0.020, showing that this SEC-purified material, which was used in all subsequent analyses, was monodisperse. Based on the angular dependence of the scattered light intensity, molar mass values were measured across this peak (see distribution in the right panel of Fig. 1B), allowing a weight-averaged molecular mass (MW) of 344 ± 7 kDa to be calculated for rhPTX3. From this experimentally determined value, and assuming that each PTX3 protomer has a molecular mass of ~42.5 kDa (i.e. based on the calculated mass of the polypeptide (40.165 kDa) along with the average mass of the N-linked glycans of ~2.5 kDa (28)), it can be concluded that the major species in the rhPTX3 preparation is composed of eight protomers (42.5 kDa × 8 = 340 kDa). A similar analysis of peak 2 (Fig. 1A) gave an estimated MW of 702 ± 15 kDa, indicating that this species is composed of 16 promoter subunits (i.e. resulting from the non-covalent association of two octamers); very high MW material was eluted in peak 1, which probably corresponds to aggregated protein and other particulate contaminants.

Given that mass values from light scattering can be affected by glycosylation (39), we also determined the Mw (the polypeptide molecular mass) of rhPTX3 using a three-detector method that is based on the measurement of the protein concentration from its UV absorbance at 280 nm (see supplemental material) (i.e. the Mw value does not include the mass contribution from attached glycans). This gave a value of 319 ± 4 kDa for the weight-averaged Mw consistent with the theoretical polypeptide molecular mass of a PTX3 octamer (40.165 kDa × 8 = 321.32 kDa). Moreover, sedimentation equilibrium experiments by AUC, performed on SEC-purified rhPTX3, gave an Mw value of 330 ± 3 kDa, similar to that measured by SEC-MALLS (Table 1 and supplemental Fig. 2A).

Taken together, these data conclusively demonstrate that the major species in the rhPTX3 preparation is an octamer. Electrophoretic and SEC-MALLS analyses performed on native PTX3 purified from human fibrosarcoma 8387 cells (nPTX38387) gave mass measurements consistent with those obtained for the recombinant protein (Mw of 340 kDa from PAGE and Mw of 325 ± 5 kDa from SEC-MALLS; see supplemental Fig. 3, A and B). Therefore, it can be concluded that the octameric oligomerization of the recombinant PTX3 is representative of the native human protein.

The PTX3 Octamer Is an Elongated Molecule with Two Structurally Distinct Domains—The overall shape of the rhPTX3 molecule was studied in solution using quasielastic light scattering, as part of the SEC-MALLS analysis, and sedimentation velocity AUC. This gave values of 8.6 ± 0.3 nm for the hydrodynamic radius (Rh) and 8.62 ± 0.16 S for the protein sedimentation coefficient (s20,w) (see Table 1). Based on these values, a frictional ratio (f/f0) of 1.9 was calculated using Sednterp (where f is the measured frictional ratio, and f0 is the frictional ratio of an anhydrous sphere of the same mass), indicating a marked shape asymmetry of the rhPTX3 molecule. Notably, the Rh determined for the native PTX3 (8.5 ± 0.2 nm) by SEC-MALLS (supplemental Fig. 3B) is very similar to that observed for the recombinant protein.

To further investigate the quaternary structure of the PTX3 octamer, we used TEM and SANS. Monodisperse rhPTX3 specimens (i.e. purified from SEC-MALLS) were imaged by

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**TABLE 1**

| Protein          | Mw (kDa) | s20,w (S) | f/f0 | Rh (nm) |
|------------------|----------|-----------|------|---------|
| rhPTX3           | 344 ± 7  | 8.62 ± 0.16 | 1.9  | 8.6 ± 0.3 |
| nPTX38387        | 325 ± 5  | 8.6 ± 0.3   | ND   | 8.5 ± 0.2 |
| N_PTX3           | 325 ± 5  | 8.6 ± 0.3   | ND   | 8.5 ± 0.2 |
| C_PTX3           | 325 ± 5  | 8.6 ± 0.3   | ND   | 8.5 ± 0.2 |

* a Mw from MALLS (mean of six determinations ± S.E.).
  b Mw from sedimentation equilibrium AUC (mean of three determinations ± S.E.).
  c s20,w from sedimentation velocity AUC (mean of three determinations ± S.E.).
  d Rh from QELS (mean of six determinations ± S.E.).
  e ND, not determined.
  f s20,w from sedimentation velocity AUC under dissociative conditions (i.e. in 1 M NaCl; mean of three determinations ± S.E.).

**FIGURE 1.** SEC-MALLS analysis of rhPTX3. A, SEC separation of rhPTX3 (3 μl) with detection by differential refractive index (RI) (left); the main signals (peaks 1–3) are numbered according to the elution time. Right, an expanded view of the refractive index chromatogram for peaks 2 and 3, including the molar mass (M) of the proteins (black data circles and right axis). B, peak 3 (0.3 μl) from A (i.e. the major component) was reanalyzed by SEC-MALLS (left panels) with detection by light scattering (LS) at a 90° angle (top) and refractive index (bottom); expanded views of the refractive index chromatogram are shown in the right panels with overlaid plots of M and Rg.
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Although a number of different relative orientations of these two regions were observed, most classes show the protein particles in such an orientation that the 4-fold symmetry axis of the tetrameric domain is essentially perpendicular to the EM grid (i.e. rhPTX3 adopts a preferential orientation). Therefore, although the acquired TEM images revealed important features of the rhPTX3 structure, we could not use them for three-dimensional reconstructions. TEM analyses of rhPTX3/II/II/II (222 particles) indicate that the native protein displays similar structural characteristics to those described above for rhPTX3 (see supplemental Fig. 3, C and D).

SAXS experiments performed on rhPTX3 solutions allowed a low resolution model for the PTX3 octamer to be determined. From these scattering data (Fig. 3A), the $R_g$ and $D_{max}$ were determined using GNOM (8.2 ± 0.1 and 25.5 ± 1.3 nm, respectively); the molecular weight derived from $I(0)$, which was estimated by comparison with the $I(0)$ from a known concentration of bovine serum albumin, was 357 kDa (i.e. in good agreement with the values determined by SEC-MALLS and AUC). The distance distribution function, shown in Fig. 3B, displays a sharp peak at ~5.5 nm and a shallow peak in the range 16–22 nm, which indicates the presence of two separated domains. Based on the assumption of 4-fold molecular symmetry, as indicated by TEM, an ab initio model of the rhPTX3 envelope was generated using DAMMIN (Fig. 3, C and D). This model is composed of two domains (one large and one small) that are connected by a short stalk (45 Å in length); the 20 individual models (Fig. 3C), on which the final average model (Fig. 3D) was based, are all asymmetric and of similar shape (with a mean normalized spatial discrepancy value of 0.955). Furthermore, it should be noted that an elongated and asymmetric model also resulted when no symmetry constraints (P1) were imposed (not shown). Hydrodynamic simulations run on the SAXS three-dimensional envelope (i.e. that depicted in Fig. 3D) using HydroPro (38) provided theoretical values for the sedimentation coefficient (8.25 S) and radius of gyration (8.5 nm), which are in good agreement with those determined experimentally from AUC and GNOM (8.62 S and 8.2 nm).

negative staining TEM (Fig. 2A). Classification and summation of 5,109 picked particles (Fig. 2, B and C) indicate that the protein is composed of four globular domains (each with a diameter of ~4 nm) arranged into tetramers with apparent planar square symmetry and an overall diameter of ~10 nm. A small (~1.6-nm) electron-poor region is observed at the tetramer center; however, it is not clear whether this represents an empty volume in the protein structure or is due to differential local staining. In some classes, an additional domain protrudes from the tetramer (see arrows in Fig. 2B), suggesting that the rhPTX3 protein is composed of two structurally distinct regions.
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respectively) (i.e., supporting the reliability of the model). The dimensions of the SAXS structure indicate that in the EM analysis, the PTX3 molecules are oriented on the grid such that they are being viewed down the long axis of the protein (e.g. as seen in the left-hand image of Fig. 3D). Presumably, it is the smaller domain that is seen to project out from the larger domain in certain EM classes (Fig. 2B), where this may depend on the flexibility of the stalk region or be due to conformational perturbations caused by the staining and/or absorption of the protein onto the grids (40).

PTX3 Oligomerization Is Mediated by the N-terminal Region of the PTX3 Protomer.—To determine the contributions of the N- and C-terminal domains of PTX3 in the assembly of the octamer, we extended our investigations to recombinant fragments corresponding to these regions (i.e. N_PTX3 and C_PTX3; see Fig. 4A). Under non-reducing conditions on SDS-PAGE, the N_PTX3 preparation is composed of two species with estimated masses of 82 and 42 kDa (Fig. 4B); it migrates as a single band of 20 kDa under reducing conditions, consistent with the theoretical molecular mass of the domain (18,163 Da, as calculated from the protein sequence). Thus, the 82- and 42-kDa species probably correspond to disulfide bond-linked tetramers and dimers of N_PTX3, respectively. However, MALLS and sedimentation equilibrium AUC analyses performed under non-denaturing (and non-reducing) conditions showed N_PTX3 to form predominantly tetramers, with less than 5% of total protein present as dimers (Fig. 4C, Table 1, and supplemental Fig. 2A). Therefore, in solution phase, the N_PTX3 preparation is a mixture of covalently linked tetramers and covalent dimers, where the latter associate non-covalently (i.e., to form a tetramer made up of a dimer of dimers); from the relative staining intensity of the 42- and 82-kDa species (Fig. 4B), it would appear that the two different types of tetramer are present in approximately equal amounts. In this regard, sedimentation velocity experiments carried out in 1 M NaCl (supplemental Fig. 4) (i.e., to dissociate the non-covalent tetramer) revealed two distinct species with $s_{20, w}$ of 3.62 ± 0.15 S and 2.73 ± 0.09 S (Table 1), corresponding to the disulfide-linked tetramer and disulfide-linked dimer, respectively, both of which are elongated ($\beta f_0 = 1.8$ and 1.5).

To analyze further the role of non-covalent interactions in the oligomerization of the PTX3 N-terminal domain, we made single, double, and triple mutants of N_PTX3, where cysteine residues at positions 103, 47/49, and 47/49/103 (see supplemental Fig. 1) were replaced by serine. The triple mutant revealed two non-covalent interactions (68 ± 2 and 74 ± 4 kDa, respectively) for a monomer on SDS-PAGE under non-reducing conditions (as expected for a protein with no disulfide bonds), whereas the single and double mutants both ran as a mixture of dimers and monomers (Fig. 5A). SEC-MALLS analysis (Fig. 5B) under non-denaturing conditions revealed that the triple mutant is composed entirely of dimers ($M_w = 33 ± 1$ kDa), despite having no cysteine residues; similarly, a mutant of the full-length rhPTX3, lacking the entire set of cysteines involved in interchain disulfide bonds, retained a dimeric assembly (26). Therefore, in this case, non-covalent associations between N_PTX3 monomers mediate dimerization. The single and double mutant proteins both corresponded to a mixture of tetramers (68 ± 2 and 74 ± 4 kDa, respectively) and dimers (34 ± 1 and 32 ± 1 kDa, respectively) on SEC-MALLS (Fig. 5B). SDS-PAGE analysis of these species (separated by SEC) revealed the tetramers to be composed of disulfide-linked dimers, whereas the dimeric fractions contained both monomers and disulfide-linked dimers (Fig. 5C). Thus, it can be concluded that in order to form a tetramer of the N-terminal domain (i.e., under non-denaturing conditions), at least one disulfide bond is required.

SDS-PAGE analysis of C_PTX3, which has a predicted molecular mass of ~25 kDa (based on a peptide mass of 22,591 Da and an N-linked glycan of ~2.5 kDa (see supplemental Fig. 5)), revealed a single band with apparent molecular mass of ~27 kDa under both reducing and non-reducing conditions (Fig. 4B). Furthermore, MALLS and AUC analyses dem-
disulfides (i.e. in different protomers (26)) and are required for oligomerization of the protomer subunits (see supplemental Fig. 1). However, in the isolated C_PTX3 domain, these cysteines were found by mass spectrometry to form only intrachain disulfides (supplemental Fig. 6), consistent with this construct being monomeric. Therefore, we conclude that the interchain disulfides linking the C-terminal regions of PTX3 are only formed in the presence of the N-terminal domain, which assembles into tetramers and thus contains the major structural determinants that mediate oligomerization.

A New Model for the Quaternary Organization of the PTX3 Octamer—As described above, our biophysical analyses of PTX3 demonstrate it to be composed of eight identical protomer subunits, which form an elongated and asymmetrical octamer, where the N-terminal region has an essential role in oligomerization. In particular, the asymmetric shape determined by SAXS (i.e. comprising a small and large lobe connected by a stalk) cannot be readily explained by the symmetric arrangement of disulfide bonds that we proposed previously, which would result in two identical domains, each composed of four protomers (see supplemental Fig. 1 and Ref. 26). The subtle reorganization of this disulfide bond pattern, as shown in Fig. 6A, still consistent with the experimental data of Inforzato et al. (26), would explain such an asymmetry. Here, Cys103 (in the N-terminal domain) would support two different disulfide arrangements in the intact PTX3 molecule such that either two or four protomers were connected via this cysteine; Cys317/Cys318 in the C-terminal domain would then link these “tetramers” and “dimers” into an octamer. This hypothesis is consistent with the finding that a mixture of disulfide-linked tetramers and dimers was present in the isolated N-terminal construct, where the dimers associated into tetramers via non-covalent interactions (see Fig. 4B and supplemental Fig. 4). In keeping with this, our previous analysis of rhPTX3 in which both Cys317 and Cys318 were changed to serine revealed that this mutant forms a mixture of disulfide-bonded tetramers and dimers (26). From the frictional ratios of the tetramers and dimers present within the N_PTX3 preparation, it can be concluded that these have different shapes and therefore are likely to have a different molecular organization.

As noted in the Introduction, part of the N-terminal region of PTX3 is predicted to form coiled-coil structures from three α-helical segments that lie between amino acid residues 78 and 170 (11); circular dichroism analyses of N_PTX3 indicate that α-helices comprise 30–40% of its secondary structure (data not shown). It is plausible that these helices could be organized into two distinct structures in the disulfide-linked tetramers and dimers, which may account for the asymmetry of the PTX3 molecule (Fig. 6B). In the tetrameric form of the N-terminal domain, a coiled-coil structure involving the parallel association of four protomers would lead to the generation of an elongated stalk and small globular domain with apparent 4-fold symmetry (Fig. 6C). From the estimated lengths of the helical regions (and the model derived from SAXS analysis; Fig. 3D), it seems likely that the coiled-coil structure would involve all three α-helices from each chain, where it is Cys103 that connects the four chains (i.e. located in the short non-helical segment between the α1 and α2 helices). Based on the dimensions

![Diagram](image-url)
PTX3 Quaternary Structure

![Diagram of PTX3 quaternary structure](Image)

**FIGURE 6.** Model of the PTX3 octamer; arrangement of protomer subunits. A, disulfide bond organization of the PTX3 octamer in which Cys\(^{103}\) can either stabilize the protomers into tetramers (right), as suggested previously (26), or into dimers (left) (i.e. based on the recombinant N-terminal domain (N\_PTX3) forming a mixture of disulfide-linked tetramers (green box) or disulfide-linked dimers (black box), where the latter associate non-covalently into tetramers). The N-terminal domain (yellow) is composed of an N-terminal segment (amino acids 18-70), which is probably globular, followed by three \(\alpha\)-helices (residues 78-97, 109-135, and 144-170; estimated to be 29, 39, and 39 Å in length, respectively, on the basis of 1.45 Å per residue (51)). Cys\(^{317}\) and Cys\(^{318}\) in the C-terminal pentraxin domains (denoted in red) link the protomers (i.e. a tetramer and two dimers) into octamers. B, the \(\alpha\)-helical regions of the N-terminal domains, predicted to form coiled-coil-like structures (11), are hypothesized here to adopt two distinct structural arrangements, either an extended \(\alpha\)-helical segments of the N-terminal domain, which either form one interchain four-helix bundle or a compact organization (left), where each protomer self-associates to form an anti-parallel three-helix bundle. In the former, a long tubelike structure (\(-110\) Å in length and \(-30\) Å in diameter) would be generated, around which both the N-terminal globular domains (yellow ovals) and C-terminal pentraxin domains (red ovals) would be arranged, with an intervening stalk region of 45 Å. C, a comparison of the SAXS model with a schematic model for PTX3 based on the two different organizations proposed for the N-terminal domain above; the \(\alpha\)-helical segments of the N-terminal domain, which either form one interchain four-helix bundle or four intrachain three-helix bundles, are depicted as yellow rods. The position of the C-terminal pentraxin domains (red) in the schematic model is consistent with the dimensions derived from the three-dimensional homology model proposed previously on the basis of the CRP crystal structure (see Ref. 26).

of parallel four-helix bundles for which structures have been determined (42), the tetrameric coiled-coil proposed here would have a diameter of \(-30\) Å, which matches that seen for the stalk in our SAXS envelope. On the other hand, in the dimeric form of the N-terminal domain, three helices from each protomer would form an intrachain coiled-coil (i.e. in an anti-parallel arrangement; Fig. 6B), leading to the generation of a more compact structure, where two protomers would be linked via Cys\(^{103}\). The non-covalent association of two such dimers (as indicated in Fig. 6C) would probably form a more globular structure (compared with the disulfide-linked tetramer) with pseudo-4-fold symmetry (consistent with our TEM analysis).

In our model, the differently structured N-terminal region tetramers (yellow in Fig. 6) would be positioned on either side of a central region composed of the C-terminal domains (depicted in red). This is based on the previous observation that disulfide bridges linking the C-terminal domains are responsible for bringing together PTX3 tetramers into octamers (26) (i.e. the above), it can be concluded that PTX3 contains two FGF2-binding sites (i.e. a combined \(M_w\) of \(-365\) kDa); these AUC analyses were performed at a rhPTX3 concentration more than 20-fold higher than the dissociation constant of the PTX3-FGF2 interaction (\(K_d \approx 18\) nM; adapted from Camozzi et al. (29) and based on a stoichiometry of 2 (see above)) to ensure full saturation of the FGF2 binding sites. Furthermore, SEC-MALLS analysis of the rhPTX3-FGF2 complex gave an \(M_w\) value (353 ± 8 kDa) consistent with two FGF2 molecules binding to the PTX3 octamer (Fig. 7B).

The inhibitory effect of PTX3 on FGF2-induced chemotaxis of human aortic smooth muscle cells (17) was investigated in order to understand the FGF2-PTX3 interaction in a more cellular context. As illustrated in Fig. 7C, rhPTX3 inhibited FGF2-mediated migration in a dose-dependent manner, where a maximal response was reached at a 1:2 molar ratio of PTX3/FGF2; suboptimal inhibition was seen at 1:16 to 1:4 ratios. Therefore, these data are consistent with the 2:1 stoichiometry described above for the interaction of FGF2 with PTX3.
FIGURE 7. FGF2 binding to PTX3. A, analysis of FGF2-rhPTX3 complexes using sedimentation equilibrium AUC at different FGF2/rhPTX3 molar ratios (rhPTX3 at 0.4 μM). The molar masses of the complexes (right-hand axis) are shown relative to the size of PTX3 alone and to the masses predicted for a complex of PTX3 bound to one or two molecules of FGF2 (left-hand axis and dashed lines); values are the mean of two determinations ± S.E. (error bars). B, UV chromatograms from SEC-MALLS of rhPTX3 (0.6 μM) alone (top) or with FGF2 at a 4:1 (FGF2/rhPTX3) molar ratio (bottom), including overlaid plots of the Mₚ value as a function of the elution volume (open data circles and right-hand axis). Average Mₚ values (mean of three determinations ± S.E.) are indicated in boldface type. SDS-PAGE analysis of species I and II showed them to contain PTX3 alone or both PTX3 and FGF2, respectively (data not shown). C, human aortic smooth muscle cells (hAOSMC) were seeded in the upper compartment of a Boyden chamber and incubated with 6 nM FGF2 in the presence of rhPTX3 (at rhPTX3/FGF2 molar ratios of 1:16 to 1:1); data are expressed as percentage inhibition of cell migration in the absence of rhPTX3 (mean of eight determinations ± S.E.). D, the binding of rhPTX3, N_PTX3, or N_PTX3C47S/C49S/C103S (T) to immobilized FGF2; the PTX3 proteins were applied at a range of concentrations, and their binding was detected using an anti-PTX3 antibody. Values are plotted as mean absorbance at 405 nm (n = 6 ± S.E.). E, the inhibitory activity of rhPTX3, N_PTX3, or N_PTX3C47S/C49S/C103S on the binding of bPTX3 to FGF2-coated wells; bPTX3 (1.5 nM) was incubated with the unlabeled PTX3 proteins at a range of concentrations, where its binding was detected with streptavidin-alkaline phosphatase. Data (mean of six determinations ± S.E.) are expressed as percentage inhibition of bPTX3 binding in the absence of competitors. F, binding of N_PTX3, which is composed of disulfide-linked tetramers (4-mer) and dimers (2-mer), to bFGF2. N_PTX3 in the absence or presence of bFGF2 was incubated with streptavidin-agarose (SA-agarose), and the bound N_PTX3 species (i.e. 4- and 2-mers) were detected by Western blot using an anti-PTX3 antibody; a gel that is representative of two independent experiments is shown.
PTX3 Quaternary Structure

A putative FGF2-binding site has been described previously in the N-terminal domain of PTX3 (29). Here we found that the N_PTX3 and rhPTX3 proteins interact with FGF2-coated wells in a dose-dependent manner when analyzed using a microtiter plate-based binding assay (Fig. 7D), confirming that FGF2 does interact with the N-terminal domain of PTX3. Interestingly, the N_PTX3 triple mutant, composed entirely of non-covalent dimers (see above), did not support the binding of FGF2 (Fig. 7D), whereas the N_PTX3C103S single mutant (that contained 44% tetramers under non-denaturing conditions) retained ~50% of the activity of the wild type N_PTX3 protein (see supplemental Fig. 7A). Furthermore, unlike N_PTX3 and rhPTX3, which have IC_{50} values of 106 and 46 nM, respectively, the triple mutant was unable to compete for the interaction of bPTX3 with immobilized FGF2 (Fig. 7E); the N_PTX3C103S mutant could inhibit this interaction, albeit less effectively than the wild type protein (supplemental Fig. 7B). Therefore, it seems likely that PTX3 tetramers are the functional units in FGF2 binding. To test this further, bFGF2 was incubated with the N_PTX3 preparation, and bFGF2-containing complexes were extracted using streptavidin-agarose beads. Western blot analysis of the bound complexes (Fig. 7F), using an anti-PTX3 antibody, revealed that both the disulfide-linked tetramer (4-mer) and disulfide-linked dimer (2-mer), where the latter associates in solution into non-covalent tetramers, bound to bFGF2; there was no binding of N_PTX3 to the beads in the absence of bFGF2. These data indicate that both types of tetramer in the N_PTX3 preparation can support the binding of FGF2.

Therefore, these studies revealed that the structural elements necessary for formation of the PTX3 octamer also correlate with the functional domains that mediate the interaction with FGF2 and thus underpin the antiangiogenic function of PTX3.

DISCUSSION

In this study, we have demonstrated that human PTX3 is a disulfide-linked octamer where the N-terminal domain of the protomer acts as the main structural determinant supporting protein multimerization. This oligomerization state and the asymmetric shape observed in solution make PTX3 unique among pentraxins (24, 25); i.e. it has 4-fold symmetry, in sharp contrast to the typical pentameric structure of the classical short pentraxins (43, 44). The only other pentraxin that forms an 8-mer is SAP from *Limulus polyphemus*, which has been reported to fold into a doubly stacked octameric ring (45). In this regard, the amino acids that mediate oligomerization in the pentameric pentraxins (i.e. CRP and SAP) are not conserved in the pentraxin domain of the PTX3 protomer (27), consistent with the finding here that the isolated C-terminal domain is monomeric. A major conclusion of the present study is that the N-terminal domain of PTX3 mediates the association of protomers into tetramers; the equivalent regions of other long pentraxins have also been implicated in oligomer formation (46, 47). In PTX3, we have hypothesized that the three α-helices, predicted to be present in the N-terminal segments (11), form a parallel four-helix bundle that drives the assembly of the disulfide-linked tetramer (see schematic model in Fig. 6); in addition to this extended structure, one of the α-helical regions, in the context of an intrachain three-helix bundle, could support the tetramerization of the dimer of dimers (i.e. a higher order association of coiled-coils (48, 49)). Coiled-coils are also found in the N-terminal domains of other long pentraxins, such as the neuronal pentraxin 1 (46) and 2 (47), as well as some collectins (50), where they have been shown to have a key function in protein oligomerization (51). Thus, the PTX3 quaternary structure is based on protein tetramers whose formation is driven by structural determinants localized in the N-terminal domain. Given that the isolated C-terminal domain (C_PTX3) is monomeric and that mutants of full-length PTX3 where Cys^{317} and Cys^{318} were altered to serine form 4-mers (26), it seems likely that the association of PTX3 tetramers into octamers and their linkage through disulfide bonds occur via a chaperone-mediated process (i.e. during biosynthesis).

An unexpected finding of this study was that the PTX3 octamer only has the capacity to bind two FGF2 molecules, where the interaction surfaces are located in the tetramers formed by the N-terminal domains. It had been anticipated that each protomer of PTX3 could provide an independent ligand-binding site (8), allowing the intact protein to bind simultaneously to up to eight identical ligands. As noted in the Introduction, the binding of PTX3 to FGF2 inhibits the proangiogenic activity of this growth factor by blocking receptor interactions (16, 17). Therefore, the relatively high concentration of PTX3 needed for its antiangiogenic function in *in vivo* and *in vitro* assays (16) can be explained, at least in part, by the low stoichiometry of the PTX3-FGF2 complex. From our interaction analysis, it is apparent that both types of tetramer in the N_PTX3 preparation can support the binding of FGF2 (see Fig. 7F). Therefore, it seems probable that the disulfide-linked tetramer and the tetramer made up of a dimer of dimers can each bind to a single FGF2 molecule. On the basis of the PTX3 model shown in Fig. 6 and the functional data described above, it is reasonable to suggest that the FGF2-binding sites are located at the interface of the four globular subdomains found in the N-terminal regions (i.e. at either end of the PTX3 octamer). However, it should be noted that a previous analysis of PTX3 peptides indicated that FGF2 bound to a linear region of PTX3 protein (residues 97–110) that corresponds to the segment between the α1 and α2 helices (29, 52); this peptide, which would be disulfide-linked together in the intact protein via Cys^{103}, acted as a competitive inhibitor of the PTX3-FGF2 interaction when it was monomeric, which is inconsistent with our evidence of a composite binding site on PTX3 for FGF2. In this regard, functional analysis of the C103S N_PTX3 mutant revealed that it retained substantial FGF2-binding activity (supplemental Fig. 7), indicating that Cys^{103} does not play a major role in the interaction of PTX3 with FGF2. However, further structural/biophysical analysis of the PTX3-FGF2 complex is needed to fully resolve this discrepancy.

The complement component C1q has been demonstrated to bind to the C-terminal domain of PTX3 (21, 25). From our schematic model (Fig. 6), it can be seen that these pentraxin domains are all clustered in the central portion of the PTX3...
octamer. Although the stoichiometry of the PTX3-C1q complex is not currently known, in previous studies it has been found that the monomeric C-terminal domain did not support the C1q interaction and that oligomerization (i.e. through chemical cross-linking) was required for binding (25). Therefore, it seems likely that multiple pentraxin domains are necessary for this interaction. The observation that C1q and FGF2 can both bind simultaneously to PTX3 (29) indicates that the interaction surfaces for these ligands are non-overlapping. This is consistent with our PTX3 model in which the C-terminal pentraxin domain and the globular N-terminal segment are well separated. Furthermore, the complement-regulatory protein, component factor H, which binds to both the N- and C-terminal regions of PTX3 (22), is a highly elongated protein, where the individual binding sites in complement factor H (i.e. in complement control protein modules 19–20 and 5–7, respectively) have the potential to be far apart (53).

PTX3 has been identified as a key component of the hyaluronan-rich cumulus extracellular matrix, which forms around the oocyte prior to ovulation (8, 9). In our previous work, we demonstrated that the multimeric organization of PTX3 is essential to this activity in that protein tetramers are the minimal functional units required for cumulus matrix organization (26), in line with its suggested role of forming a “node” where multiple hyaluronan chains are brought together. Clearly, these tetramers must contain at least two ligand-binding sites (e.g. for heavy chains of inter-α-inhibitor, which binds to the N-terminal domain of PTX3 (9)) if PTX3 is to perform this cross-linking function. Although the N-terminal domain preparation used in the present study (composed of a mixture of disulphide-linked tetramers and a dimer or dimers) has not been evaluated for its effect on cumulus matrix formation, it seems probable that at least one of the tetramer types must contain two binding sites for the heavy chains. Thus, the position of the heavy chain interaction surface on the N-terminal domain of PTX3 is likely to be distinct from that of FGF2, although these interactions can both be inhibited by the same monoclonal antibody (9, 29).

In conclusion, this study has provided the first three-dimensional model of the long pentraxin PTX3 based on data from several biophysical techniques. The protein was demonstrated to be composed of eight protomer subunits, forming an asymmetric and elongated molecule in solution, in which two structurally distinct domains are interconnected by a linker region. We have also identified the N-terminal domain of the protomer as the main structural determinant supporting PTX3 oligomerization and have shown that this domain mediates the binding to FGF2, where two molecules of the growth factor bind to a PTX3 octamer. Thus, these studies provide novel insights into PTX3 and pave the way for further investigations into the structure/function interrelationships of PTX3 and the long pentraxins in general.

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PTX3 Quaternary Structure

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