Molecular Weight of Poly(C9)

12 TO 18 C9 MOLECULES FORM THE TRANSMEMBRANE CHANNEL OF COMPLEMENT*

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Poly(C9), the tubular 27 S complex forming the transmembrane channel of the membrane attack complex of complement, was purified to homogeneity by gel filtration and sucrose density gradient ultracentrifugation. The molecular weight of poly(C9) was determined by two independent methods in addition to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. First, sedimentation equilibrium analysis using 0.2% SDS or 1% deoxycholate containing buffer as solvents yielded a point weight average molecular weight exclusive of bound detergent of 0.9 to 1.3 x 10^6 and a weight average molecular weight of all poly(C9) complexes of 1,050,000 ± 40,000 (S.D.). SDS and deoxycholate binding to poly(C9) was measured in an air-driven ultracentrifuge and was determined to be 0.53 ± 0.065 (S.D.) g of SDS and 0.26 ± 0.015 (S.D.) g of deoxycholate/g of poly(C9), respectively. Second, the mass of 27 S poly(C9) devoid of bound detergent was determined by electron scattering of unstained specimens in the scanning transmission electron microscope. The molecular weight obtained by this method was 1,078,000 ± 194,000. The inner diameter of poly(C9) tubules imaged in top view projections by negative staining electron microscopy varied between 9 and 12 mm. The accumulated data suggest a true heterogeneity of the molecular weight of poly(C9) due to polymers with varying protomer numbers.

Using a mean value of 73,500 for the molecular weight of monomeric C9, the protomer number of poly(C9) tubules appear to vary between 12 and 18 C9 subunits. Approximately 50–75% of the tubules have 14 to 16 subunits as deduced from the mass distribution determined by electron scattering and from ring size measurements.

It is suggested that poly(C9) tubules with various protomer numbers may arise due to limited flexibility in the C9-C9 interaction.

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The membrane attack complex of complement may be defined as an association product of poly(C9) with C5b-8 (1, 2). The C5b-8 complex is assembled on target membranes by the recognition and activation steps of the classical or alternative pathway of complement. The function of C5b-8 is to direct C9 to the target membrane and to promote C9 polymerization (1). Poly(C9) assembled by C5b-8 or by spontaneous polymerization (3, 4) apparently forms a transmembrane channel and disrupts membranes by reorganization of the lipid bilayer (5–8).

Isolated poly(C9) formed by spontaneous polymerization of purified C9 has characteristic properties that are shared by poly(C9) in the MAC. Poly(C9) is a tubular complex with 9- to 12-nm internal diameter and 16- to 18-nm length. The tubule is rimmed on one end by a torus of ~21-nm outer and ~10-nm inner diameter (3). The opposite end of the tubule is terminated by a ~4-nm long hydrophobic domain (3). Electron microscopical projections of the poly(C9) tubule appear as rectangles (side views) or ring structures (top views) (3), and these images are characteristic also for the cylindrical part of the MAC (2, 4, 9, 10). Monomeric C9 is hydrophilic and water soluble. Polymerization of C9 is accompanied by a hydrophobic-amphiphilic transition that may be mediated by restricted unfolding of C9 and expression of hydrophobic domains. The hydrophobic-amphiphilic transition during C9 polymerization permits membrane insertion of poly(C9) followed by the release of membrane-entrapped markers (4, 5). Poly(C9) in isolated form and in the MAC contains increased α-helix (5) and expresses C9-specific neoantigens (1) suggesting conformational rearrangement upon C9 polymerization.

Poly(C9) in the MAC, as well as isolated poly(C9), is resistant to dissociation by 5-min boiling in 2% SDS even under reducing conditions (1, 12). Resistance to dissociation by SDS is a property of circular poly(C9), whereas linear (noncircular) poly(C9) is susceptible to SDS dissociation (12).

Monomeric C9 has a molecular weight of 71,000 as determined by SDS-polyacrylamide gel electrophoresis (13) and of 79,000 as calculated from its diffusion and sedimentation constants (14). Homogeneous circular poly(C9) sediments with a sedimentation coefficient of 27 ± 1 S in detergent solution and migrates on SDS-polyacrylamide gels with an apparent molecular weight of ~1.1 x 10^6 (12). The precise knowledge of the molecular weight of poly(C9) and of monomeric C9 is not only of importance for the determination of the protomer number of poly(C9) but also for the evaluation of the structure of the MAC. Previous molecular weight
determinations of the MAC have given conflicting results (10, 11, 15, 16).

**MATERIALS AND METHODS**

C9 was purified and polymerized as described previously (10, 12, 17). SDS-resistant circular 27 S poly(C9) was separated from other C9 polymers exactly as described (12). [3H]deoxycholate and [3H]sodium deoxycholate were obtained from Amersham Corp.

Deoxycholate—0 to 80 µg of poly(C9) were mixed with 220 µl (final volume) of 0.2% [3H]deoxycholate (final concentration) using a vacuum generator (STEM HB5 interfaced to a minicomputer as determined in preliminary experiments with 125I-poly(C9). When 0.2 mM EDTA, 0.02% NaN3, pH 8.1. In some experiments monomeric C9 was substituted for poly(C9). [3H]SDS was used at 0.2% final concentration in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4. SDS-containing samples were incubated for 5 min at 100 °C. Deoxycholate-containing samples were incubated for 1 h at 37 °C. 50-µl aliquots were then withdrawn and counted for radioactivity. The remaining 170 µl were pipetted into centrifuge tubes and centrifuged for 25 min at 20 °C at 100,000 rpm in an Airfuge (Beckman Instruments). This time was sufficient to completely sediment 27 S poly(C9) as determined in preliminary experiments with 125I-poly(C9). When monomeric C9 was polymerized to 13 ± 3 S polymers by incubation with deoxycholate, as described in Ref. 3, a 60 min centrifugation time was necessary for complete sedimentation. After centrifugation, a 50-µl aliquot was withdrawn from the top of the supernatant layer and again counted for radioactivity. The diminution of radiolabeled detergent in the 50-µl aliquot after centrifugation was used as a direct measure of protein-bound detergent cosedimenting with poly(C9). The values in Table I were corrected for the small amount (<5%) of detergent lost during due to sedimentation of detergent micelles in the absence of protein.

**Sedimentation Equilibrium Analysis**—A Beckman analytical ultracentrifuge equipped with a xenon light source and a photoelectric scanner was employed for sedimentation equilibrium measurements. 27 S poly(C9) in 1% deoxycholate and Tris-buffered saline, pH 8.1, and in 0.2% SDS and TBS, respectively, was diluted to an optical density at 280 nm of 0.3 corresponding to approximately 0.3 mg/ml (1). 60 µl of these solutions were filled in double-sector cells. The second sector received the corresponding buffer without protein. In each run purified IgM (provided by Dr. H. Ziccardi of this department) was included as a control. Molecular weight for IgM was 905,000 ± 40,000 in all experiments. The samples were centrifuged at 3400 rpm and 20 °C for 1 to 3 days in an An-J rotor. Monomeric C9 in TBS was first checked for its homogeneity by a sedimentation velocity run (αw0.5 = 4.5 S) and then centrifuged in an An-G rotor at 10,000 to 13,000 rpm. The temperature was kept at 19 °C to avoid C9 polymerization. After equilibrium was attained, the protein concentrations in the cells at the various radial positions were determined by measuring the absorbance at 280 nm with the photoelectric scanner. Subsequently the base-line was established by sedimenting poly(C9) or monomeric C9, respectively, at high speed and scanning the 280-nm absorbance of the protein-free buffer solution. The molecular weight of monomeric C9 was calculated from a least squares fit of the absorbance versus the square of the radial distance using a computer program. Due to heterogeneity of poly(C9), this method was not applied for the determination of the weight average molecular weight of poly(C9). Instead, the molecular weight was determined by determining the concentration of the protein at the meniscus and the bottom as suggested by Lansing and Kraemer (25). Point number average molecular weights were calculated by computing the slope at the position of the central point of five points from a least squares fit through the 25 points as discussed in Ref. 26. A partial specific volume of 0.72 ml/g for C9 was calculated from its amino acid and carbohydrate composition (18). Correction for detergent binding of poly(C9) was done according to Tanford et al. (19) and is described under "Results." 

**RESULTS**

**Molecular Weight Determination of Circular Poly(C9)**

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A Chromatix (Chromatix, Eching, FRG) 632.8-nm laser light-scattering apparatus was employed for the determination of the molecular weight of monomeric C9. The intensity of the scattered light was measured at an angle of 6–7° with respect to the incident laser beam allowing the direct evaluation of the molecular weight of a particle.

C9 aggregates were separated from monomeric C9 by high performance gel filtration (22) after dialysis in 0.1 M acetic acid, 20 mM CaCl2. The use of this buffer instead of TBS resulted in a better separation of aggregates. An 0.4-ml sample of C9 (0.5 mg/ml) was pumped onto a LKB Ultratrace TSK G 3000 SW column which was directly connected to a photometer reading the absorbance and to a refractometer measuring the refractive index of the effluent. The sample was then passed through an 0.1-µm filter to the light-scattering cell. The molecular weight M of the protein was calculated by using the following equation (25),

\[ M = \frac{MR}{Nk_c} \]

where \( k \) is an optical constant (as defined in the instrument manual), \( R \) the relative intensity of scattered light, and \( c \) the weight concentration of the protein. \( c \) was calculated from both the absorbance at 280 nm and the refractive increment of C9 as measured directly before the sample was pumped into the light-scattering apparatus. The refractometer was calibrated with bovine serum albumin and in controls its molecular weight varied between 66,000 and 67,000.

**Measurement of the Inner Diameter of Poly(C9) Tubules—Poly(C9)**

was applied to electron microscopy grids and, after washing to remove detergent micelles, negatively stained with 2% uranyl formate. Images of poly(C9) were obtained at 60,000-fold direct magnification in a Hitachi 12A microscope. Top view projections (ring structures) of individual poly(C9)s on several micrographs were selected based on symmetrical round appearance, and the inner diameters measured in two directions at right angles. The mean value of both diameters was used for the histogram in Fig. 6.

**RESULTS**

**Molecular Weight Determination of Poly(C9) and C9 by Sedimentation Equilibrium Analysis—Poly(C9) formed by spontaneous polymerization of C9 at 37 °C was incubated with 1% SDS for 5 min at 100 °C, and SDS-resistant tubular poly(C9) was isolated by gel filtration and sucrose density gradient ultracentrifugation as described (12). Fig. 1 shows the ultrastructure, SDS-polycyclamide gel electrophoresis, and sedimentation velocity analysis in the analytical ultracentrifugation of a typical poly(C9) preparation used for the subsequent molecular weight determinations. It will be referred to as 27 S poly(C9). Since 27 S poly(C9) is an amphiphilic complex it requires the presence of detergents for its solubility. Preliminary experiments showed that 1% (w/v) sodium deoxycholate at pH 8.1–8.7 or 0.2% SDS at pH 7.2 were suitable to keep 27 S poly(C9) in solution without apparent aggregation or dissociation. On the other hand, 0.1 M octylglucoside and 1% Triton X-100 did not completely prevent aggregation of poly(C9) and resulted in part of poly(C9) sedimenting in a broad distribution at greater than 27 S.

Unambiguous molecular weight determinations of amphiphilic proteins by sedimentation equilibrium analysis in detergent solution require knowledge of the partial specific volume of the protein (\( \psi_p \)) and of the detergent (\( \psi_D \)) in addition to the amount of protein-bound detergent (\( \psi_0 \)). [3H]SDS and [3H]deoxycholate binding to 27 S poly(C9) was determined in the air-driven centrifuge which has a k factor of 11 at 100,000 rpm. Thus, the clearing time (i.e. the time for complete
sédimentation) for 27 S poly(C9) is 25 min at 100,000 rpm in this centrifuge. The detergent-binding assay used is based on the cosedimentation of bound detergent with poly(C9) resulting in the partial depletion of detergent from the upper part of the centrifuge tube due to the differential sedimentation velocity of free and bound detergent micelles. Fig. 2 and Table I show the result of several experiments measuring deoxycholate (Fig. 2, top) and SDS (Fig. 2, bottom) binding to 27 S poly(C9). Deoxycholate binding was also determined with monomeric C9 by centrifugation for 60 min (Fig. 2, top, open circles). Incubation of monomeric C9 with deoxycholate caused formation of noncircular C9 polymers that sediment with 13 ± 3 S (3). These C9 polymers bound similar amounts of deoxycholate on a weight basis as 27 S poly(C9) (Table I). Deoxycholate binding to both 27 S and 13 S poly(C9) was in the range of 0.25 to 0.28 g/g of poly(C9). SDS binding to 27 S poly(C9) was determined similarly after 2-min boiling of poly(C9) with 0.2% [35S]SDS. The values obtained ranged from 0.47 to 0.6 g of SDS per g of poly(C9) (Table I). In contrast to deoxycholate, SDS does not induce polymerization of monomeric C9, and SDS binding to monomeric C9 could not be determined by this method because a substantial amount of free SDS micelles sedimented during the period it took (~3 h) to centrifuge down monomeric C9 in SDS solution.

For sedimentation equilibrium analysis 27 S poly(C9) that had been checked for homogeneity by a sedimentation velocity run (see Fig. 1) was adjusted to an optical density at 280 nm of 0.3 and exhaustively dialyzed against 1% deoxycholate containing buffer at pH 8.1 or with 0.2 M SDS in TBS at pH 7.2. Two parallel samples containing poly(C9) were run simultaneously with a third sample containing purified IgM as control. After equilibrium was established, the optical density determined with an optical scanner at 280 nm was plotted versus the radial position (Fig. 3A). A curvature was observed in the dependence of \( \ln c \) on \( r^2 \) suggesting heterogeneity in the poly(C9) sample. These data points could not be analyzed...
Analysis of monomeric C9 by inelastic light scattering and sedimentation equilibrium analysis of monomeric C9 at 10°C in the absence of detergents yielding a value of 77,000 ± 4,000. Analysis of monomeric C9 by inelastic light scattering immediately subsequent to high performance gel filtration to remove C9 aggregates gave a molecular weight value of 71,000 ± 4,000 (Table II). Table II summarizes the molecular weight of monomeric C9 obtained by various techniques. Mass Determination of Poly(C9) in the STEM—Purified 27 S poly(C9) in deoxycholate solution was absorbed to a thin carbon film and after washing and air drying imaged in the STEM without negative contrasting. Fig. 4, A and B, shows the low dose dark field image of a typical preparation. Globular structures of circular or slightly elongated appearance with a diameter of approximately 25 nm are seen which may be interpreted as top views or side views of poly(C9), respectively. Some larger and consequently brighter structures suggest the presence of poly(C9) aggregates possibly formed during the grid preparation. Individual particles with diameter close to 25 nm were selected for further analysis as indicated by boxes in Fig. 4B. The mass values from 220 particles were accumulated in a histogram (Fig. 4C), and 75% of all particles evaluated are displayed in Fig. 4D. The average molecular weight (1,078,000) corresponds to 14–15 subunits per poly(C9) complex. An estimate of the statistical error due to shot noise and carbon film noise yields 50,000 under the recording conditions in these experiments. Thus, the standard deviation of 194,000 indicates a remarkable heterogeneity of the sample, in agreement with results from the sedimentation equilibrium analysis.

A heterogeneous protomer number as suggested from the molecular weight determinations should also affect the diameter of the poly(C9) tube. To test this hypothesis, top views of negatively stained poly(C9) (Fig. 5) were selected and their inner diameters measured in two directions at right angles. The inner diameter was between 10 and 11 nm in 76% of all poly(C9) rings analyzed (Fig. 6). Rings exhibiting a diameter of 9 and 12 nm had a frequency of 6 and 18%, respectively.

**Discussion**

Molecular weight determinations of amphiphilic proteins by standard techniques such as gel filtration, sedimentation analysis, or SDS-PAGE are notoriously unreliable. The problem arises from the binding of detergent which affects the hydrodynamic properties of the amphiphile by increasing the molecular weight and by altering the partial specific volume. Molecular weight determinations by SDS-PAGE are unreliable due to the often anomalous binding of SDS to hydrophobic proteins. In the case of poly(C9), these problems are aggravated by the large size of the poly(C9) tube which requires polycrylamide gels of high porosity. The lack of suitable high molecular weight markers for the calibration of SDS-polycrylamide gels allows only a rough estimate of the molecular weight of poly(C9) by this technique.

Alternative techniques for a precise molecular weight determination of poly(C9) were, therefore, sought, and the results of two independent methods are described here. The combination of sedimentation equilibrium analysis and measurement of detergent binding described by Tanford et al. (19) allows a good approximation of the molecular weight of poly(C9) corrected for the effect of detergent binding. In addition, the amount of protein-bound detergent gives information on the size of the hydrophobic membrane-binding domain of poly(C9). We found that poly(C9) binds 0.25 ± 0.015 g of deoxycholate/g of protein, corresponding to 720 deoxycholate molecules/27 S poly(C9) complex. This indicates that poly(C9) has a sizable hydrophobic domain in agreement with phospholipid-binding studies (24) and with previous analyses indicating that 4 nm of the poly(C9) tube opposite to the torus constitute the hydrophobic domain of poly(C9). The combined molecular weight of the deoxycholate-poly(C9) complex is thus approximately 1.4 × 10^6. The binding of SDS to 27 S poly(C9) was determined to be 0.53 ± 0.0165 g/g of protein. SDS binding, however, occurs not only to the hydrophobic domain but in all probability also to the poly(C9) torus (12) and to other sites. The ultrastructural analysis described in a previous publication (12) indicates
that the poly(C9) torus is unfolded by SDS, thus transforming the 16-nm long poly(C9) to 22-nm long tubules (compare also Figs. 1 and 4). Despite the differential binding of deoxycholate and SDS to poly(C9) with the accompanying effects on the partial specific volume, the values for the weight average molecular weight of poly(C9) exclusive of bound detergent by sedimentation analysis in both detergents are in good agreement and further support the validity of the results.

Weight average molecular weights calculated at different points within the cell showed a concentration dependence in all cells analyzed. The point average molecular weight varied in the range of 0.9 to $1.3 \times 10^6$ indicating that the poly(C9) complexes are not homogeneous.

Mass determination of proteins by electron scattering of
FIG. 5. Negative staining electron micrograph of “native poly(C9)” not incubated with SDS. Boxed poly(C9) complexes imaged in top views were selected for determination of the inner diameter. Scale bar, 114 nm.
unstained specimens allows the measurement of the molecular weight of individual complexes. Averaging the large numbers of complexes gives a precise molecular weight of poly(C9) in addition to the distribution of the molecular weights among individual poly(C9) rings. The resolution of the ultrastructural image, however, is lower than that obtained by the negative staining technique due to the lack of contrast in the absence of heavy metals, due to the poor preservation of the sample in absence of embedding salt, and due to the low recording dose employed. The histogram in Fig. 4 shows that the majority of 27 S poly(C9) complexes have a mass of $1.01 \times 10^6$ (14-15 C9 monomers) but that complexes in the range from $8 \times 10^5$ to $1.4 \times 10^6$ (19 C9 subunits) are found. Since the complex under analysis is a polymeric product of C9, it is suggested that the protomer number in poly(C9) in fact is variable to the extent of 11-19 subunits.

The inner diameters of poly(C9) rings imaged by the negative staining technique suggest less variation in the poly(C9) protomer number. Since this method depends on the selection of symmetrical ring structures for measurement it may be biased toward a narrower range of protomer numbers.

Additional evidence for a variable subunit number in poly(C9) was obtained by SDS-PAGE analysis reported in an earlier paper (12). It is consistently found that the $M_r = 1.1 \times 10^6$ band of isolated poly(C9) or of poly(C9) in the MAC upon SDS-polyacrylamide gel electrophoresis consists of 2-5 closely spaced bands which may be interpreted to indicate different sizes of poly(C9) due to the presence of complexes with different subunit numbers. The accumulated data can be interpreted with great confidence to indicate that the majority of 27 S poly(C9) has a protomer number of 14-16 with a range of 11-19 C9 molecules/tubule. This value is in excellent agreement with measurements of the binding of (C9) molecules to C5b-8-bearing target cells (1). In these studies it was found that a single C5b-8 complex can bind 12-15 C9 molecules and that C9 binding is accompanied by the formation of the ring-like structure characteristic for the classical complement membrane lesion. A dodecameric composition of poly(C9) was deduced previously by visual inspection of negatively stained specimens (4). This value, in the light of the present results, represents only one of the possible forms of poly(C9).

The molecular weight of poly(C9) is of importance not only for the structure of the transmembrane channel that it forms but also for considerations of the structure of the MAC. The MAC may be described as a poly(C9) channel associated with one or more C5b-8 complexes (2). The calculated molecular weight of (C5b-8)poly(C9) is $1.6 \times 10^6$ and of (C5b-8)poly(C9), $2.1 \times 10^6$. The molecular weight of 1 million for the monomeric C5b-9 complex containing all subunits upon SDS-PAGE and showing a typical tubular structure as reported by Bhakdi et al. (15) is at variance with our data and remains to be explained. Higher molecular weights of the MAC as reported by other laboratories (16) may be interpreted to represent fusion products of several C5b-8 complexes with poly(C9) or as larger polymerization products of several poly(C9)s with several C5b-8 complexes. In any event, it is clear that the elucidation of the structure of the MAC formed on natural membranes under physiological conditions requires a careful re-evaluation, taking into account the characteristic properties of poly(C9) reported here and in preceding publications.

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