Small angle neutron scattering studies of C8 and C9 and their interactions in solution

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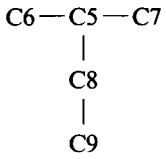
ABSTRACT Small angle neutron scattering (SANS) results revealed that contrary to most reports C9 is not a globular protein. Its radius of gyration (R_g) at pH 8 and an ionic strength of 0.5 is 32.2 ± 1.4 Å increasing to 35 Å at physiologic ionic strength. In contrast, C8, which has a 2.2-fold larger mass, has a similar R_g value [34.6 ± 1.6 Å]. Calibration plots of R_g vs. M_r indicate that native C8 is a spherical protein whereas native C9 is elongated. From previous reports it was known that native C8 and C9 associate in solutions of low ionic strength. SANS results confirmed this observation but also demonstrated that C8-C9 heterodimers are already formed at physiologic ionic strength. The dimeric complex is globular [R_g = 40 ± 0.8 Å] indicating that the proteins associate side-by-side rather than end-to-end. In contrast, in presence of the drug Suramin, a potent inhibitor of the assembly of the C5b-9 complex, C9 forms a complex with twice the molecular mass that is still elongated (R_g = 48.8 ± 0.8 Å), suggesting that in this case the protein dimerizes end-to-end via a bridging Suramin molecule.

INTRODUCTION

The studies of Kolb and co-workers established the central role of complement proteins C5, C6, C7, C8, and C9 in the formation of the cytolytic C5b-9 complex, also known as the membrane attack complex (MAC) of complement (1, 2). A key observation in these early studies was the finding that these proteins, in their native form, would interact with each other provided that the ionic strength of the solution was much less than physiological. Using sedimentation rate centrifugation in sucrose density gradients it was found that C8 would interact with C5 and C9 but not with C6 or C7, C9 would interact only with C8, and C6 and C7 only with C5 but not with each other. From such an interaction pattern Kolb et al. (2) concluded that the MAC had the following subunit structure:



These results were verified by Sodetz and colleagues (3–5) who also established that the C8α chain interacts with C9 and the C8β chain with C5 and that the binding site on C5 for C8β is located on the C5b fragment. Using analytical ultracentrifugation Podack et al. (6) determined a one-to-one stoichiometry for the C8-C9 interaction in solution, which was an important observation since multiple C9 molecules can be present in the membrane-bound C5b-9 complex (7). C8α and C9 are Ca^{2+} binding proteins, however, the interaction between the two proteins is not dependent on the presence of the ion (8).

A caveat inherent in interpreting the results from all these studies is the fact that the experiments had to be carried out at reduced ionic strength in order to observe the interactions. Apparently the binding constants for the formation of these protein pairs at physiological conditions are too low to withstand the force generated during centrifugation. We have now used small angle neutron scattering (SANS) to study the interaction between C8 and C9 under physiological conditions. This method has the advantage that no extraneous forces are present during the experiment that might perturb weak protein-protein interactions. Furthermore, the technique allows detection of molecular shape changes as a result of complex formation that are otherwise difficult to observe (9, 10). We have limited our study to C8-C9 interactions since these two proteins are responsible for the cytolytic function of the complex, and more importantly, it has been postulated that C9 doubles in length after it binds to C8 on a membrane and enters the hydrophobic lipid bilayer (11). A preliminary account of this work has been presented (12).

MATERIALS AND METHODS

Proteins. Human C8 and C9 were purified either from Cohn Fraction III (Cutter Laboratories) or from ACD plasma as described by Esser and Sodetz (13). C9 was also isolated from plasma as described but substituting the final hydroxyapatite chromatography step in the C9 isolation procedure with ion exchange chromatography on FPLC Mono S and Mono Q columns (Pharmacia, Piscataway, NJ) and keeping 0.5 mM Ca^{2+} ions in all buffers. C9 and C8α are calcium-binding proteins (8) and especially C9 is more stable in the presence of Ca^{2+} ions (14). All protein solutions, however, were gel filtered on Sephacryl S-200 equilibrated with 10 mM Mops, 150 mM NaCl, pH 7.0 just before the SANS studies. Both proteins were better than 99% pure based on SDS PAGE analysis and staining with Coomassie blue. C8 and C9 concentrations were determined by absorbance measurements at 280 nm using extinction coefficients of ϵ 1%/cm = 14.9 for C8 and ϵ 1%/cm = 9.6 for C9 (13) and M_r values of 150,000 for C8 (13) and of 70,000 for C9 (13).

Neutron scattering. Small angle neutron scattering (SANS) experiments were done on the D11 camera (15) at the Institut Laue-Langevin in Grenoble. Neutron wavelength, λ, was 10 Å with an 8% spread

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and sample detector distance was 2.8 m to give a Q range from 0.01 to 0.08 $Å^{-1}$, where $Q = 4\pi \sin \Theta/\lambda$, and Θ is half the scattering angle. Samples were contained in 1.00 mm path length quartz cuvettes (Hellma, Jamaica, New York) thermostated at 6°C. Protein concentration was determined by measuring the absorbance at 280 nm of the protein solution in the same cuvettes used for SANS studies and the measurements were repeated after the experiment to verify the absence of solvent evaporation. Protein aggregation during the experiment was assessed by comparing the ratio of A_{250}/A_{280} before and after the experiment.

The intensity, $I(Q)$, of neutrons scattered by the protein particles in solution was obtained from the sample scattering after subtraction of the buffer contribution and was corrected for detector response and put on an absolute scale by normalizing by the incoherent scattering of 1.00 mm of H_2O (16). The neutron scattering contrast radius of gyration, R_g, and forward scattered intensity, $I(0)$, were obtained from the $I(Q)$ data at the smaller Q values, by applying the Guinier approximation (17):

$$\ln I(Q) = \ln I(0) - (1/3)R_g^2Q^2.$$

Data were plotted as $\ln I(Q)$ vs. Q^2 and straight lines were fitted to the chosen Q range by least squares. Typical Q ranges corresponded to $0.5 < QR_g < 2$. The molar mass **M** [g/mol] of the solution particle was calculated from the $I(0)/c$ values (where c is protein concentration in mg/ml) by using the formula given in Jacrot and Zaccai (16), and the scattering length per mole of protein was calculated from its chemical composition. In the case of a polydisperse solution of particles of the same chemical composition, the apparent molar mass, **M**, calculated from $I(0)/c$ is given by: $\mathbf{M} = c_1\mathbf{M}_1 + c_2\mathbf{M}_2 + c_3\mathbf{M}_3 + \cdots$, where there are c_1 [mg/ml] of particles of molar mass $\mathbf{M}_1$, c_2 of $\mathbf{M}_2$, etc.

RESULTS

SANS data were collected from protein solutions in H_2O buffers with concentrations close to 1 mg/ml. This is the smallest concentration possible for SANS studies and no studies were performed at lower concentrations. Under these conditions, the weak signal to noise ratio did not allow measurements to be made beyond Q of about $3/R_g$, and only two parameters (**M** and R_g), calculated from the Guinier plots, could be determined. Nevertheless, these are two extremely useful independent quantities, because they are obtained on an absolute scale and they are very sensitive to the size and shape of the solution particles and therefore of their interactions. Furthermore, because of the very small coherent scattering of H_2O, the analysis on an absolute scale is insensitive to assumptions concerning the partial specific volumes of the particles (16). The hydrodynamic analogy is that of an experiment in vacuum, in which there is no buoyancy term.

Table 1 lists the R_g and **M** values calculated for human C9 and C8 under different environmental conditions and Fig. 1 shows some of the recorded experimental data from which the tabulated values were calculated. The **M** values are in excellent agreement with molecular weight determinations for C8 and C9 using other techniques. For example, molecular weight values for monomeric C9 range from 79,000 as determined from S and D (18), to 70,000 ± 5,000 measured by light scattering (19), and the value calculated from the amino acid sequence and the amount of N-linked sugars is 66,000 (11). There are also O-linked sugars in C9 but the total amount is not known (11). Careful sedimentation equilibrium centrifugation yields an M_r value of 70,000 ± 3,000 (Esser and Nagel, unpublished results). Previous molecular weight values for C8 are all very close to 150,000; for example, Sodetz and colleagues reported 151,000 ± 8,000 by sedimentation equilibrium ultracentrifugation (20) and the value calculated from the amino acid composition and the amount of N-linked sugars (21) is 146,240.

TABLE 1 **Radius of gyration and molecular mass of C8 and C9**

Protein	pH	μ	Addition	R_g [Å]	M [Da]
C8	8.0	0.5	—	34.6 ± 0.5	150,000 ± 2,100
C8	8.0	0.16	—	35.5 ± 0.4	176,000 ± 2,000
C9	8.0	0.5	—	32.2 ± 1.4	73,900 ± 3,800
C9	8.0	0.16	—	35.2 ± 1.6	73,600 ± 3,300
C9	8.0	0.16	Suramin	42.8 ± 1.0	155,900 ± 4,000
C8 + C9	8.0	0.16	—	39.9 ± 0.8	206,700 ± 5,200
C8 + C9	8.0	0.5	—	34.4 ± 0.7	126,400 ± 2,500
C8	7.2	0.16	—	37.1 ± 0.5	221,000 ± 2,500
C9	7.2	0.16	—	38.1 ± 1.3	75,600 ± 3,100
C9	7.2	0.16	Suramin	48.8 ± 0.8	187,000 ± 3,300
C8 + C9	7.2	0.16	—	40.9 ± 0.6	242,500 ± 3,800

From the work of Tschopp and Masson (22) it is known that the polyanionic drug Suramin inhibits formation of the MAC. Inclusion of 10-fold molar excess of Suramin to C9 solutions results in an increase of R_g to 43 Å and a doubling of **M**, indicating that C9 forms a dimer under these conditions (Table 1 and Fig. 2). When C8 and C9 are mixed in buffers of physiological ionic strength they form a one to one complex with a mass of about 207 kD and complex formation is inhibited when the ionic strength is raised to 0.5 (Table 1 and Fig. 3). Surprisingly, there is only a small increase in the radius of gyration for the C8-C9 complex and the complex remains globular (Fig. 4). Dialysis of C9 into D_2O buffers results in severe aggregation of the protein (Fig. 5).

DISCUSSION

The molecular weights of C9 and C8 as determined by SANS are in excellent agreement with previously published values using different techniques. Therefore, considering the large difference in mass between the two proteins it is striking that they have the same radius of gyration at pH 8 and physiological ionic strength, that is 35.5 ± 0.4 and 35.2 ± 1.6 Å, respectively. Both proteins appear to become slightly more compact at higher ionic strength since these values decrease to 34.6 ± 0.5 Å and 32.2 ± 1.4 Å. Thus, C9 is not a compact globular protein. This interpretation is corroborated by results from a sufficient number of SANS studies on other globular proteins. As was pointed out by Zaccai and colleagues (23), for globular proteins a good correlation exists between M_r and R_g, that is, a doubling of R_g requires an approxi-

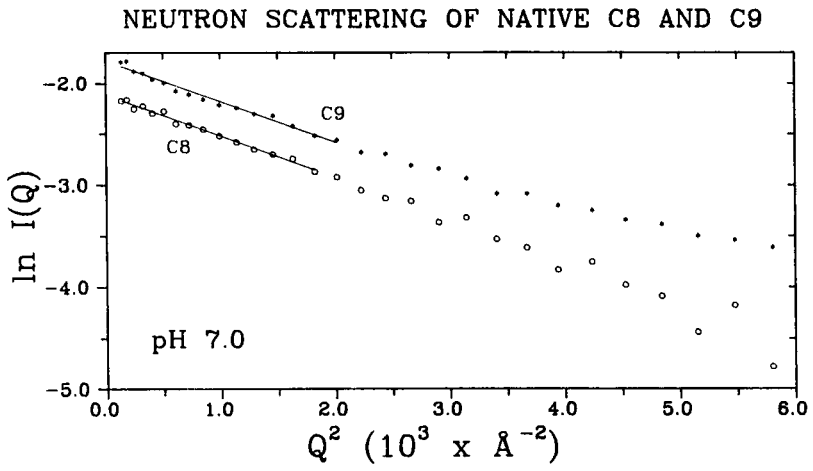

FIGURE 1 Neutron scattering data plotted in the Guinier approximation for native C8 (○) at 1.6 mg/ml and C9 (*) at 1.2 mg/ml at 6°C, in 10 mM Mops, 150 mM NaCl buffer, pH 7.0. See text for further experimental conditions.

mate five fold increase in M_r, and one can construct a "calibration" curve relating M_r to R_g for globular proteins (Fig. 4). Based on this correlation one can classify C8 as a globular protein. This conclusion is unexpected since C8 is a three chain protein consisting of α, β and γ polypeptides with molecular masses of 64, 64, and 22 kD that are made from different genes (21). Multisubunit proteins of this size are mostly not globular. In contrast, the single chain C9 protein, which has frequently been considered to be a globular protein, is unquestionably elongated. Monomeric C9 is thought to elongate when it enters a membrane during formation of the MAC (11, 19, 24). Our results raise the possibility that such changes might be far less drastic than had been envisioned previously.

More parameters could have been calculated from $I(Q)$ to larger Q values (25), but for such data to be reliable, protein concentrations of ~10 mg/ml are required. Unfortunately, C9 protein solutions were aggregated beyond about 2 mg/ml in agreement with many other studies that reported a tendency of C9 to polymerize when concentrated (26–28). Our own previous studies had shown that C9 aggregation is retarded at higher ionic strength (8) and at higher pH (14) and for these reasons most studies were performed at pH 8, and statistically the most reliable R_g value for C9 was obtained at an ionic strength of ~0.5.

Another useful approach would have been to study proteins in D_2O buffers, in which the signal to noise ratio is greatly enhanced (29). The problem with such studies, however, is the fact that D_2O stabilizes hydrophobic interactions and thereby can promote protein aggregation. This was observed for many of the complement proteins studied thus far and was now encountered likewise for C9 (Fig. 5), making this approach very hazardous. In this respect our data on C9 differ significantly from those reported by Perkins and colleagues (30, 31). They report SANS data obtained for C9 in 100% D_2O and at protein

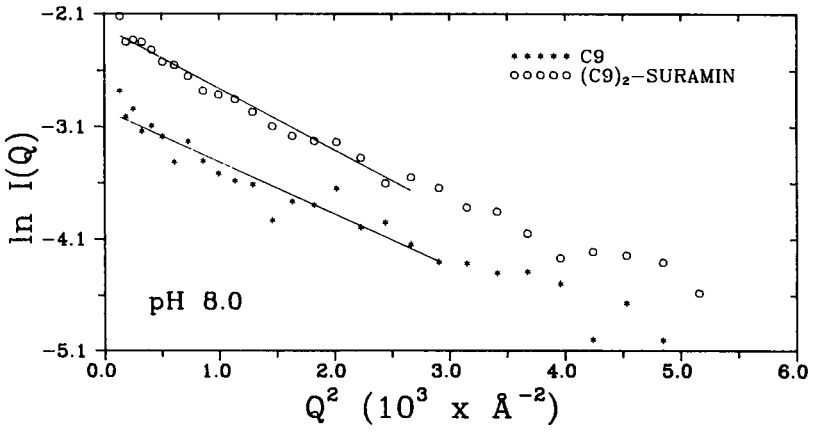

FIGURE 2 Guinier plots for native C9 (1.4 mg/ml) with (*) and without (○) a 10-fold molar excess of Suramin in 10 mM Mops, 150 mM NaCl buffer, pH 8.0.

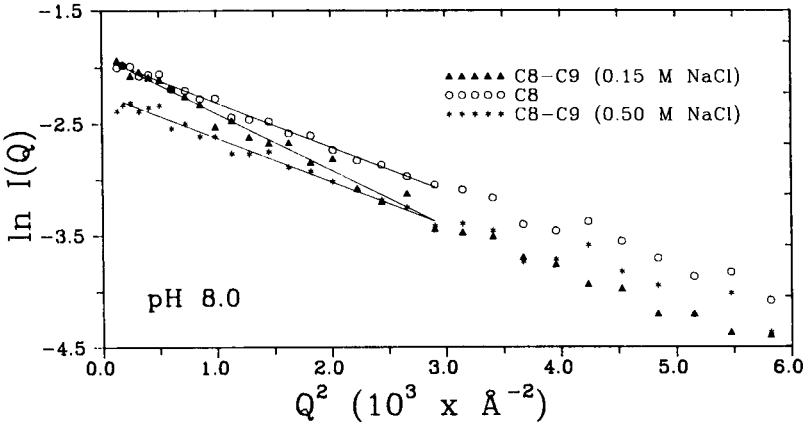

FIGURE 3 Guinier plots for equimolar mixtures of C8 and C9 in (○, ▲) 10 mM Mops, 150 NaCl, pH 8.0 buffer or in (✱) 10 mM Mops, 150 mM NaCl, 200 mM Na_2SO_4, pH 8.0 buffer.

concentrations as high as 7.6 mg/ml. Our C9 preparations could not be concentrated to more than 2 mg/ml and substitution of H_2O with D_2O resulted in immediate aggregation of C9. We used several techniques, such as slow dialysis of C9 into D_2O buffers, or quick exchange using spinner columns, exchange at room temperature or at 4°C, but none were successful and C9 could not be examined in D_2O buffers. This result is not too surprising since C9 is known to be amphipathic (28) and the hydrophobic character of the protein would be enhanced in D_2O. The reason for these differences between our data and those of Smith et al. (31) is unknown, although a major difference is the inclusion of 1 mM EDTA in the buffers used by them. Therefore, while it is clear that their values are representative of the apo-protein and not the native, Ca-containing protein, it is, however, doubtful that 1 mM EDTA would be able to prevent C9 aggregation either at high protein concentration or in D_2O buffers. It is known that EDTA causes formation of C9 aggregates (28) or tubular polymerized C9 at higher protein concentration (27). Smith et al. (31) indicate that their protein was ~95% pure whereas we could not detect impurities in the C9 preparations used for this work on Coomassie blue-stained SDS polyacrylamide gels. It may be possible that unknown impurities prevented C9 association in their experiments. In the present study, therefore, we interpreted the M and R_g values from different solutions of ~1 mg/ml of protein, all in H_2O buffers and with well defined pH and salt content, and we have refrained from modeling studies aimed at providing molecular details of proteins that are beyond the resolution of the technique and the accuracy of the experimental data.

How the three C8 subunits combine to form a globular protein remains an interesting question especially con-

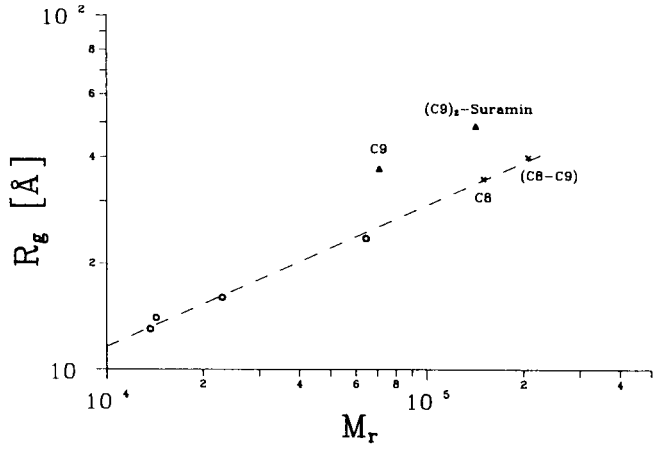

FIGURE 4 Dependency of the radii of gyration on their molecular wights. The line (R_g^3 = constant × M_r) in logarithmic coordinates is for globular proteins (○) such as ribonuclease (R_g = 13; M_r = 13,900), lysozyme (R_g = 14; M_r = 14,300), papain (R_g = 16; M_r = 23,000) and hemoglobin (R_g = 23.5; M_r = 64,500). For further details see reference (23).

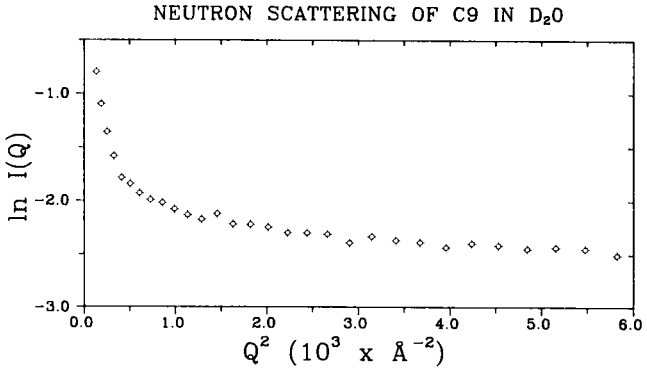

FIGURE 5 Guinier plots for C9 (1.2 mg/ml) in a D_2O buffer containing 10 mM Mops, 150 mM NaCl, pD 7.0.

sidering the fact that C8 α, C8 β, and C9 are evolutionary closely related polypeptides. One would expect them to have a similar three dimensional folding since they share a mosaic structure in the sense that they are made from similar domains or modules:

C8α: NH_2—T—L—(lb)—G—T—COOH

C8β: NH_2—T—L—(lb)—G—T—COOH

C9: NH_2—T—L—(lb)—G—COOH.

The nomenclature for these modules (T, L, G) was offered by Baron et al. (32) who also proposed that the structure of such domains is preserved in different proteins; (lb) is a putative lipid binding domain but not necessarily a module.

Even more intriguing is the fact that when C9 associates with C8 the resulting complex remains globular (Fig. 4). Careful experiments by Sodetz and colleagues (3–5) revealed that in solution C9 has an affinity only for C8α and not for C8β nor C8γ. This result together with the observation that the C8-C9 dimer is globular would indicate that C8 is in close contact with C9 along the latter's longer axis otherwise the dimer would not be globular. At higher ionic strength C8 and C9 do not associate as indicated by the fact that R_g is unchanged and that the measured mass corresponds to the mass proportions of the two proteins in the solution (Fig. 3 and Table 1). Previously, the association of C8 and C9 could only be observed at reduced ionic strength, but based on the ionic strength dependency of the interaction, Stewart and Sodetz (4) proposed that the two proteins should be in association under physiological conditions and our SANS studies confirm this prediction. This is not a trivial result since some of the intermediates formed during MAC assembly, for example, C5b or C5b-7 have only a very short lifetime and they must find another reaction partner, such as C6 or a membrane, in a short time. Association of C8 and C9 under physiological conditions, and by analogy, the other proteins that form the MAC, assures that they can interact much more efficiently as would be otherwise possible.

The absence of multimers (Fig. 3) also indicates that a SANS study of the C8-C9 complex might be possible at higher protein concentrations that is otherwise not feasible because of the tendency of C9 to aggregate. This would allow a more precise modeling of the dimer structure using Debye spheres (25). A second possibility to elucidate the structure of C9 more precisely by SANS could come from the use of Suramin at pH 8. This drug interacts strongly with terminal complement proteins (22) and as our results indicate the compound produces a complex with twice the mass of monomeric C9 and again there is no indication of higher protein aggregates. Suramin is a symmetrical molecule with a hydrophobic aromatic core and two negatively charged naphthalene trisulfonate side chains. Thus, it is most likely that there is only one binding site per C9 for Suramin and that two C9 molecules are bridged by Suramin via the negatively charged sulfonates. Since the resulting dimer remains elongated (Fig. 4), the most plausible structure is an end-to-end rather than a side-to-side association of the two C9 molecules.

In summary, our results indicate that C8 is a globular protein while C9 is not and that the two proteins associate under physiological conditions. They also provide evidence that a future neutron scattering study of C8-C9 heterodimers and of C9 dimers formed in the presence of Suramin at larger scattering angles and at higher protein concentration might allow a more precise modeling of the solution structure of these proteins.

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