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Small-Angle Neutron Scattering Study of Bence–Jones Protein Mcg: Comparison of Structures in Solution and in Crystal†

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ABSTRACT: Immunoglobulins and immunoglobulin fragments are composed of globular domains linked by extended polypeptide segments. The molecular flexibility inherent in this arrangement allows for significant potential differences between structures observed in the crystalline state and those attained in solution. Small-angle neutron scattering measurements in dilute solution were performed on the Mcg Bence–Jones protein dimer, for which accurate atomic coordinates have been determined by crystallographic methods [Edmundson, A. B., Ely, K. R., Abola, E. E., Schiffer, M., & Panagiotopoulos, N. (1975) *Biochemistry 14*, 3953–3961; Schiffer, M. (1980) *Biophys. J. 32*, 230–232]. The measured radius of gyration (R_g) in H_2O buffer is 24.0 ± 0.4 Å and in D_2O buffer is 23.3 ± 0.1 Å; the calculated value of R_v (R_g in vacuo) is 24.0 Å. The above values compare well with the calculated R_g value of 23.6 Å when refined coordinates of the trigonal crystal form of the Mcg Bence–Jones protein are used. On the basis of a match point of 44.2% D_2O concentration, the experimental partial specific volume is 0.74 cm^3/g. The experimentally derived molecular weight of 47 000 is in very good agreement with that (45 500) calculated from the amino acid composition. For comparisons with different Fab's (antigen binding fragments) exhibiting various "elbow bends" due to the flexibility of the switch peptide between variable and constant domains of the immunoglobulin chains, calculation of the R_g value of the Mcg dimer was performed as a function of the elbow bend. The R_g varied from 22.8 to 26.0 Å as the elbow bend was opened from 100° to 180°; the maximum radius of gyration of the particle was 26.5 Å with the switch peptide stretched by separating the variable and constant domains by an additional 1.5 Å at an elbow bend of 180°.

The correspondence of the functional structure of a molecule in solution and the structure determined by X-ray crystallography has been the subject of continued discussion. This question is especially pertinent for immunoglobulins (Ig's), which are composed of several globular domains linked by extended polypeptide segments. The domain structure imparts an inherent flexibility to the Ig molecule and its Fab (antigen binding) and Fc components. This flexibility may be necessary for the multifunctional nature of the protein, and thus, in contrast to most globular enzymes, the Ig's might be expected to differ between the crystalline and solution states in their gross conformation. Complete IgG immunoglobulins and their fragments have been studied by single-crystal X-ray diffraction (Poljak et al., 1973; Segal et al., 1974; Matsushima et al., 1978; Navia et al., 1979; Silverton et al., 1977; Marquart et al., 1980) and by small-angle X-ray (Pilz et al., 1973, 1975, 1976, 1977) and neutron scattering (Cser et al., 1976; Gilmour et al., 1981) in dilute solution. The overall dimensions of the molecules derived by these two methods differ significantly, the values obtained in solution being larger.

The IgG molecules consist of two heavy and light chains; the light chains and the N-terminal half of the heavy chains form the antigen binding (Fab) fragments. Each chain with the Fab's consists of an N-terminal variable and a C-terminal constant domain connected by the "switch" peptide. Light chains also form dimers and are known as Bence–Jones proteins. The Mcg Bence–Jones protein consists of two chemically identical human λ-type light chains and resembles an Fab both in its three-dimensional structure and in its ability to bind small molecule haptens (Schiffer et al., 1973; Edmundson et al., 1974). The structure of the Mcg dimer in the trigonal-crystal form was determined at atomic resolution (Edmundson et al.,

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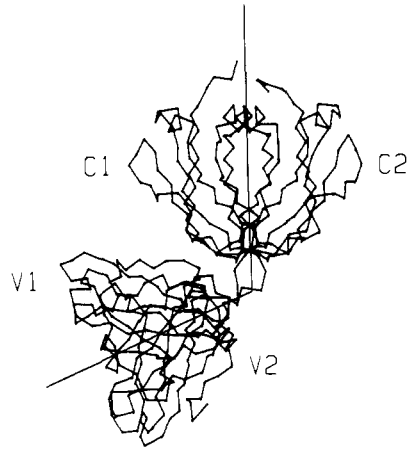

FIGURE 1: Polypeptide backbone of the Mcg Bence–Jones dimer as determined from crystal structure analysis of its trigonal crystal form. The local 2-fold axes between the constant C_1 and C_2 domains and variable V_1 and V_2 domains are shown. The angle between these two axes defines the elbow bend of the particle. In this figure the two axes are in the plane of the paper.

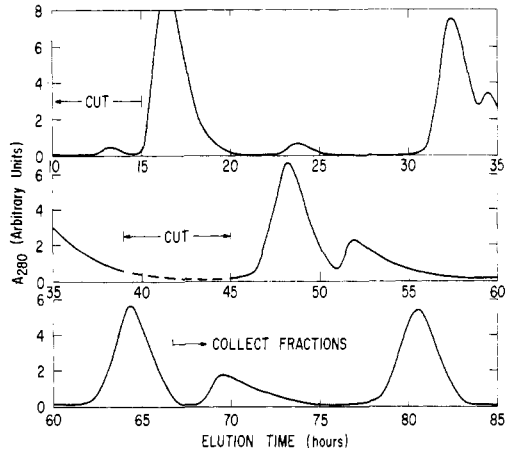

FIGURE 2: Removal of noncovalently linked light chain from Mcg covalent dimer preparation by recirculating gel filtration under nondissociating conditions. The Mcg protein sample (1 mL, 36 mg/mL) containing both covalent and noncovalent dimers was applied to a column of Sephadex G-75 (Superfine grade) of dimension 2.5 × ~90 cm and operated in the ascending mode at a flow rate of 15 mL/h. The column was maintained at 4 °C. The column eluant was monitored continuously at 280 nm. For removal of high molecular weight contaminants, fractions were collected until the Mcg protein was observed to begin elution. Low molecular weight contaminants were removed after the second passage. Resolution of monomer and dimer was observed to be complete after the fourth passage at which point the monomer was removed and the dimer then collected.

1975; Schiffer, 1980). In the crystal the two chains have different conformations; one chain resembles the light chain and the other the heavy chain of an Fab.

In the Mcg Bence–Jones protein and the Fab's, the main interactions between the component chains are very similar and are primarily lateral through their like domains, while the longitudinal interactions between variable and constant domains are less extensive. The antibody binding site is formed through the interaction of the variable domains of the two chains. The two variable domains and the two constant domains are related by local 2-fold axes. These local 2-fold axes define the so-called "elbow bend" of the molecule. The elbow bend is 113° for the Mcg light chain dimer (Figure 1) crystallized in the trigonal form, but it is 132° in the orthorhombic form determined at 6.5-Å resolution (Abola et al., 1980). The elbow bends of the Fab's (Poljak et al., 1973; Segal et al., 1974; Matsushima et al., 1978; Navia et al., 1979) vary from 131° to 166°, reflecting the different longitudinal contacts and the flexibility of the molecules as known from crystallographic studies.

Since the relationship of the structures in the crystal and in solution is of major importance, small-angle neutron scattering studies were undertaken of the Mcg Bence–Jones dimer for which accurate atomic coordinates have been determined by crystallographic methods. These data have been compared with calculations based on the refined coordinates. Further calculations were performed by using the atomic coordinates of the antigen binding fragment derived from the human myeloma protein New.

Materials and Methods

Preparation of Protein. The Mcg Bence–Jones protein was prepared from the urine of a patient with multiple myeloma. The protein was stored frozen in an ammonium sulfate paste and contained both covalent dimer and noncovalent dimer. The dimerization constant of the Mcg protein is $\sim 10^6$ M^{-1} (F. J. Stevens et al., unpublished observation). At the concentration of monomer present in the ammonium sulfate paste, most of the light chains are associated in the form of noncovalent dimer and could not be separated from the covalent dimer by routine gel filtration. Two methods for preparing the pure covalent dimer were used.

(1) The ammonium sulfate paste was dialyzed against deionized water and was chromatographed on Sephadex G-100 in 0.4 M propionic acid at 4 °C. For further purification, the peak fractions of the dimer were collected, concentrated, and rechromatographed. The protein was then dialyzed into 0.05 M Tris[1]–0.10 M NaCl, pH 7.2, buffer. Since in this method the two chains of the covalent dimers are probably dissociated, there is a possibility that the native conformation of the protein is not recovered.

(2) So that this possible complication could be avoided, separation under "nondissociation" conditions was effected by chromatography at 4 °C on Sephadex G-75 (Superfine grade) in 0.05 M Tris–0.10 M NaCl buffer, pH 7.2. The protein was dialyzed into the running buffer and was applied to the column. For separation of the covalent and the noncovalent dimer, the column was operated in a recirculating mode. On the first round the fractions that contained high molecular weight protein eluting before the dimer peak were removed. After the second pass through the column, fractions with low molecular weight protein were removed. At the fourth pass, as can be seen in Figure 2, the covalently and noncovalently bound dimers were resolved. The fractions containing the noncovalently bound dimer were collected at this point, and the covalent dimer was recirculated one more time before collection. The pooled fractions of covalent dimer were checked for purity by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Material prepared by method 2 was used in most of the scattering experiments. The sample was concentrated to ~20 mg/mL by ultrafiltration, taking $E_{1\%}^{280}$ as 12.5 for the Mcg dimer. Half the sample was subsequently dialyzed against the Tris–NaCl buffer prepared with D_2O. To obtain the samples for the measurements at different D_2O concentrations, we mixed the Mcg protein samples in D_2O and H_2O buffers in different proportions.

Atomic Coordinates. The crystallographic refinement of the crystal structure of the Mcg Bence–Jones protein is in progress (Schiffer, 1980). The coordinates used in the model

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

calculations were based on 2.3-Å resolution diffraction data that were refined to an R factor of 29%. Crystallographic refinement consisting of cycles of structure factor calculations and real space refinement was used. The positions for 3000 of the 3220 nonhydrogen atoms of the molecule were determined. An eight-residue segment of one of the chains for which there was no electron density in the crystal (Schiffer, 1980) was generated from the other chain with a rotation about the noncrystallographic 2-fold axis. In solution it is expected that both chains have the same conformation in this region. The hydrogen positions were generated by Herman's model building program. The coordinates of Fab New (Saul et al., 1978) were obtained from the Brookhaven data bank.

Measurement and Data Analysis. The scattering experiments were carried out at the high flux beam reactor at Brookhaven National Laboratory by using the small-angle neutron spectrometer at the H4S satellite station. The instrument and experimental arrangement have been described by Schoenborn et al. (1978). Neutrons of wavelength 2.37 Å ($\Delta\lambda/\lambda = 0.02$ full width at half-maximum) were used with an incident flux on the sample of 4.5×10^5 n/(cm^2·s). A low efficiency detector was used to monitor the incident-beam intensity. The sample-to-detector distance was 209 cm, and both the collimator and the detector snout were flushed with ^{4}He. Samples and buffers were contained in 2 mm thick quartz cuvettes mounted in an automatic sample changer and kept at 7 °C during the experiment. Samples, buffers, an empty cell, and an incoherent scatterer were repeatedly exposed to the beam in sequence for a preset number of monitor counts to eliminate the effects of intensity fluctuations. Samples were monitored spectrophotometrically in the sample cuvettes before and after exposure to neutrons. No denaturation was observed and no precipitation of protein from solution occurred during the course of the experiments.

A two-dimensional position-sensitive neutron detector (Alberi et al., 1975) was used; it encodes the positions of counts and sums the scattered neutrons into a 128×128 array. Data from the repetitive experiments on a given sample were then summed, radially integrated over concentric rings, and normalized to constant monitor counts to give the intensity as a function of Q, the amplitude of the scattering vector. Here $Q = 4\pi \sin(\theta/\lambda)$ where 2θ is the scattering angle and λ is the neutron wavelength. Data for the incoherent scatterer (8.1% D_2O) treated in the same way showed that the detector response was uniform over the angular range that was used to determine the radii of gyration. The intensity scattered by the solute is then

$$I(Q) = I_S(Q)/T_S - (1 - \bar{\nu}c)I_B(Q)/T_B - \bar{\nu}cI_E(Q)/T_E \quad (1)$$

where $\bar{\nu}$ is the partial specific volume (assumed to be 0.74 for immunoglobulins), c is the weight fraction of the proteins, and the I's represent the measured scattered intensities and the T's the fractions of the beam transmitted by each sample (S), each buffer (B), and the empty cell (E). The T's were measured for each cell by attenuating the direct beam, removing the beam stop, and counting for a set number of monitor counts.

The radius of gyration (R_g) of the protein was determined from $I(Q)$ by using the Guinier (1939) approximation:

$$I(Q) = I(0) \exp(-R_g^2Q^2/3) \quad (2)$$

at different D_2O concentrations. The forward scattered intensities $I(0)$ were obtained by extrapolation to $Q = 0$. $I(0)$ is proportional to the protein concentration c and to the square of the contrast $\bar{\rho}$. The contrast is the difference between the mean scattering length density of the protein ρ_{mean} and the scattering length density of the solvent ρ_s; thus

$$I(0)/c \propto (\rho_{mean} - \rho_s)^2 \quad (3)$$

The match point of the protein [where $I(0)/c$ equals zero and ρ_s equals ρ_{mean}] was determined from $I(0)$'s over a range of D_2O concentrations. Further, the dry volume of the protein determined at the match point is

$$V = \sum_i b_i/\rho_{mean} \quad (4)$$

where b_i is the scattering length of the ith atom of the protein. Since the amino acid sequence of the Mcg is known, the b_i's were summed, allowing for the equilibrium of exchangeable protons. The partial specific volume was determined from the relationship

$$\bar{\nu} = N_A V/M \quad (5)$$

where N_A is Avogadro's number and M is the known molecular weight. The molecular weight was also independently determined from scattering data in different contrast buffers according to the relationship (Wise et al., 1979)

$$\partial[I(0)/c]^{1/2}/\partial X = (\Phi_0 AZ\Delta\Omega/N_A)^{1/2}\bar{\nu}M^{1/2}[\partial(\rho_{mean} - \rho_s)/\partial X] \quad (6)$$

where Φ_0 is the incident neutron flux, A is the cross-sectional area of the beam at the sample, Z is the sample thickness, $\Delta\Omega$ is the solid angle of the detector, and X is the volume fraction of D_2O in the buffer.

Model Calculations. Radius of Gyration. The R_g's of the Mcg dimer and Fab New were calculated from the refined atomic coordinates by using the equation

$$R_g = (\sum_{i=1}^{N} b_i R_i^2 / \sum_{i=1}^{N} b_i)^{1/2} \quad (7)$$

where b_i is the atomic scattering length of the ith atom and R_i is its distance from the centroid of the dimer.

The radius of gyration of the Mcg Bence–Jones dimer was also calculated as a function of the elbow bend. In order to change the elbow bend, we transformed the coordinates of the two variable domains in such a manner that the 2-fold axes were kept in the same plane.

Scattering Curve. The scattering curves for the light-chain dimer Mcg and the Fab New were calculated from the coordinates of the nonhydrogen atoms by using the Debye relationship (Debye, 1915):

$$I(Q) = \sum_{i=1}^{N}\sum_{j=1}^{N} b_i b_j [\sin(QR_{ij})/(QR_{ij})] \quad (8)$$

where R_{ij} is the distance between ith and jth atoms in the molecule.

Results

The experimental and calculated radii of gyration of the Mcg dimer are in excellent agreement; their values are summarized in Table I. Figure 3 shows scattering curves plotted as ln $I(Q)$ vs. Q^2 for different contrast buffers. Protein concentrations ranged from 15.9 to 18.1 mg/mL in 100%, 87%, 13%, and 0% D_2O buffers. The range of Q^2 over which the curves were linear was similar for a curve calculated by using eq 8 and the crystal coordinates for the Mcg Bence–Jones protein. [A calculated scattering curve was also used by McDonald et al. (1979) to determine the linear range of the experimental scattering curve of yeast hexokinase.] The R_g derived from the slope of the calculated scattering curve by using points to $Q^2 = 4.9 \times 10^{-3}$ Å^{-2} was found to agree with that obtained by using eq 7. This value of Q^2 was used as the

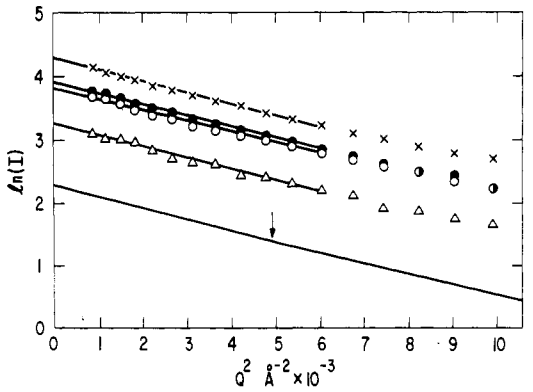

FIGURE 3: Small-angle neutron scattering curves of the Mcg Bence–Jones dimer in buffers containing different concentrations of D_2O: 100% (×), 87% (○), 13% (△), and 0% (●). The bottom curve is the scattering curve calculated with the refined crystallographic coordinates, on an arbitrary scale. The calculated scattering curve is linear to a Q^2 of 4.9 × 10^{-3} Å^{-2}, indicated by the arrow. The lines through the experimental points were derived by a least-squares fit through points with a Q^2 of (1.4–4.7) × 10^{-3} Å^{-2}.

Table I: Measured and Calculated Radii of Gyration

sample	D_2O (%)	R_g (Å)
measured		
Mcg BJP	0	24.0 ± 0.4
Mcg BJP	13	23.9 ± 0.7
Mcg BJP	87	23.1 ± 0.2
Mcg BJP	100	23.3 ± 0.1
Mcg BJP[a]	100	23.4 ± 0.2
calculated		
Mcg BJP		23.6 (23.5)[b]
new Fab		24.5[b]

[a] Sample purified in 0.4 M propionic acid. [b] R_g calculated with the nonhydrogen atoms of the molecules.

upper limit of the linear region of the experimental scattering curves. The lower limit was chosen to exclude values that were attenuated due to low-angle interference of the beam stop.

The linear regions of the curves in Figure 3 were extrapolated to $Q^2 = 0$; the $[I(0)/c]^{1/2}$ values were used to determine the contrast match point as shown in Figure 4. By application of a least-squares procedure, the contrast match point of the Mcg Bence–Jones was determined to be at 44.2 ± 0.2% D_2O concentration, corresponding to a mean scattering length density of 2.52 × 10^{14} cm/Å^3. By use of eq 4, the dry volume of the dimer was calculated to be 55 800 ± 250 Å^3, assuming all non-carbon-bound hydrogens exchange and are in equilibrium with the hydrogen–deuterium composition of the buffer. The partial specific volume was then calculated to be 0.738 ± 0.003 cm^3/g by using M = 45 460 based on the amino acid composition. This estimate may be slightly high because of the generous exchange assumption. If, in fact, only 90% of the non-carbon-bound hydrogens exchange, this value would be reduced to 0.720 cm^3/g, a low value for globular proteins. An independent determination of the molecular weight using eq 6 and the data in Figure 4 gives $M = 4.7 \times 10^4$. Together, the good agreement of $\bar{v}$ with values for other globular proteins (Smith, 1966) and of M with that known from the amino acid sequence suggests the assumption of equilibrium exchange is a good one.

The calculated radius of gyration of the Mcg Bence–Jones dimer in the trigonal crystal form was R_g = 23.5 Å by using the nonhydrogen atoms determined by high-resolution crystallographic studies. When the hydrogen atoms were also included in the calculation, the result was R_g = 23.6 Å. For comparison with the calculated value of the radius of gyration,

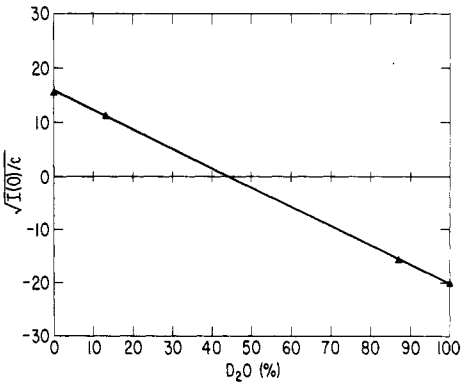

FIGURE 4: Determination of match point of the Mcg Bence–Jones dimer. The square root of the extrapolated zero-angle intensity $I(0)$ divided by the protein concentration c is plotted as a function of the D_2O concentration. Concentrations of protein were 15.9, 16.2, 17.8, and 18.1 mg/mL for protein in 100%, 87%, 13%, and 0% D_2O buffer. The scattering length density of the Mcg protein is equivalent to a 44.2% D_2O buffer.

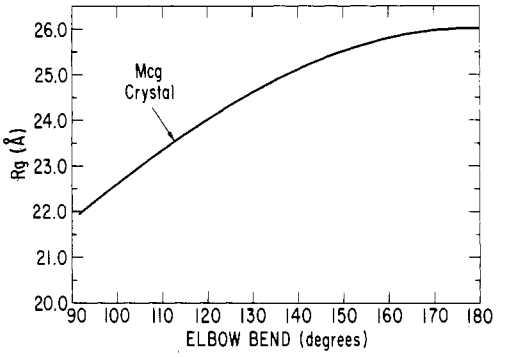

FIGURE 5: Radius of gyration of the Mcg particle as a function of elbow bend. The elbow bend is the angle between the local 2-fold axes relating the constant and variable domains of Bence–Jones proteins or Fab fragments (see Figure 1). The elbow bend of the molecule was changed in model calculations by moving the variable domains in such a manner that the axes remained in the same plane.

the radius of gyration in vacuo (R_v) was determined by interpolation of the measured values: R_v = 24.0 ± 0.4 Å (at ρ_s = 0 for 8% D_2O). There is a slight dependence of the radius of gyration on contrast. This dependence is apparent if R_g^2 is plotted against $1/\bar{\rho}$ and has been observed for other globular proteins (Ibel & Stuhrmann, 1975).

Because of the existence of the flexibile switch peptide connecting the variable and the constant domains of the light chains, calculations of the R_g of the Mcg dimer as a function of the elbow-bend angle were performed. As shown in Figure 5, the radius of gyration varied from 22.8 to 26.0 Å as the elbow bend was opened from 100° to 180°. When the switch peptide was stretched by separating the variable and constant domains by an additional 1.5 Å at an elbow bend of 180°, a maximal R_g of 26.5 Å was obtained.

The radius of gyration was also calculated for Fab New by using the crystallographically determined nonhydrogen atom coordinates (Saul et al., 1978) and was found to be 24.5 Å. Calculations of the scattering curves for both the Mcg dimer and Fab New to Q = 0.4 Å^{-1} correspond closely, as expected from their similar three-dimensional structures. In both cases, the calculated scattering curves did not show the pronounced dip that was observed at Q = 0.28 Å^{-1} in X-ray scattering studies of the Fab fragment Kol (Pilz et al., 1976).

Discussion

The radius of gyration in vacuo (R_v = 24.0 ± 0.4 Å) of the Mcg Bence–Jones light-chain dimer as determined by neutron

scattering was in excellent agreement with that calculated (R_g = 23.6 Å) by using the refined atomic coordinates of the trigonal crystal form. The experimental molecular weight of 4.7×10^4 and partial specific volume of 0.738 cm^3/g were also in good agreement with expected values. An R_g of 26.3 Å and a partial specific volume of 0.736 cm^3/g determined by small-angle X-ray scattering have been reported for a different Bence–Jones protein by Holasek et al. (1963). Apparently the choice of the two methods used here to purify the dimer is not critical, as the protein treated with propionic acid had an R_g almost identical with that of the non-acid-treated protein (see Table I).

The Mcg Bence–Jones dimer has been crystallized in two crystal forms. The basic structural distinction was at the elbow bend of the molecule; in the trigonal form crystallized from 1.9 M ammonium sulfate (Edmundson et al., 1975) the elbow bend is 113° whereas it is 132° in the orthorhombic form crystallized from distilled water (Abola et al., 1980). This potential flexibility in solution was incorporated into the model structures to assess the effect of varying the elbow bend on the calculated R_g. The range of bends was assumed to be limited to 100°–180° by steric hindrances. The observed in vacuo radius of gyration R_v = 24.0 ± 0.4 Å corresponds to an elbow bend of 120 ± 6°, suggesting a conformation or average conformation intermediate between the 113° and 132° elbow-bend conformations observed in the two crystal forms. Although the precision of this determination does not eliminate either as the sole conformation in solution, it does suggest that the crystalline structures are representative of the solution conformation and that flexibility in solution is limited to the lower half of the total range of 100°–180°. We have in fact observed that at larger angles than the Guinier region, in the shape region of the scattering curve, experimental curves fall more slowly than the calculated one. The difference between the observed and calculated scattering curves above the Guinier region might be due to the presence of molecules having different conformations.

The Mcg light-chain dimer has a conformation very similar to that of Fab fragments, which consist of a light chain and the N-terminal half of a heavy chain. This structural similarity was also shown by the similarities of the calculated scattering curves of human Fab New and the Mcg Bence–Jones dimer; the calculated R_g's of the two proteins are 24.5 and 23.5 Å. The difference reflects mainly the change of elbow bend from 131° for protein New (Poljak et al., 1973) to 113° for protein Mcg. Crystallographic determination of the structures of other Fab fragments show a range from 135° to 166° in the elbow bend (Segal et al., 1974; Matsushima et al., 1978; Navia et al., 1979). In contrast to the agreement for the Mcg protein between particle sizes as determined in the crystal and measured in solution, the measured R_g of the different Fab's was much larger than the maximum calculated value of 26.5 Å. Measured values have been reported as 27.4 Å (Gilmour et al., 1981) and 31.5–34.5 Å (Pilz et al., 1975, 1976; Cser et al., 1976). Since the Fab's and the light-chain dimer Mcg are very similar in three-dimensional structure, there is no obvious structural explanation for the large radii of gyration observed for Fab's in solution.

Comparison of the experimentally derived R_g in our experiments with that calculated from the refined crystallographic coordinates shows that the Mcg Bence–Jones protein does not have a grossly different conformation in solution compared with the structure observed in its crystalline forms. The data also indicate that the molecule may have flexibility, but there is no indication of a large conformational change.

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