

Studying Antibody Conformations by Ultracentrifugation and Hydrodynamic Modeling

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1. Introduction

Intact immunologically active antibodies are too large for high-resolution structural analysis by nuclear magnetic resonance (NMR) spectroscopy. The flexibility between the Fab and Fc domains at the hinge region also makes structure determination by X-ray crystallography generally difficult, except for a limited number of cases (antibodies that possess short hinge regions, hinge-deleted mutants, or those stabilized by complexation with antigen). Hydrodynamic methods—such as measurement of the translational frictional ratio by ultracentrifugation or dynamic light scattering—have no such limitations. However, two caveats exist. First, the structures obtained are only low-resolution models. Second, hydrodynamic parameters, such as the frictional ratio, are often at least as sensitive to the volume of the particle as to conformation. This is tricky, since this volume includes a significant contribution from any solvent associated with the antibody; and this “solvation” or “hydration” is a complex dynamic process. Nonetheless, if the hydration problem can be adequately dealt with, useful information about domain orientation can be obtained, and in particular how orientations differ between different antibody classes and subclasses, and between wild-type and mutant or engineered structures. This information may ultimately assist with strategies for antibody engineering, particularly if we can understand which sequences are responsible for “compact” as opposed to open structures. Domain orientation and flexibility are intimately linked with an antibody’s ability to crosslink antigen and bind to the effector system, and are also related to its stability (*1*).

By combining the measured frictional ratio data (from measurements of sedimentation or diffusion behavior) of the individual domains and of the

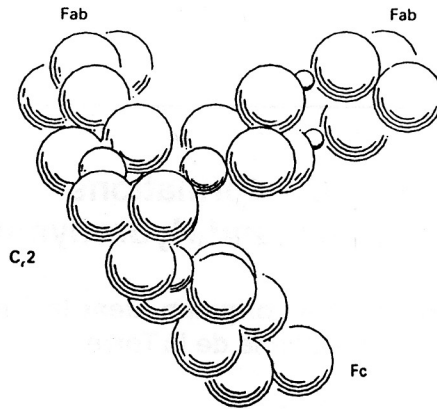


Fig. 1. The cusp-shape conformation of IgE was first discovered using hydrodynamic methods in 1990 (from **ref. 3**).

intact antibody, with information calculated from the known crystallographic structures of the domains, the possible conformations—in terms of the domain orientation—of intact antibody molecules can be given, *without ambiguities through hydration*. This combination of hydrodynamics and crystallography was introduced recently as “crystallohydrodynamics” (2). An earlier form of this approach was reported in 1990 with the first demonstration that the antibody IgE was cusp-shaped in solution (3) (**Fig. 1**).

Essentially, the time-averaged apparent hydration (expressed as the amount of water associated with the protein on a mass/mass basis) is dealt with by comparing hydrodynamic properties with the crystal structures of those antibody forms with crystal structures that are known. This can then be used to represent the hydration of other structures with conformations that are being sought. In the case of **ref. 2** “those antibody forms” were the known structures of the Fab’ and Fc domains of IgG. In the case of the earlier **ref. 3**, “those antibody forms” were the hingeless mutant antibody IgGMcg, one of the few intact (although not immunologically active) antibodies with structures that have been solved.

This chapter focuses on the measurement and use of the translational frictional ratio for representing the conformation of antibodies and other multi-domain structures in solution, in terms of the low-resolution orientation of the domains.

1.1. The Sedimentation Coefficient, Frictional Ratio, and the Perrin “Frictional Ratio due to Shape”

1. In simple terms of boundary sedimentation, the sedimentation coefficient, s , (second, or Svedberg units, S, in which $1\text{ S} = 10^{-13}\text{ s}$) of a macromolecule is defined as

the velocity of sedimentation, v (cm/s), of a macromolecule per unit centrifugal field (cm/s²):

$$s = v/\omega^2 r \quad (1)$$

where ω is the angular velocity ($=2\pi$.rpm/60) in rads/s and r (cm) is the radial position of the sedimenting *boundary*. NB: The full description of the change in concentration with time and radius across the centrifugal cell is given by a much more complicated differential relation known as the Lamm equation, which also takes into account boundary spreading caused by diffusion (*see ref. 4*). The Lamm equation forms the basis of modern software considered here.

2. The Svedberg unit, S , is often used instead of seconds: $1S = 10^{-13}$ s. Increase in mol wt tends to increase s . Increase in asymmetry or hydration tends to decrease s .
3. The sedimentation coefficient of a macromolecule measured in an arbitrary buffer solution or solvent will depend on the intrinsic properties of the macromolecule as well as on the viscosity and density of the solution it is sedimenting through. We therefore normalize to standard solvent conditions—namely, the density and viscosity of water at 20.0°C (*see ref. 4*):

$$s_{20,w} = \{(1 - \bar{v} \cdot \rho_{20,w}) / (1 - \bar{v} \cdot \rho_{T,b})\} \cdot \{\eta_{T,b} / \eta_{20,w}\} \cdot s_{T,b} \quad (2)$$

where $s_{T,b}$ is the measured sedimentation coefficient in the buffer and at the temperature used; $s_{20,w}$ is the corresponding corrected value at 20.0°C in water; $\rho_{T,b}$ and $\eta_{T,b}$ are the densities and viscosities of the buffer; $\rho_{20,w}$ and $\eta_{20,w}$ are the corresponding values at 20.0°C in water; \bar{v} is the partial specific volume, which can be reasonably estimated from the amino acid and carbohydrate content: the program *SEDNTERP* will do this (*see Subheading 1.2.3*).

4. The sedimentation coefficient is corrected for non-ideality effects by extrapolation to zero concentration to give $s^0_{20,w}$ according to the relation:

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

where k_s is known as the concentration dependence parameter or the Gralén coefficient (mL/g).

5. $s_{20,w}$ depends not only on the conformation but the mol wt and (hydrated) volume of the macromolecule. We need a parameter that can be explicitly given in terms of conformation alone. The first step is to combine sedimentation coefficient with the mol wt to give the *translational frictional ratio* (ff_o) (the ratio of the frictional coefficient of the macromolecule to a spherical particle of the same mass and hydrated volume):

$$(ff_o) = M(1 - \bar{v} \cdot \rho_{20,w}) / [N_A 6\pi\eta_{20,w} s^0_{20,w} \{(3\bar{v} \cdot M) / (4\pi N_A)\}^{1/3}] \quad (4)$$

where M is the mol wt (g/mol) and N_A is Avogadro's number (6.02×10^{23} mol⁻¹).

6. The final step is to define a "frictional ratio due to shape" or "Perrin function" P , by combining the frictional ratio with the hydrated volume of the macromolecule

$$P = (ff_o) \cdot \{1 + (\delta_{app}/\bar{v} \cdot \rho_{20,w})\}^{-1/3} \quad (5)$$

where δ_{app} is the apparent hydration. As stated in the previous section, hydration is a complicated, dynamic process. For the purpose of hydrodynamic modeling represents a time-average, and δ_{app} represents an "apparent" hydration because it considers other non-shape contributions toward the frictional ratio.

7. The P value obtained from experimental data in this way can then be compared with the P values computed from models of the antibody shape—using, for example, the computer program SOLPRO as described under **Subheading 1.2.3**.

1.2. Choice of Modeling Methods: Ellipsoids, Beads, and Shells

1.2.1. Ellipsoids

The simplest representation of the shapes of proteins in solution is as an ellipsoidal shell (**Fig. 2**). An ellipsoid is a symmetric structure, and there are three types. There is the general "tri-axial" ellipsoid with three unequal axes, $a > b > c$, with a, b, c the semi-axial dimensions. Its shape is specified by two axial ratios (a/b , b/c). The general triaxial ellipsoid is symmetric about the center.

There are two simpler ellipsoids that are symmetric about an *axis*: as in the situation when two of the three axes are forced to be equal. The prolate ellipsoid has $a > b = c$ —e.g., it has two equal shorter axes and one longer axis, and its shape is specified by the axial ratio a/b . The oblate ellipsoid has $a = c > b$, and its shape is also specified by a single axial ratio a/b . The prolate ellipsoid resembles a rugby ball, whereas the oblate ellipsoid resembles a "smartie" or an "M&M" shape. In the limit $a = b = c$, the shape is a sphere. The advantage of ellipsoids is that their hydrodynamic properties can be calculated exactly (5); the disadvantage is that they can only be applied directly to reasonably symmetrical structures. In fact, antibody Fab domains approximate a prolate ellipsoid and Fc domains approximate an oblate ellipsoid. However, intact immunologically active antibodies are not symmetric, and cannot be represented by a single ellipsoid structure. Unfortunately, the hydrodynamic properties of three ellipsoids joined together cannot be calculated. Fortunately, however, the hydrodynamic properties of arrays of spheres can be calculated to a high degree of accuracy.

1.2.2. Beads and Shells

The hydrodynamic and other solution properties of macromolecules or particles arbitrary shapes can be calculated using a methodology that is known as bead modeling (*see*, for example, **ref. 6**). This approach has two variants (2). One of them is bead modeling in a strict sense, in which the particle is represented as an assembly of spherical elements of arbitrary shape, with the only

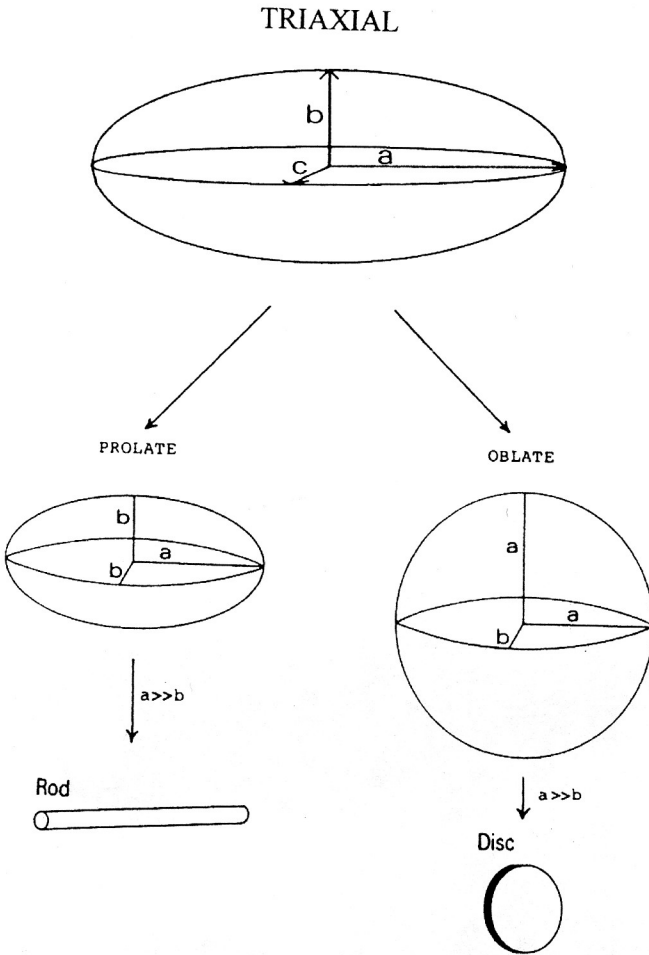


Fig. 2. Ellipsoids: general tri-axial (three unequal axes), prolate (two equal short axes and one longer axis), oblate (two equal longer axes and one shorter axis). A Fab domain can be approximated by a prolate ellipsoid, an Fc domain by an oblate. In the extremes $a \gg b$ (not antibodies), a prolate approaches a rod, an oblate approaches a disk.

condition that the overall size and shape of the particle must be as close as possible to that of the particle. The other variant is shell modeling, in which the *surface* of the particle is represented by a shell of small elements (“minibeads”); the results are extrapolated to a zero minibead radius. This procedure is slightly more computing-intensive than bead modeling, but it is hydrodynamically more rigorous and avoids problems (such as bead overlapping) that had usually plagued the use of the first method. **Figs. 3–6** have been

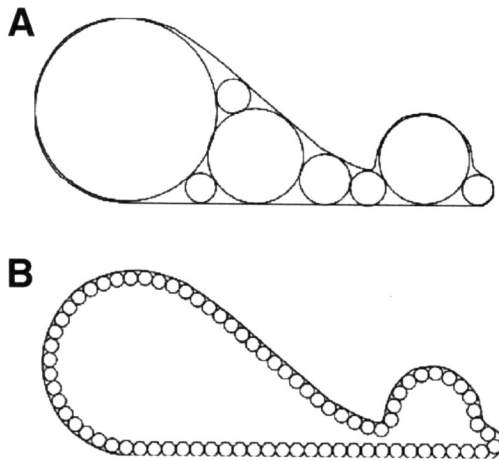


Fig. 3. Schematic representation of (A) a bead model in a strict sense and (B) a shell model.

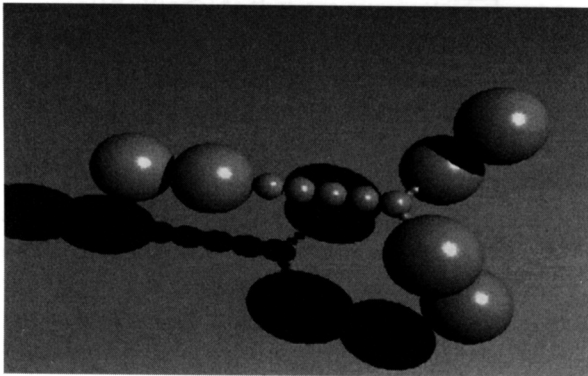


Fig. 4. A bead model for the human antibody molecule IgG3.

included to illustrate both conventional bead and bead-shell models for various proteins (and a glycoprotein in the case of IgG3).

1.2.3. *Bead and Shell Modeling Software*

We now describe the basic aspects of those programs that can be most useful for hydrodynamic modeling of antibodies and related molecules (7-13). See **Note 1** for details of how to access the software described.

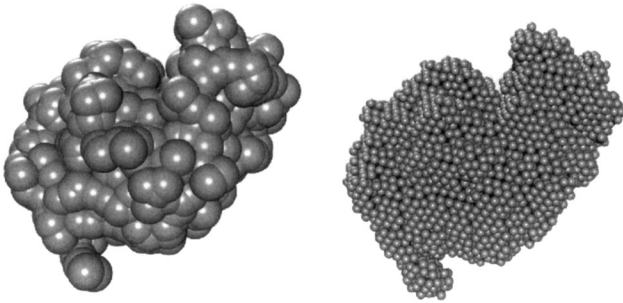


Fig. 5. **Left:** A primary bead model of lysozyme, showing bead overlap. **Right:** A shell model, produced by HYDROPRO.

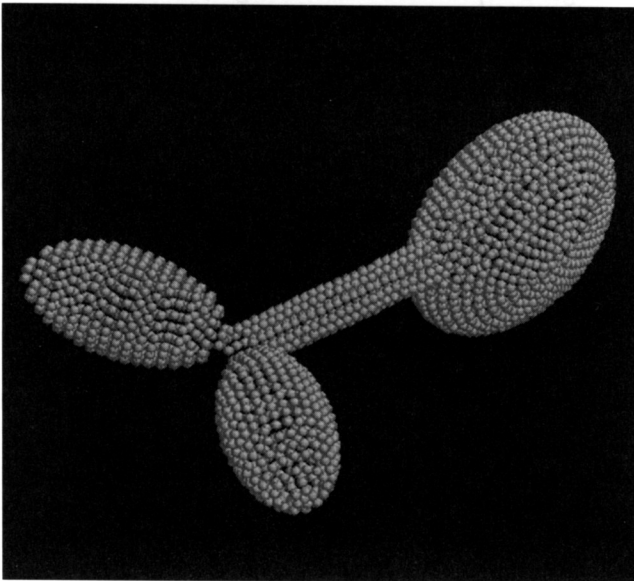


Fig. 6. A shell model for an IgG3 molecule, generated with HYDROSUB showing the two prolate Fab domains, the oblate Fc subunit and the long (nearly rodlike) hinge. The molecule is the same as that in **Fig. 4**: note the difference between a bead model and a shell model.

1. HYDRO is a program for the calculation of hydrodynamic coefficients and other solution properties of rigid macromolecules, colloidal particles, etc., that employs bead models in the strict sense, as mentioned previously. The user of HYDRO has to first build the bead model, which will be specified as a list of Cartesian coordinates and radii of the beads. An example of a bead model (for rat IgE) has already been given, in **Fig. 1**. Another (for human IgG3) is given in **Fig. 4**.

2. HYDROPRO computes the hydrodynamic properties of rigid macromolecules (e.g., globular proteins and small nucleic acids) from their atomic-level structure, as specified by the atomic coordinates taken from a Protein Data Bank (PDB) file supplied by the user, from which the proper hydrodynamic model is built by the program itself. This program employs the shell-modeling method. In the primary hydrodynamic model, each non-hydrogen atom is represented by a sphere of radius approx 3 Å. In order to avoid the inconveniences associated with bead overlapping, this model is replaced by a shell model, in which small “minibeads” are placed at the surface of the primary hydrodynamic model. HYDROPRO calculates the basic hydrodynamic properties: translational diffusion coefficient, sedimentation coefficient, intrinsic viscosity, and relaxation times, along with the radius of gyration. Optionally, HYDROPRO computes also other solution properties such as the co-volume (related to the second virial coefficient) and scattering related properties such as the angular dependence of scattering intensities and the distribution of intramolecular distances.
3. HYDROSUB is intended for the calculation of solution properties of a macromolecular or supramolecular structure modeled as an assembly of subunits that have ellipsoidal (prolate, spherical, or oblate) and/or cylindrical shapes. The subunits are represented using a variation of the bead-shell method, which is particularly suitable for axially symmetric structures. The program builds the shell model, stacking rings of tangential minibeads. The radius of the rings is fixed for cylinders and decreases from the equator to the poles in the case of ellipsoids. This is done internally by the program; the user just has to specify, for each subunit, the coordinates of their centers and the polar angles of their symmetry axis.
4. SOLPRO. The results from HYDRO, HYDROPRO, and HYDROSUB are the primary solution properties: e.g., hydrodynamic coefficients, radius of gyration, and co-volume. SOLPRO (11,12) is an ancillary program, whose input is one of the output files produced by HYDRO, HYDROPRO, or HYDROSUB. This program takes the values of the solution properties and combines them to obtain a number of size-independent shape quantities such as the P function defined in **Equation 5**. The user should use this program according to the supplied instructions.
5. SEDNTERP. This is a general support program for researchers using analytical ultracentrifugation, which provides a very convenient interface for calculating buffer densities, viscosities from buffer/colvent composition data. It will also estimate macromolecular partial specific volumes—and in the case of proteins, extinction coefficients, the isoelectric pH and the valency on a protein for a given pH, all from chemical composition data.

1.3. Workflow Summary of Hydrodynamic Modeling

1. Measure the sedimentation coefficient, and check to determine whether it has been affected by aggregation (boundary shape, is the mol wt consistent with the value from the sequence?).
2. Calculate the experimental frictional ratio from the sedimentation coefficient and (sequence) mol wt.

3. Calculate the experimental Perrin function P from the frictional ratio and the appropriate estimate for the (time-averaged) hydration.
4. Construct candidate models using HYDROSUB or HYDRORO and calculate their P values using SOLPRO.

2. Materials

2.1. Analytical Ultracentrifugation

1. Beckman XL-I or XL-A ultracentrifuge.
2. Optical system: Ultraviolet (UV) absorption optics at 280 nm.
3. Rotor (conventionally a four-hole or eight-hole) for Beckman XL-I or XL-A analytical ultracentrifuge.
4. Ultracentrifuge cells: double sector. Multichannel may be used for sedimentation equilibrium, but not velocity.
5. Solvent: choose as appropriate. To keep problems of hydrodynamic or thermodynamic non-ideality to a minimum (by minimizing polyelectrolyte or charge effects), we suggest a minimum ionic strength of 0.1 M .
6. Sample requirements:
 - a. Sedimentation velocity: at least four concentrations in the range 0.2–1.0 mg/mL (0.3–0.4 mL each), unless reversible self-association phenomena (see **Note 2**) are present and need to be probed, in which case a wider concentration range may be appropriate.
 - b. Sedimentation equilibrium: one concentration should suffice, at ~0.4 mg/mL (0.2 mL), unless, there is a complication through self-association phenomena (see **Note 2**).

2.2. Computer

The hydrodynamic modeling programs run on a variety of platforms, including Microsoft DOS/Windows and Linux for PCs. Thus, the computational work can be carried out on inexpensive personal computers. With fast Pentium or AMD PC processors, the calculations run with a speed similar to that of more expensive machines. We recommend the use of a PC with the second- or third- fast Pentium processor (which optimize the performance/cost ratio), and a moderate amount of fast memory. At the time of writing this chapter, we are using a DELL Dimension 8200, but any computer with the following requirements should be adequate:

1. Pentium IV 2 GHz (is not the fastest Pentium, but the performance/cost ratio is rather good).
2. Memory: RDRAM is recommended. This is not common, but is very good for the matrix calculations that are within the HYDRO programs. An alternative is “DDR” memory. Although 256 Mb is usually sufficient, we advise 512 Mb.
3. A medium-range graphics card.

3. Methods

3.1. Experimental Methods

3.1.1. Sedimentation Velocity in the Analytical Ultracentrifuge

This is used to provide a check on the homogeneity and monomeric state of the antibody. It involves placing the solution and a reference solvent in a specialized ultracentrifuge cell that has end windows transparent to visible and UV light. An optical system (either UV-absorption- or refratometric-based) can be used to record the distribution of solute concentration as a function of radial position and time. The balanced cell is placed in a special rotor. Sedimentation velocity speeds typically range from 30,000–50,000 rpm, depending on the size and shape of the macromolecular system being analyzed. The primary parameter that results from a sedimentation velocity experiment is the sedimentation coefficient. After consulting the manufacturer's operation instructions and guidelines, for measurements on antibodies we suggest the following protocol:

1. Select a rotor speed of ~50,000 rev/min (**Note:** With analytical ultracentrifugation, researchers almost always refer to rotor settings in terms of revolutions per min rather than the equivalent g -force. This does not create any ambiguities, because at the time of writing there is only one commercial manufacturer, and all rotors are of standard size (just differing in the numbers of holes for cells).
2. Choose a temperature: 20.0°C is normally used, unless reproducibility of physiological conditions is necessary or there are particular temperature effects on conformation or stability you wish to probe. If the antibody is relatively fragile, choose 4°C.
3. An experimental run lasts for approx 3–4 h. This should allow the protein to sediment through most of the cell.
4. Choose the appropriate software for analysis. We recommend one of the three packages: DCDT+ (**14**), SVEDBERG (**15**), or SEDFIT (**4**). All three are downloadable from the internet (*see Note 1 and ref. 16*), SEDFIT is free, whereas DCDT+ and SVEDBERG require a nominal charge. All three come with comprehensive instructions and help files. They all follow the change in the whole concentration vs radial position with time (old methods used to follow just the sedimenting boundary), which facilitates extraction of the sedimentation coefficient to a high degree of precision.
5. Check the homogeneity (**Fig. 7A**): there should only be one (macromolecular) component. If there are multiple components present, select the component which corresponds to the monomeric antibody: you may wish to consult a hydrodynamics expert (*see Note 3*).
6. Extract the sedimentation coefficient $s_{20,w}$. The subscripts 20,w, refer to corrected values in "standard solvent conditions," namely the viscosity and density of water at 20°C. The most recent versions of the three packages DCDT+, SVEDBERG, and SEDFIT provide that correction for you: the packages ask you for the buffer/solvent details you used in your experiment, and the partial specific volume.

If you don't know the latter, then take the uncorrected sedimentation coefficient from these programs and then use the program SEDNTERP (ref. 16,17; see Note 1) to correct it—you need to enter the PDB data file for the protein and it will evaluate the partial specific volume from the amino acid (and carbohydrate) content. You can then enter the buffer details and $s_{20,w}$ will then be calculated.

7. Extrapolate $s_{20,w}$ to zero concentration, (Fig. 7B), to give $s_{20,w}^0$.
8. Also extract the translational diffusion coefficient $D_{20,w}$ (cm^2/s): the software packages referred to here also estimate this for you, from the rate of boundary spreading, and extrapolate this to zero concentration. Extrapolate $D_{20,w}$ to zero concentration to give $D_{20,w}^0$ (Fig. 7C).
9. From $s_{20,w}^0$ and $D_{20,w}^0$ calculate the mol wt from the Svedberg equation.

$$M_w = \{s_{20,w}^0/D_{20,w}^0\} \cdot \{RT/(1 - \bar{v} \cdot \rho_{20,w})\} \quad (6)$$

where R is the gas constant. Check that this is within experimental error ($\pm 5\%$) of the mol wt for the monomeric antibody from its sequence. If this is confirmed by further checks from sedimentation equilibrium studies (Subheading 3.1.2.), you can then proceed with some confidence using the $s_{20,w}^0$ and the sequence mol wt for modeling.

3.1.2. Sedimentation Equilibrium in the Analytical Ultracentrifuge

This is used to provide a further check on the mol wt of the antibody. Lower speeds are chosen compared with sedimentation velocity. At these lower speeds, the effects of sedimentation are matched by the effects of diffusion, and instead of a boundary forming, an equilibrium distribution of solute is obtained in the ultracentrifuge cell, with a lower concentration at the air/solution interface and a higher concentration at the base of the cell. Because the system is at equilibrium, shape or frictional effects do not affect the distribution, which is entirely dependent on molecular mass (provided that non-ideality effects are allowed for). After consulting the manufacturer's operation instructions and guidelines for measurements on antibodies, we suggest the following protocol:

1. Select a rotor speed of $\sim 10,000$ rev/min and the same solvent and temperature conditions as described in Subheading 3.1.1.
2. Choose a temperature: 20.0°C is normally used, unless reproducibility of physiological conditions is necessary or there are particular temperature effects on conformation or stability you wish to probe. If the antibody is relatively fragile, choose 4°C .
3. An experimental run lasts at least overnight. This is because of the long period required for sedimentation and diffusive processes to reach equilibrium.
4. Choose the appropriate software for analysis. We recommend the package MSTAR (18), which gives the mol-wt average for all the macromolecular components across the entire radial range in the cell, not just a selected region (Fig. 7D). This is also downloadable from the web (16) (see Note 1) and is free. If the value

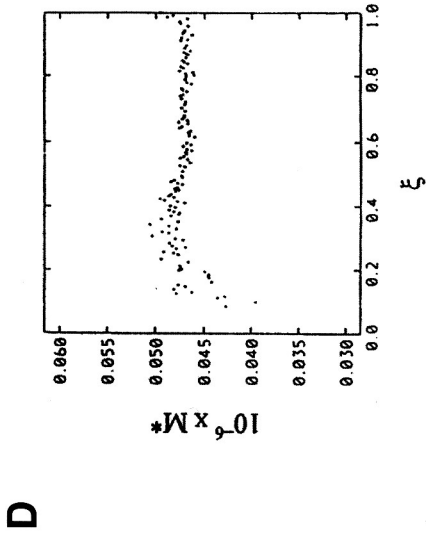
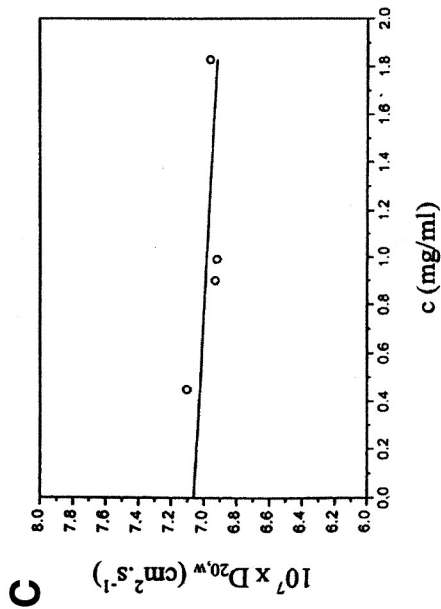
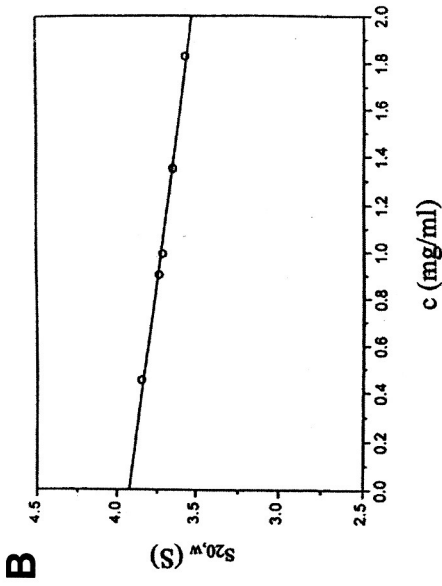
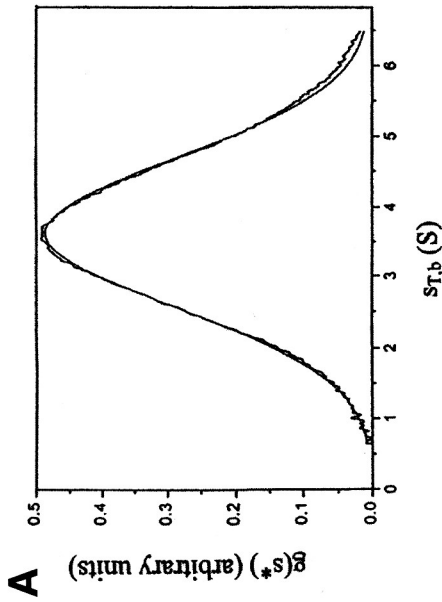


Fig. 7. Sedimentation analysis of IgGFab'. (A) Sedimentation velocity DCDT+ plot for IgGFab' at a loading concentration of 1.10 mg/mL. Rotor speed = 49,000 rpm, temperature = 20.0°C. $g(s^*)$ is the apparent (e.g., not corrected for diffusion or non-ideality) distribution of sedimentation coefficients. The shape of the peak gives information of homogeneity (this sample is monodisperse). The center of the peak gives $s_{T,b}$; the width, for a monodisperse peak, gives $D_{T,b}$. (B) Gralén plot to obtain $s^{\circ}_{20,w}$. The line fitted is to **Equation 3**. (C) Plot of $D_{20,w}$ vs c to obtain $D^{\circ}_{20,w}$. (D) Extraction of M from sedimentation equilibrium using MSTAR analysis. M^* is an operational point average mol wt whose value extrapolated to the bottom ($\xi=1$) of the ultracentrifuge = the weight average mol wt of all the macromolecular components in the centrifuge cell M . ξ is simply a normalized radial displacement squared parameter = $(r^2 - a^2)/(b^2 - a^2)$, with r =radial distance from the rotor center, and a, b are the corresponding distances of the air/solution meniscus and the cell bottom, respectively.

for M (a weight average) is also within experimental error of the mol wt of the monomeric antibody from its sequence, you can use with complete confidence the $s^{\circ}_{20,w}$ and the sequence mol wt for modeling.

3.1.3. Calculation of the Frictional Ratio and the “Frictional Ratio due to Shape”

1. Obtain the sedimentation coefficient $s^{\circ}_{20,w}$ for your antibody from the analytical ultracentrifuge as described in **Subheading 3.1.1**.
2. Obtain the sequence mol wt, M .
3. Use s , M , and the partial specific volume (\bar{v}) to calculate the frictional ratio ff_o of the antibody according to **Equation 4**. In that formula, the value you use for the density and viscosity of solvent should be that of water at 20°C, since you have corrected your sedimentation coefficient to $s^{\circ}_{20,w}$. The program ELLIPS_PRIME (20) (see **Note 1**) can perform this calculation for you.
4. ff_o cannot be used directly to model the shape, since it also depends also on the volume of the macromolecule: this volume has to take into account “swelling” through association of water/solvent with the protein. Although this association is a complicated dynamic process, for the purpose of overall shape modeling, a time-averaged “hydration” value, δ will suffice (**Subheading 3.2.1**). Then, the “frictional ratio due to shape,” P can be estimated (**Equation 5**), and compared with values computed from the shape models using HYDROSUB and SOLPRO as described in the following section.

3.2. Modeling Methods

3.2.1. δ -Value

1. Taking a so-called average protein value of 0.3–0.4 is inadequate for antibodies, primarily because they are glycoproteins and sugar residues show a higher affinity for water association than amino acid residues do.

2. δ can be estimated from hydrodynamic data, providing that the crystal structures of the Fab (or Fab') and Fc domains are also known (this is the case for human IgG and IgE). This procedure involves taking the crystal structure for the domains and obtaining the corresponding value of P using bead modeling. Then, this is compared with the experimental value for ff_0 for the domains (from the sedimentation coefficient and mol wt), and δ can be obtained. An average δ for the intact antibody can then be estimated from a simple average:

$$\delta_{\text{app}} = \{2 \times \delta_{\text{app}}(\text{Fab}') + 1 \times \delta_{\text{app}}(\text{Fc})\}/3 \quad (7)$$

where δ_{app} means "apparent" hydration in the sense it takes into account other non-shape contributions towards the frictional ratio. For human IgG a value of ~ 0.59 has been obtained (12).

3.2.2. Surface Shell Representation of the Fab and Fc Domains and Hinge: HYDROSUB and SOLPRO

P values for domain orientations of the Fabs and Fc in an antibody (taking into account the hinge) can be calculated using the program HYDROSUB with SOLPRO. However, before this can be done, a way of specifying i) the shapes of the domains; ii) relative length of any hinge; and iii) the positions of the domains relative to each other needs to be achieved. This may be the most laborious part of the modeling procedure.

1. The Protein Data Bank (PDB) file is taken for the domains and a surface ellipsoid is fitted using the program ELLIPSE used by X-ray crystallographers (21). This provides the shape of the ellipsoid in terms of the two axial ratios a/b , b/c (Fig. 8).
2. Conveniently, the crystal structure of Fab approximates well to a "prolate" ellipsoid (two equal shorter axes, and one longer axis), and the Fc domain approximates an oblate ellipsoid (two equal long axes, and one shorter axis) as described in **Sub-heading 1.1.** and illustrated in **Fig. 2.**
3. The equivalent axial ratio (a/b) is then given for the Fab and Fc domains (in terms of a prolate and oblate ellipsoid, respectively) (Fig. 9).
4. Once the axial ratios are known, in preparation for piecing together the domains, we need to know the individual values of a and b for each domain: this can be done by expanding the ellipsoid fit to the surface of the crystal structures during the application of the ELLIPSE routine. A surface bead model to each ellipsoid domain is then generated using HYDROSUB. We are now ready to piece the molecule together.

3.2.3. Piecing the Molecule Together to Calculate P for the Intact Antibody (see **Notes 3-6**)

1. To generate the shell model for the pieced together antibody the user just has to specify, for the two Fabs and the Fc, the relative coordinates of their centers and the angles that define the orientation of the symmetry axis (Fig. 10), together, when appropriate, with the relative length of the hinge.

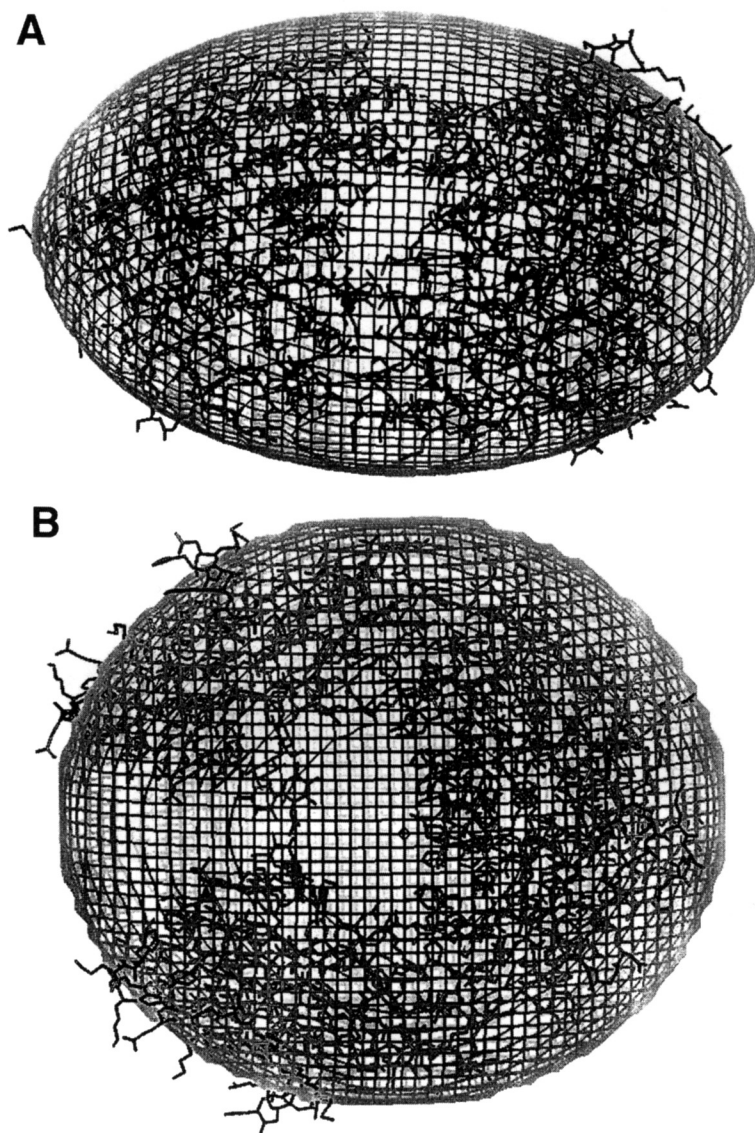


Fig. 8. General triaxial shape fits to the surface of the crystal structures of (A) IgGFab' and (B) IgGFc using the ELLIPSE algorithm (21). Instructions for fitting an ellipsoidal surface to the crystal structure of an antibody domain can be found in ref. 2.

2. The coordinates and angles of each subunit are given in the main input data file for HYDROSUB (for more details on the convention for angles, file format, etc. see the HYDROSUB user guide). SOLPRO is subsequently run to calculate P and all

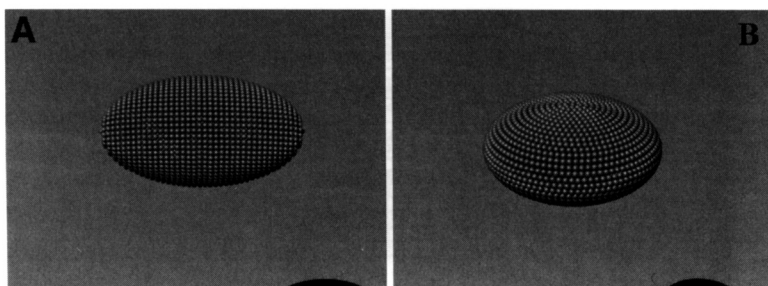


Fig. 9. Bead shell models for (A) the equivalent prolate ellipsoid fit to **Fig. 3A** for IgGFab and (B) the equivalent oblate ellipsoid fit to **Fig. 3B** for IgGFc. The procedure for generating the bead coordinates is described in the documentation for HYDRO-SUB.

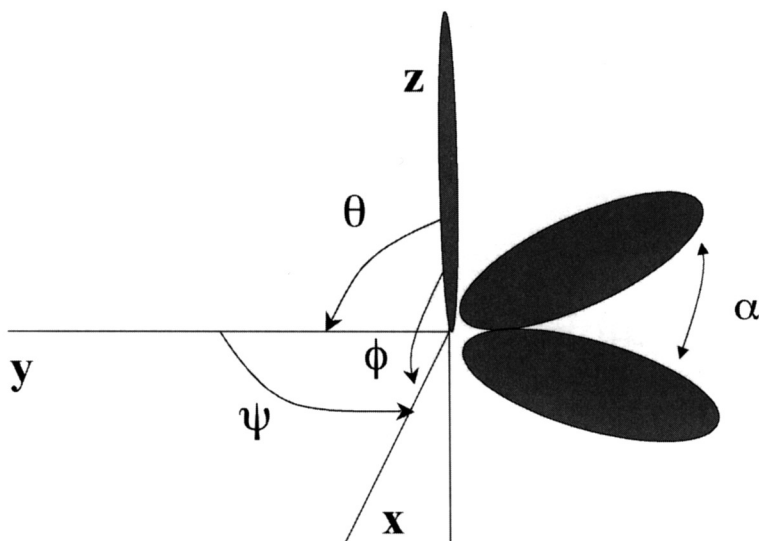


Fig. 10. Orientation angles between Fab' and Fc domains relative to an xyz Cartesian frame. α : angle between Fab domains; θ : non-coplanarity angle of the Fc domain to the Fab's. $\theta = 90^\circ$ corresponds to a planar structure; ϕ : twist angle (swivel of the Fc face); ψ : bend angle (bending up of Fc toward one of the Fab arms).

the other shape functions. A value of P is returned for a given arrangement of the domains and hinge length. The value of P does not depend on the absolute dimensions of the domains or hinge, merely on their dimensions relative to each other—e.g., the overall shape.

3. The values of P for various domain orientations are compared with the experimental value obtained from **Subheading 3.1**. The most appropriate models are selected by matching the P values for the models with the experimental P value after taking into account experimental error. **Fig. 11** gives some examples.

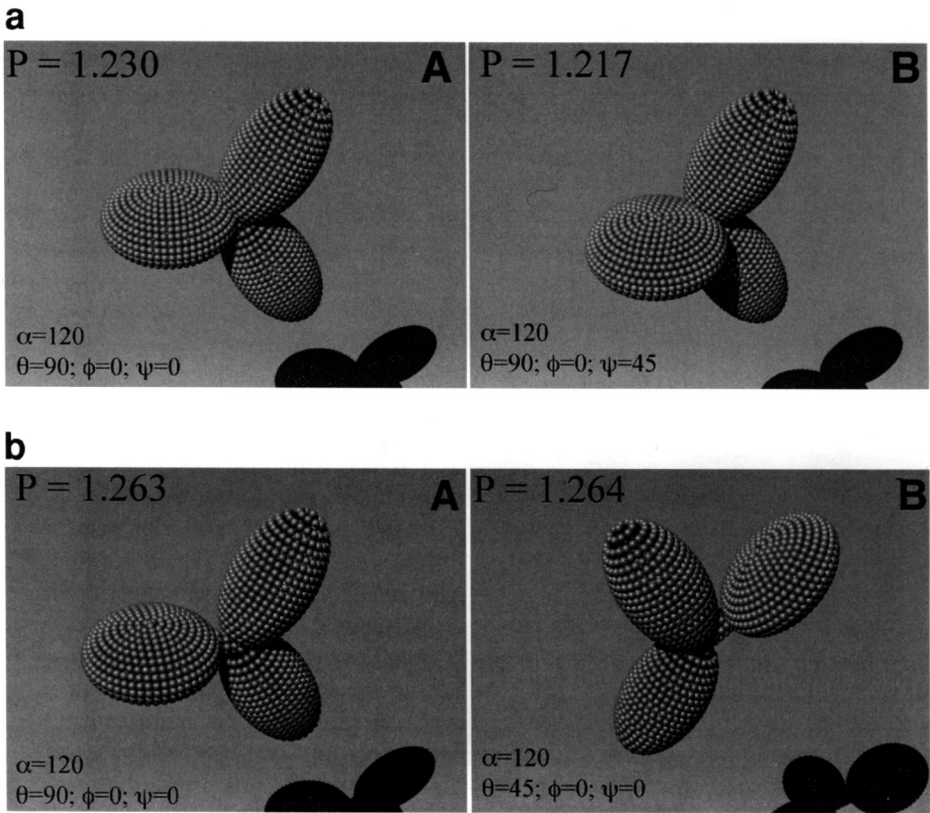


Fig. 11. Examples of domain representations for IgG subclasses of human antibody (a) Open Y-models for human IgG2 and IgG4. Experimental $P = 1.22 \pm 0.03$ for IgG2 and 1.23 ± 0.02 for IgG4. Experimentally, it is impossible to distinguish these two subclasses on the basis of sedimentation properties alone. Model A ($P = 1.230$): coplanar Y shape; Model B ($P = 1.217$) distorted (non-coplanar) Y. Compact models with the Fab arms folded down yield P values that are too small compared with the experimental values, and are ruled out. (b) Open Y-models for human IgG1. Small cylindrical hinge is necessary to model the experimental value of $P = 1.26 \pm 0.03$. Model A, Coplanar hinged Y ($P = 1.263$); Model B, Non-coplanar hinged Y ($P = 1.264$). (c) Coplanar T and Y-models for the hingeless mutant IgGMcg. The experimental value of $P = (1.23 \pm 0.03)$ is best satisfied by either Model A (coplanar Y shape, with twist angle, $\phi = 90$, $P = 1.215$), or with Model B, a coplanar T-shape, with twist $\phi = 0$ and $P = 1.194$. Although immunologically inactive, this hingeless mutant is one of the few intact antibodies whose crystal structures are known: that structure is consistent with the planar hydrodynamic models shown here. (d) Bead shell model for human (Fab)₂. Experimental Perrin function $P = (1.23 \pm 0.02)$. Modeled $P = 1.208$ (linear arrangement of domains: other arrangements yield lower values). (*Figure continues.*)

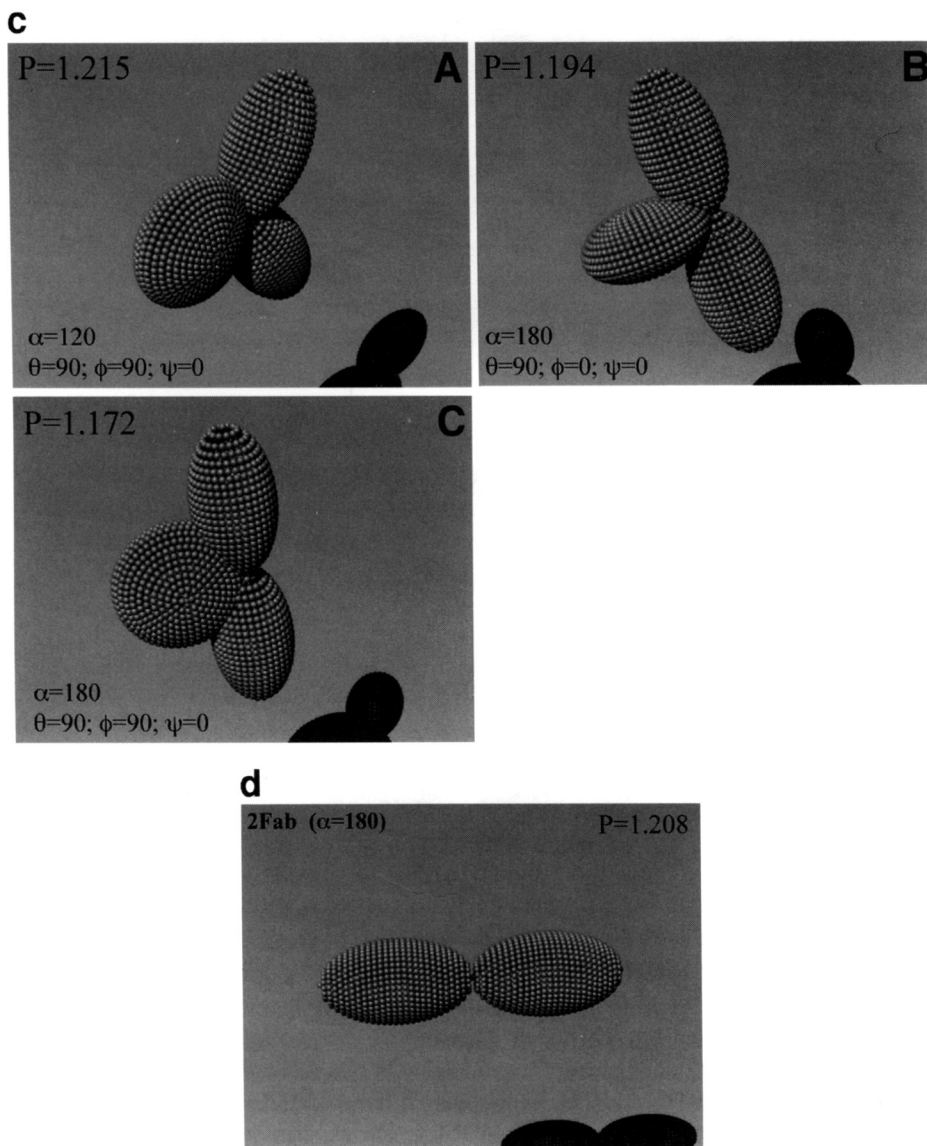


Fig. 11. (Continued.)

4. Notes

1. HYDRO, HYDROPRO, HYDRONMR, and SOLPRO (executables for several platforms, sample files and user guides) are available from the Internet web site <http://leonardo.fcu.um.es/macromol>. ELLIPS_PRIME is available from <http://www.nottingham.ac.uk/ncmh/unit/method.html#Software>; MSTAR and SED-NTERP are available from <http://www.bbri.org/RASMB/rasmb.html>.

2. The symptoms of this are that the measured sedimentation coefficient increases with increase in concentration; and that the measured mol wt > sequence molecular weight, and which also increases with concentration.
3. Experimental advice can be obtained from SEH (steve.harding@nottingham.ac.uk). For specific inquiries with regards the running of HYDROSUB, HYDROPAR, and SOLPRO, please contact JGT (jgt@um.es). General problems over the ultracentrifuge you can post your enquiry to rasmb@server1.bbri.org, where a host of experts will be willing to help.
4. It will be clear from **Fig. 11** that several models can give equally good fits to experimental values for P , the frictional ratio resulting from shape. This is known as the uniqueness problem: using just P as a hydrodynamic modeling parameter limits the detail that can be specified. For example, in the case of IgG1 we can distinguish open from compact conformations, but to be more specific as to which type of open conformation in terms of specific domain angles more information is required. This is the subject of ongoing research: researchers are currently investigating the virtue of additional information from viscosity, fluorescence anisotropy decay methods, and solution X-ray scattering.
5. Other sources of hydrodynamic information are intrinsic viscosity and rotational hydrodynamic parameters. The equivalent shape parameter to P from the intrinsic viscosity is the Einstein-Simha viscosity increment ν , which has a value of 2.5 for spheres and greater for other shapes (22). It is a much more sensitive function of shape than P , but until recently, much higher concentrations have been required for its measurement (>5 mg/mL). A new generation of viscometer based on a measurement of the pressure difference between flow of a solution and flow of the corresponding pure solvent offers the possibility of measurements at much lower concentrations (for example, *see* ref. 22). Fluorescence anisotropy relaxation times (including the harmonic mean) are also much more sensitive to shape than P , but have the disadvantage of requiring the presence of a suitable chromophore which does not rotate with respect to the rest of the molecule. The range of hydrodynamic methods that could be used is considered in **ref. 6**.
6. Bead models can also be calculated from solution X-ray scattering data. X-ray scattering is more sensitive to shape than the translational frictional ratio, but again there are problems, namely in the form of irradiation damage to the protein: this is particularly serious for antibodies because of the extra susceptibility of the hinge region. Neutron scattering is a further alternative, but sources are not readily accessible, and the hydration corresponding to an antibody in aqueous solution may be quite different in a solution of deuterium oxide.

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