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ARTICLE

Joachim Behlke

Recent developments of analytical ultracentrifugation in biopolymer research

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Abstract The development of the analytical ultracentrifuge as a full on-line computer controlled device enables an ever broader application of these ultracentrifuge methods for characterizing macromolecular substances. In this short review some of the numerous possibilities for analyzing biopolymers with respect to solution conformation, conformational changes, association behavior, and homologous and heterologous interactions (including the thermodynamics) are discussed. Most of the results presented here are from the last five years and should be helpful for researchers to get an insight into the structure function relationships of biopolymers.

Key words Analytical ultracentrifuge · Biopolymer structure · Modeling · Interaction · Association constants

Introduction

A powerful tool for the characterization of different kinds of polymers is the analytical ultracentrifuge. This technique allows one to determine the molecular mass, density and size of polymeric substances and in the case of heterogeneous substances also their distributions. Techniques for estimating the sedimentation or diffusion behavior have been widely used for the study of synthetic polymers, as well as biological macromolecules, with sizes varying between several hundreds to tens of millions of Daltons. Moreover, researchers interested in getting information about the shape, gross conformation and conformational changes of biopolymers in solution or other parameters such as subunit stoichiometry, the mechanism of assembly and disassembly of macromolecular complexes, the equilibrium constants or thermodynamic data of association reactions can obtain valuable results. In this way some insight into the structure function – relationships of biopolymers can be deduced from such ultracentrifuge experiments. The number of scientific contributions on biopolymers in this field, particularly after the introduction of the new Beckman analytical ultracentrifuge XL-A, is too large to be discussed here. In this article we will try to indicate some of the most important developments to demonstrate the many applications of ultracentrifuge methods used to characterize biopolymers. This will hopefully act as a stimulus for workers not so familiar with these techniques.

J. Behlke (✉)
Max Delbrück Center for Molecular Medicine,
Robert Rössle Strasse 10, D-13122 Berlin, Germany

Homogeneity and size

In order to characterize structure – function relations of biopolymers (proteins, nucleic acids, polysaccharides and lipids or their complexes) it is important to know whether these macromolecules are "pure", i.e. whether they are monodisperse or contain associates or aggregates, because the latter can cause quantitative as well as qualitative deviations in their properties (Benndorf et al. 1994). One possible way of identifying such behavior is molecular mass determination, for example by the sedimentation equilibrium technique. Data analysis of the radial concentration distribution c(r) of the form

$$c(r) = c(r_m) \exp\left[M_w (1 - \rho \bar{v}) \omega^2 (r^2 - r_m^2)/2RT\right] \quad (1)$$

where r_m represent the radial meniscus position, $(1 - \rho \bar{v})$ the buoyancy factor, ω the angular velocity, R the gas constant and T the absolute temperature, respectively, or the d lnc /d (r^2) values derived from Eq. (1) and using the program MSTAR (Harding et al. 1992; Cölfen and Harding 1997) enables us to determine a whole cell weight average molecular mass M_w (after adequate correction for thermodynamic non-ideality effects, if necessary) from the extrapolation of a special type of molecular mass function known as "M*" to the cell bottom. A linear lnc (r) versus ξ plot ($\xi = (r^2 - r_m^2)/(r_b^2 - r_m^2)$ means the normalized radial displacement parameter) is suggestive but not conclusive

proof of the absence of additional oligomers or strong non-ideality effects.

Monodispersity can be recognized also from sedimentation velocity experiments:

$$s = M\,(1-\rho\,\bar{\nu})\,D/RT \qquad (2)$$

with D the diffusion coefficient, showing a single symmetrical moving boundary. The regression of the sedimentation coefficient (s) or its reciprocal on solute concentration (c) can be characterized by a linear coefficient, k_s in the relation

$$1/s_{20,w} = 1/s^0_{20,w}\,(1+k_s \cdot c) \qquad (3)$$

where $s^0_{20,w}$ means the limiting value of the sedimentation coefficient for c = 0 corrected to water as solvent at 20°C. Positive k_s data according to Eq. (3) imply the absence of self-association. The parameter k_s depends not only on molecular mass, but also on the macromolecular shape. The Wales van Holde ratio $k_s/[\eta]$ = 1.6 ([η] = intrinsic viscosity) observed for sphere-like substances is lower for more extended compounds. The application of this parameter to describe the gross conformation of different chitosans has been discussed recently (Errington et al. 1993).

Sedimentation velocity runs can also be combined with experiments to determine the diffusion coefficients (D) using a synthetic boundary cell. After overlaying the macromolecular solution with solvent (buffer) at low speed (4000–6000 rpm) and measuring the time dependent boundary spreading for the determination in D in a subsequent high speed experiment allows one to obtain the sedimentation coefficient. This procedure permits the quick determination of the molecular mass (M) using Eq. 4,

$$M = s \cdot R \cdot T/D\,(1-\rho\,\bar{\nu}) \qquad (4)$$

with ρ solvent density and $\bar{\nu}$ the partial specific volume of the solute. A program which fits sedimentation velocity concentration profiles by non-linear least squares techniques to models derived from approximations of the Lamm equation has been presented (Philo, 1994). This method works well in situations where the boundaries are significantly spread by diffusion. Both s and D can be determined simultaneously by this method. However, this method seems to be limited to proteins with molecular masses >10^4 Dalton.

Frictional coefficient and radius of spherical macromolecules

The molecular mass is related to the sedimentation coefficient by

$$s_{20,w} = M\,(1-\rho\,\bar{\nu})/N_A \cdot f_s \qquad (5)$$

with N_A the Avogadro number. The translational friction coefficient for sedimentation f_s equals $6\,\pi\,\eta\,R_s$, where η means the solvent viscosity and R_s the Stokes radius. It is obvious that the latter parameter exceeds R_0, the radius of the anhydrous particle,

$$R_0 = (3\,M\,\bar{\nu}/4\pi \cdot N_A)^{1/3} \qquad (6)$$

from the anhydrous molecular volume V_0.

The hydration δ is necessary to calculate the volume V of the hydrated sphere

$$V = V_0\,[(\bar{\nu} + \delta)/\bar{\nu}\,] \qquad (7)$$

From comparable sedimentation and small angle X-ray scattering experiments for proteins with a molecular mass M<10^5 Da (Pessen and Kumosinski, 1985) an average hydration δ = 0.28 g/g protein was deduced. According to the Stokes law the translational friction coefficient f_{sphere} of sphere-like particles is given by

$$f_{sphere} = 6\,\pi\,\eta\,R_{sphere} \qquad (8)$$

Asymmetric macromolecules possess a frictional coefficient larger than those of spherical substances when taking into account the same volume. So, the frictional ratio f/f_{sphere} is often used to approximate macromolecular structures either to prolate or oblate ellipsoids and to estimate the size and ratio of their semiaxes. As has been demonstrated on human blood clotting enzyme VIIa and complexes the soluble tissue factor (Waxman, 1993) additional time-resolved fluorescence anisotropy decay measurements (see Fig. 1) provide information to eliminate one of the above mentioned possible models.

Beside shape, compactness of macromolecules also reflects the frictional coefficient which is inversely related to the sedimentation coefficient. As demonstrated for many single-domain proteins and also for the homodimer creatinase from *Pseudomonas putida* (Schumann and Jaenicke 1993) the transition from neutral to acidic pH (pH 2) leads to formation of a molten globule state of these proteins. The decrease in sedimentation coefficients at the same molecular mass can be explained by assuming a slight expansion of the hydrodynamic volume and increased frictional coefficient according to Eq. (5). Similar effects have been observed for plasmid DNA with positive or negative superhelix density (ΔLk) which sediments faster than the relaxed form (ΔLk = 0), and appears in the least compact conformation available to a circular DNA molecule (Clark et al. 1993).

Modeling

For calculation of model-dependent hydrodynamic properties the computer program HYDRO (Carcia de la Torre et al. 1994) can be recommended. It is based on the geometry of beads which has to be considered for domains or single molecules in supramolecular structures. The program requires the radii and Cartesian co-ordinates of the centers of beads and further input data such as molecular mass, buoyancy factor, solvent viscosity and temperature. In this way translational diffusion coefficient, sedimentation coefficient and further hydrodynamic parameters can be obtained. Possible models can be selected e.g. for myosin derivatives when fitting the calculated parameters ob-

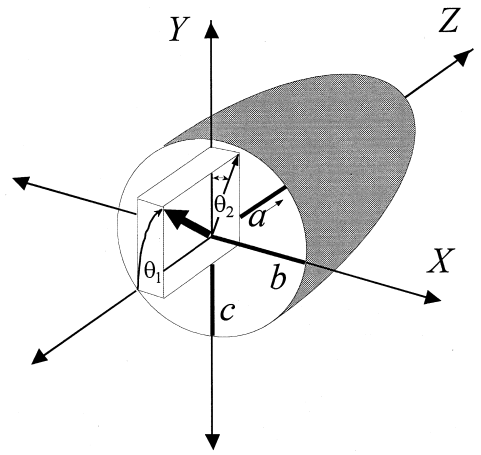

Fig. 1 Ellipsoidal structure with semiaxes a > b = c. The angles θ_1 to the major semiaxis a and θ_2 to the semiaxes b and c define the orientation of the transition dipole moments to the molecular axis. Rotational correlation time measurements on asymmetric molecules allow to differentiate between prolate and oblate ellipsoids according to Waxman et al. (1993)

tained from different arrangements of beads to the experimentally determined data (Byron 1995). Also a good approach to hydrodynamic and electron microscopic results of the ring-like structure of small heat shock protein Hsp25 was achieved recently by this technique (Behlke, Dube et al. 1995). A more recently adapted version of HYDRO called SOLPRO (Garcia de la Torre et al. 1997) permits the modeling of macromolecular shape independent of size, using the so-called "universal shape function". An equivalent series of programs (ELLIPSI-4) for ellipsoid modeling equivalent to SOLPRO has also been given (Harding et al. 1997).

Flotation

Sedimentation occurs only when macromolecules possess a positive buoyancy term $(1-\rho\,\bar{\nu})$. Because of the partial specific volume ~1,0 ml/g the buoyancy value of lipoproteins in salt-containing solutions becomes negative and consequently these substances float. From the moving boundary in opposite direction negative sedimentation or flotation coefficients can be determined. Using a synthetic boundary cell and a buffer density of 1.21 g/l lipoproteins can be separated in at least six fractions of different composition (Okajima et al. 1993). Both flotation velocity and equilibrium has also been used to good effect for the analysis fo micelle forming synthetic polymers (Morgan et al. 1990).

Macromolecular interactions

Proteins can form specific complexes, either with identical proteins (homooligomers) or with other proteins, small ligands, or other macromolecules such as polysaccharides, nucleic acids or lipids. These assemblies are clearly defined structural elements of living cells and play and essential role in cellular events. Determination of the stoichiometry and energy of such interactions is the first stage of analysis.

Self-assembly and disassembly

Many proteins form higher associates from similar but non-identical monomers frequently denoted by Greek letters. The first step in self-association is usually a dimerization reaction of monomers, e.g. in the entropical driven association of α and β tubulin (Sacket and Lippoldt 1993), the charge-charge interactions of α and β chains of human spectrin (Cole and Ralston 1992) or the ion-specific association of yeast phosphofructokinase (Bär et al. 1988). Short column sedimentation equilibrium is the method of choice for the analysis of these macromolecular interactions which are consistent either with a "co-operative isodesmic" model or closed associations stopping at the level of quaternary or oligomeric structure. In enzymes the analysis of partial concentrations of different subunits in comparison to enzymatic activity allows one to determine which of the species in the association equilibria are active (Bär et al. 1988).

If there are strong tendencies for self-association molecular mass experiments have to be carried out in very dilute solution conditions to prevent aggregation effects. As an example, using novel fluorescence detection procedures in isopycnic CsCl density gradient centrifugation (Schmidt et al. 1992) have determined the molecular mass of cyclosporing synthetase to be $M=1.4 \cdot 10^6$ Da at concentrations of 10–50 nM.

Self association plays an essential role in nucleation events as a pre-requisite for protein crystallization. The analysis of these association processes and their thermodynamic consideration can be used as a method for early recognition of crystal growth (Behlke and Knespel 1996).

Membrane proteins in the lipid- or detergent-free state exist as aggregates which are stabilized by hydrophobic forces. Increased amounts of detergents (as monitored by the detergent/protein ratios r_{DP}) provoke a disruption of hydrophobic contacts and lead to a dissociation of protein clusters as can be demonstrated by an exponential decrease of sedimentation coefficients. Semi-logarithmic plots $s_{20,w}$ values versus log r_{DP} permits one to estimate the detergent concentration or "r-values" necessary for dissociation. The efficiency for disrupting protein aggregates is proportional to the critical micelle concentration, as has been demonstrated for cytochrome P450 (Behlke 1992).

Heterologous association

Many proteins are able to form specific complexes with other proteins, polysaccharides or nucleic acids. To analyze such equilibria sedimentation velocity or sedimentation equilibrium experiments are possible. The association between coagulation factor X_a and factor V_a was quanti-

fied by utilizing changes in s-values obtained by the second moment method for different protein mixtures and fitting the data using a non-linear least squares iterative computer program (Pryzdial and Mann, 1991). To analyze interacting macromolecules from sedimentation velocity experiments (Stafford 1992) has developed new methods for computing sedimentation coefficient distribution based on the classical "sedimentation concentration distribution" g(s) approach of Goldberg (1953). The apparent sedimentation coefficient distribution function g (s*), versus s*, where s* is the apparent sedimentation coefficient defined as $s^* = \ln(r/r_m)/\omega^2 t$ and g (s*) has units proportional to concentration per Svedberg. A plot of g (s*) versus s* obtained by UV scanning and Rayleigh interferometric optical systems of the analytical ultracentrifuge (see Fig. 2) is similar to the corresponding plot of dc/dr versus r using the Schlieren optical system, however the latter is more sensitive by several orders of magnitude. Boundaries with concentrations as low as 10 µg/ml can be readily visualized with the Rayleigh optical system. This allows one to analyze interacting macromolecules in a concentration range previously inaccessible with the analytical ultracentrifuge. The higher sensitivity has been achieved by a combination of new analytical techniques that make use of the time derivative of the concentration profile and new instrumental techniques applying a rapid acquisition video-based Rayleigh optical system (Liu and Stafford 1992). By means of the time derivative an automatic background correction is achieved. The video system allows signal averaging of the sedimentation patterns resulting in a higher signal to noise ratio.

A more direct method to get quantitative information about macromolecular association is based on the sedimentation equilibrium technique either by molecular mass or concentration analysis (Kim et al. 1977; Minton 1990). The former variant requires a concentration dependent molecular mass analysis with respect to each component involved in the equilibrium. For high affinity complexes the stoichiometry of reacting components can be derived from the sum of molecular masses which frequently have been used, e.g. for Interleukin-6 interaction with its receptor (Ward et al. 1994). For direct concentration analysis different models have to be considered which simultaneously incorporate association and sedimentation conditions. This has to be carried out to calculate measurable concentration-dependent equilibrium properties depending on radial position and fixed speed or temperature, respectively (Kim et al. 1977; Minton 1990). The models have to be fitted directly to the experimental results (radial concentration distributions) by non-linear least squares procedures. Therefore a confirmation of the proposed stoichiometry and determination of the corresponding association constants can be achieved. When analyzing the radial absorbance distributions at different wavelengths and considering the mass conservation by comparing the loading concentration with those obtained by integrating the areas under the concentration curves (see Fig. 3), reliable data for protein-DNA or protein-protein interactions with respect to stoichiometry and association constant can be obtained (Kim et al. 1994; Behlke et al. 1994).

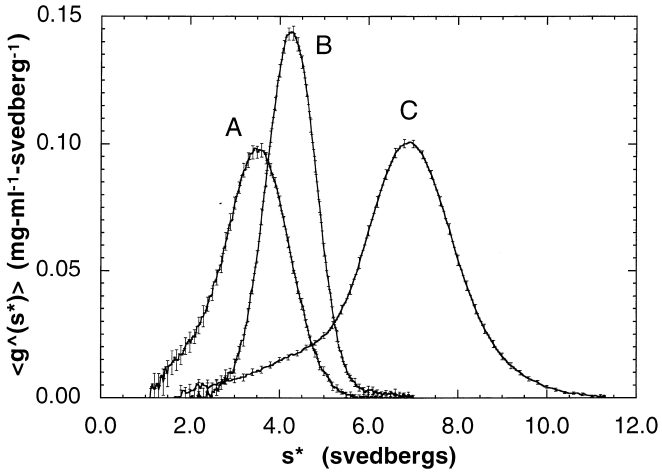

Fig. 2 Sedimentation analysis using the time derivative technique to study an antigen-antibody interaction. (**A**) dimeric single chain Fv (741F8 $(sFv')_2$) (53.0 kDa; s = 3.4 S) directed against the extra-cellular domain of the product of oncogene c-erbB-2. (**B**) recombinant extra-cellular domain (ECD) of c-erbB-2 (91.0 kDA; s = 4.3 S). (**C**) reaction boundary resulting from complex formation between 741F8 and ECD according to the mechanism $(sFv')_2 + 2\,ECD = [(sFv')_2 - ECD] + ECD = [(sFv')_2 - (ECD)_2]$. Subsequent analysis has shown that this system is characterized by a microscopic association constant of $K_a = 1 \cdot 10^7\ M^{-1}$. The value of s for the 1:1 complex is 6 S and that for the 2:1 complex is 8 S

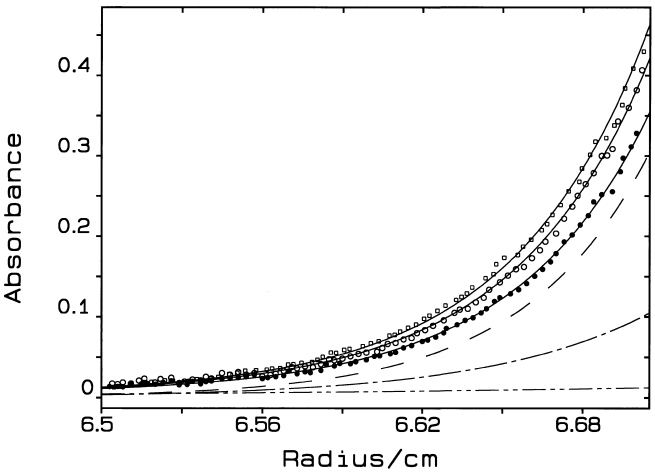

Fig. 3 Radial absorbance distribution of the reacting system adrenodoxin-reductase and adrenodoxin recorded at 378 nm (○), 415 nm (●) and 450 nm (□) at sedimentation equilibrium. The curves for the free reactants adrenodoxin-reductase (– · –), adrenodoxin (– - - –) as well as for the heterologous 1:1 complex (– –) are due to the experimental curve at 378 nm. Both reactants were mixed in an equimolar ratio, concentration 6.5 µmolar in 25 mmolar K-phosphate buffer, pH 7.4, temperature 10 °C. An association constant $K_a = 1.9 \cdot 10^6\ M^{-1}$ was derived from this experiment (Behlke, Ristau et al. 1995)

Complexes between a protein (apolipophorin III) and the lipid dimyristoylphosphatidylcholin have been determined using flotation equilibrium experiments in 2.5 M KBr. From the negative concentration gradient (Weers et al. 1994) the molecular mass of the complex, including its stoichiometry, could be deduced.

Outlook

What kind of advances can we anticipate from future work? The analytical ultracentrifuge is a widely applicable separation technique and the methods to analyze size distributions will continue to be improved. Concerning biopolymers, the analysis – including modeling – of supramolecular structures which are not crystallizable or cannot be analyzed by NMR can be a profitable task for the analytical ultracentrifuge. These techniques can be supported by electron microscopy and image analysis. The capability of modern ultracentrifuges to analyze heterologous associations with respect to stoichiometry and binding strength will have broad application. The results of such equilibria, especially under cellular conditions, are important to obtain insights about interactions under physiological as well as patho-physiological conditions. Many of the above mentioned topics have been discussed during the IX. International Symposium on Analytical Ultracentrifugation in the Max Delbrück Center for Molecular Medicine in Berlin-Buch, Germany 1995 and at the 4th UK Analytical Ultracentrifuge Users' Group Meeting 1996 in Leicester UK.

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