Methods 54 (2011) 145–156

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

The analysis of macromolecular interactions by sedimentation equilibrium

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ARTICLE INFO

Article history:
Accepted 3 December 2010
Available online 16 December 2010

Keywords:
Analytical ultracentrifugation
Sedimentation equilibrium
Interacting systems

ABSTRACT

The study of macromolecular interactions by sedimentation equilibrium is a highly technical method that requires great care in both the experimental design and data analysis. The complexity of the interacting system that can be analyzed is only limited by the ability to deconvolute the exponential contributions of each of the species to the overall concentration gradient. This is achieved in part through the use of multi-signal data collection and the implementation of soft mass conservation. We illustrate the use of these constraints in SEDPHAT through the study of an $A + B + B \rightleftharpoons AB + B \rightleftharpoons ABB$ system and highlight some of the technical challenges that arise. We show that both the multi-signal analysis and mass conservation result in a precise and robust data analysis and discuss improvements that can be obtained through the inclusion of data from other methods such as sedimentation velocity and isothermal titration calorimetry.

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1. Introduction

Macromolecular interactions, such as protein–protein, protein–oligosaccharide, protein–nucleic acid and nucleic acid–nucleic acid interactions are intrinsic to all cellular processes. Their characterization and understanding represents one of the primary endeavors of biochemistry and drug discovery. The biophysical solution studies of these interactions usually involves the determination of both the affinity of interaction and the stoichiometry of the complex through a direct or indirect measure of the concentrations of free and bound species. Traditional methods for the study of such reversible interactions include analytical ultracentrifugation, equilibrium dialysis, gel electrophoresis, size exclusion chromatography, light scattering, differential scanning calorimetry, isothermal titration calorimetry, surface plasmon resonance and various spectroscopic methods [1]. Of these, analytical ultracentrifugation is perhaps one of the oldest techniques still in use [2–4]. It is also one of the most versatile, particularly given the recent improvements in the sensitivity of the detection systems and the continuing developments in the computational methods for data analysis [5–8].

Analytical ultracentrifugation includes the methods of sedimentation equilibrium and sedimentation velocity. In both cases the macromolecular solutions of interest are subjected to a high gravitational field and the resulting changes in the concentration distribution are monitored in real time using various optical methods. Sedimentation equilibrium experiments are usually carried out at low rotor speeds such that the flux of sedimenting macromolecules is balanced by the flux of their diffusion. The time–invariant concentration gradient obtained, described simply based on first principles and equilibrium thermodynamics, can be used to provide information on the macromolecular molar mass and in the case of interacting systems the interaction affinity and stoichiometry. Conversely, sedimentation velocity experiments are usually carried out at high rotor speeds providing information on the transport behavior of the macromolecules in solution. For interacting systems, the sedimentation velocity boundaries will depend on the hydrodynamic properties of each of the macromolecular species, as well as the reaction kinetics [9–11]. Recent advances in the understanding of these systems and the deconvolution of the sedimentation velocity profiles result in a method complementary to sedimentation equilibrium [5,6,9–15].

1.1. Sedimentation equilibrium

Sedimentation equilibrium is one of the most effective methods for the characterization of macromolecular interactions – the determination of the molecular mass by sedimentation equilibrium does not depend on the macromolecular shape and the reaction kinetics does not feature in the data analysis, even though it influences the time to reach equilibrium. Applications of this method, which recently include the analysis of receptor–receptor and receptor–ligand interactions [16–18], the self-association of various regulatory proteins and receptors [19–25], and the interaction of various proteins with DNA, RNA and RNA/DNA hybrids [26–30], altogether demonstrate its usefulness for determining both the affinity and stoichiometry of interacting systems. Further illustration on the use of sedimentation equilibrium can be found in earlier

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1046-2023/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ymeth.2010.12.005

and more comprehensive reviews [8,31–35] and references cited therein.

1.1.1. Principles and considerations

In sedimentation equilibrium the flux of sedimenting macromolecules is balanced by the flux of their diffusion resulting in the establishment of a time–invariant concentration gradient. At equilibrium the chemical potential of the solution is constant, resulting in a concentration distribution that has the following exponential form for a single ideal macromolecule:

$$c(r) = c(r_o)\exp[M_2\partial\rho/\partial c_2(\omega^2(r^2 - r_o^2)/2RT)] \quad (1.1)$$

where r is the radial distance from the center of rotation, ω the angular velocity of the rotor, T the absolute temperature, R the molar gas constant, r_o an arbitrary reference point, such as the meniscus or cell bottom, M_2 the molecular mass of the macromolecule and $\partial\rho/\partial c_2$ the density increment of the macromolecule at constant chemical potential. $M_2\partial\rho/\partial c_2$ represents the buoyant molecular mass which takes into account the Archimedean displacement of a volume of water corresponding to the volume of the sedimenting species, including contributions from macromolecular hydration and macromolecular–cosolvent interactions. Using the three-component formalism developed by Eisenberg [36–39] it can be shown that at constant chemical potential:

$$\partial\rho/\partial c_2 = (1 + B_1 + B_3) - \rho(v_2 + B_1 v_1 + B_3 v_3) \quad (1.2)$$

where ρ is the solution density, v_1, v_2, v_3 are the partial specific volumes of water, the macromolecule and the buffer cosolutes, respectively, and B_1 and B_3 represent the quantities of bound or excluded water and buffer cosolutes in g/g of macromolecule, respectively. In cases where the macromolecules are not highly charged, the solution density is low and cosolutes such as glycerol or detergents are absent, the contributions of B_1 and B_3 are essentially zero and this equation reduces to the familiar:

$$\partial\rho/\partial c_2 \approx (1 - v_2\rho) \quad (1.3)$$

Traditionally, sedimentation equilibrium has been used to determine molecular mass. For proteins this has been possible through the calculation of the partial specific volume v_2 based on their amino acid composition and this parameter, along with the buffer density, can now be calculated using the program SEDNTERP [40]. Partial specific volumes can also be estimated for other macromolecules, such as carbohydrates [41–42] and glycoproteins [43–44] based on their composition, using Traube's additivity rules [45]. In addition, these rules in conjunction with the atomic partial molar volumes published by Durchschlag and Zipper [46–47] allow for estimates of the partial specific volumes of macromolecules other than proteins, carbohydrates and their conjugates. In the case of nucleic acids and other highly charged polyelectrolytes, however, care needs to be exercised as the partial specific volumes are very dependent on both the buffer composition and ionic strength [48–49]. In these cases the effective partial specific volume φ', which incorporates contributions from the B_1 and B_3 terms, needs to be determined experimentally by densimetry or sedimentation equilibrium [26–29]. For the purposes of this work, we will consider dilute solutions and buffer cosolutes such that solutes behave ideally and that $M_2\partial\rho/\partial c_2$ can be replaced simply by $M_2(1 - v_2\rho)$ or the functional $M_2(1 - \varphi'\rho)$.

1.1.2. Application to interacting systems

The study of interacting systems by sedimentation equilibrium relies on the fact that at sedimentation equilibrium the system of interest is also at chemical equilibrium and that the concentrations of the various components are related by the laws of mass action. In many respects sedimentation equilibrium, unlike other methods used to characterize interacting systems, provides a true test for whether a system is at chemical equilibrium and in fact truly reversible – in setting up the equilibrium concentration gradient one is able to assay concentration regimes in which both the complex and the free components are favored. Furthermore, reversibility can be tested by simply changing the rotor speed and perturbing the concentration gradient. The application of sedimentation equilibrium to the study of self-associating and interacting systems has been the subject of previous reviews [8,31–35]. In this work we will only consider the special case of an $A + B + B \rightleftharpoons AB + B \rightleftharpoons ABB$ interacting system, where macromolecule A has two distinct binding sites for macromolecule B. As shown in Fig. 1, the microscopic association constants K_1 and K_2 describe the binding of B to free A on either site (designated 1 and 2) to form complexes AB and BA, respectively. The microscopic association constants describing the binding of a second B species are designated $K_2(1)$ and $K_1(2)$, where $K_2(1)$ describes the binding of the B species to site 2 on A, with a B already bound to site 1 [50]. As sedimentation equilibrium only distinguishes the associating species based on their buoyant molecular mass, one cannot discriminate between complexes AB and BA, and thus between the two sets of microscopic association constants. For this reason, sedimentation equilibrium data analysis usually distinguishes between the formation of complexes AB, BA and BAB through the association constants K_{a1} and K_{a2}:

$$K_{a1} = (c_{AB} + c_{BA})/c_A c_B = c_{AB}/c_A c_B + c_{BA}/c_A c_B = K_1 + K_2$$
$$K_{a2} = c_{BAB}/(c_{AB}c_B + c_{BA}c_B) = (K_1K_2(1) + K_2K_1(2))/2(K_1 + K_2) \quad (1.4)$$

where c_A and c_B are the concentrations of free A and B, respectively, and c_{AB}, c_{BA} and c_{BAB} are the concentrations of the respective complexes. In the special case where A has two symmetric binding sites for B, the microscopic association constants K_1 and K_2 become equal, as do $K_2(1)$ and $K_1(2)$. Based on these equalities, $K_1 = K_2 = K_{01}$ and $K_2(1) = K_1(2) = K_{12}$ one can relate the microscopic association constants to the macroscopic association constants K_{a1} and K_{a2} through:

$$K_{a1} = K_1 + K_2 = 2K_{01}$$
$$K_{a2} = (K_1K_2(1) + K_2K_1(2))/2(K_1 + K_2) = K_{12}/2 \quad (1.5)$$

where K_{a1} is the association constant for the formation of complexes (AB + BA) from free A and free B, and K_{a2} is the association constant for the formation of complex BAB from (AB + BA) and B. If the binding of the first macromolecule B does not influence the binding of the second macromolecule B, then $K_1 = K_2 = K_2(1) = K_1(2)$ from which it follows that $K_{a1} = 4K_{a2}$. In a positively cooperative system, where the binding of the first species B increases the affinity for the binding of the second B species, it follows that $K_{a1} < 4K_{a2}$.

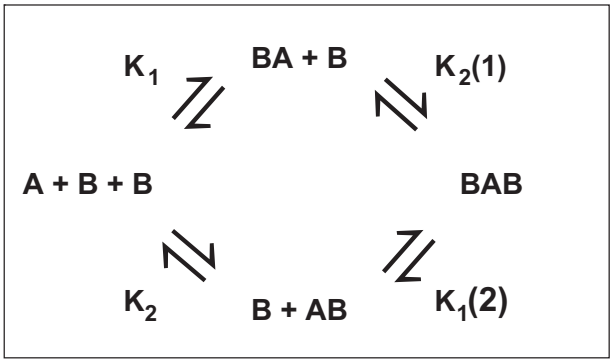

Fig. 1. Reaction scheme for a BAB system. Reaction scheme describing the association of STI (A) with α-chymotrypsin (B) in which the microscopic association constants K_1, K_2, $K_2(1)$, and $K_1(2)$ are defined.