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Self-association studies on the EphB2 receptor SAM domain using analytical ultracentrifugation

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Abstract The self-association behavior of the Eph-kinases SAM domain has been studied in phosphate buffer, pH 7.4, containing 0.14 M NaCl using concentration-dependent sedimentation equilibrium experiments. Only weak interactions typical for a monomer-dimer equilibrium up to at least 12 mg/mL were observed. Such concentrated solutions require a consideration of the non-ideality expressed by virial coefficients. A special centrifuge equation was used for the global analysis to estimate equilibrium constants based on the thermodynamic activities of the reactants. When neglecting this, the parameters deviate by about 20%. Association constants for dimerization of the EphB2-SAM domain vary between 163 M$^{-1}$ at 10 °C and 395 M$^{-1}$ at 32 °C, indicating hydrophobic forces are involved in the dimerization process. In solutions of about 12 mg/mL, less than 50% dimers are in solution and higher oligomers can be excluded.

Keywords EphB2 receptor SAM · Analytical ultracentrifugation · Self-association · Virial coefficients

Introduction

The sterile alpha motif (SAM) domain is a module of less than 80 amino acids contained in numerous signalling molecules, e.g. tyrosine and serine/threonine protein kinases, cytoplasmic scaffolding and adaptor proteins, GTPases and transcription factors (Stapleton et al. 1999). It is assumed that this module plays a functional role in self- and hetero-associations with other SAM domains. From the X-ray crystal structure of a SAM domain homodimer of the intracellular region of the EphA4 receptor tyrosine kinase it was concluded that possible collaboration on the formation of homophilic complexes could regulate signaling events at the membrane and in the nucleus (Stapleton et al. 1999).

With regard to the solution structure of the SAM domains, different results have been discussed in the literature. Whereas in dilute solutions up to 1 mg/mL corresponding monomers are described, the mode of oligomerization at moderate or higher concentrations was interpreted in contradictory ways. Thanos et al. (1999) proposed an equilibrium of monomers and dimers of the SAM domain from Eph receptor tyrosine kinase, EphB2. For the same species, Smalla et al. (1999) proposed additional tetramers, but without conclusive experimental evidence. These declarations were derived from molecular mass studies using sedimentation equilibrium experiments at different loading concentrations. A conclusion was drawn only with respect to weak associations, without statement of any equilibrium constants. Based on size exclusion chromatography and analytical centrifugation studies, Stapleton et al. (1999) have estimated a dimer dissociation for the larger EphA4 in the range of 500 μM to 5 mM. However, in all the above papers, no mention was given with respect to the thermodynamic non-ideality of such solutions. It is well known that solutions of more than 2 mg/mL solute require consideration of virial coefficients, because thermodynamically relevant association constants require activities instead of concentrations. As we demonstrated recently (Behlke and Ristau 2000), the apparent association constants can deviate considerably from the true ones based on the virial coefficients involved. Some biophysical methods (e.g. NMR) require high solution concentrations of proteins and, furthermore, knowledge about the state of association for correct data interpretation. This information can be obtained from experiments using the analytical ultracentrifuge.
Here we present data about the SAM domain from Eph receptor tyrosine kinase (EphB2) derived from sedimentation equilibrium runs that allow us to determine association constants under consideration of the virial coefficients based, in this special case, on the co-volume of the solute. Global fitting of seven radial concentration distributions derived from different loading concentrations obtained from one run in an eight-hole rotor allowed us to calculate exact association constants for the monomer-dimer equilibrium that only exist in the range up to at least 12 mg/mL. The association constants increase somewhat with increasing temperature, indicating hydrophobic interactions are involved in the complex formation.

**Materials and methods**

The SAM domain EphB2 was prepared as described by Smalla et al. (1999). The sample was dissolved in PBS buffer, pH 7.4, containing 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, and 140 mM NaCl. The concentration of the stock solution was determined by the molar extinction coefficient of 8250 M⁻¹ cm⁻¹ at 280 nm (Gill and von Hippel 1989). Molecular mass studies of the protein were carried out using an analytical ultracentrifuge (XL-II, Beckman, Palo Alto, Calif.). Either sedimentation velocity runs to estimate sedimentation and diffusion coefficients or sedimentation equilibrium experiments were performed for the direct calculation of the molecular mass. About 300 µL solution were filled in standard double-sector cells and centrifuged in sedimentation velocity runs at 50,000 rpm or in sedimentation equilibrium experiments at 26,000 rpm after 2 h overspeed at 30,000 rpm. Sedimentation and diffusion coefficients were determined from the time-dependent records of the whole boundaries using our program lamm (Behlke and Ristau 1997, 1998). Experience shows that the approximate solution from the Archibald type is particularly suited for low molecular mass compounds. Reliable results can be obtained also from moving boundaries, where the meniscus is not free of solute. From sedimentation (s) and diffusion coefficients (D) together with the partial specific volume (ν), the molecular mass of SAM was determined by the Svedberg equation. Furthermore, these parameters allow us to calculate the frictional ratio (f/f₀):

\[ f/f_0 = 10^{-8} \left( \frac{1 - \rho v}{sDv} \right)^{1/3} \]  

which is an estimate for the gross conformation.

For the determination of equilibrium constants in solutions of high protein concentration, non-ideality had to be considered. Neglecting this behavior can lead to wrong results. However, for the thermodynamic analysis of solutions at high concentration, it has to be considered that the concentration power series may not converge (Behlke and Ristau 2000). Recently we have developed an approach to overcome this problem by the following equation:

\[ c = c_0 + (K_2 - 2B_{20})c^2 + (K_3 + 6B_{30})c^3 - \frac{3}{2}B_{30}c - \frac{3}{2}B_{11}K_2c^3 + \frac{3}{2}B_{21}c^3 + (4K_1B_{12}B_{20} + K_2^2 - 2B_{21} - K_2^2B_{20} - 4B_{20}^2 + 3B_{11}K_2) \]

\[ +K_2(4B_{11}B_{21} + B_{11}^2 - B_{21}) - K_2^2B_{20} - 4B_{20}^2 + 3B_{11}K_2) \times \ldots \]  

with:

\[ z = z_0 \exp \left( \frac{M(1 - \nu \phi_0) \sigma^2 (r^2 - r_0^2)}{2RT} \right) \]  

Here \( c \) denotes the weight concentration in g/L, \( K_2, K_3, \) and \( K_4 \) are the association constants for the dimer, trimer, and tetramer associates expressed in weight concentration, and the \( B \) symbols are the true statistically defined virial coefficients. The first index is the number of monomers, the second index the number of dimers, and the third index the number of trimers involved in a molecular collision, the origin of the excluded volume. \( z \) is the activity of the monomers calculated from the activity on the reference radius with the help of the so-called \( \psi \) function (Winzor et al. 1999). \( B_{20} \) is the second virial coefficient. It is not possible to estimate the total number of virial coefficients, so the higher virial coefficients were calculated according to Bobblik and Nezbeda (1986) and defined as multiples of \( B_{20} \).

The influence of charge on the virial coefficients was neglected. When the association constants are small it is not possible to estimate \( B_{20} \) with suitable accuracy. Equation (2) has the additional advantage to be explicit in concentration. This is in contrast to the unsatisfactory relations used by Johnson et al. (1981). Simple integration of Eq. (2) according to Eq. (4) yields the theoretical loading concentration for sector cells:

\[ c_L = \frac{2}{r_0^3 - r_m^3} \int_{r_m}^{r_0} cr \, dr \]  

A program virial was written to fit the experimental data. It is able to fit simultaneously seven concentration profiles of different loading concentrations. These were obtained from a sedimentation equilibrium run using an eight-hole rotor. In order to reduce the number of fitting parameters the effective loading concentration of each cell was calculated by numerical integration of the selected part of the radial concentration distribution. From this the reference activity \( z_0 \) of each cell was determined by use of Eq. (4). The extinction coefficient for the employed wavelength was derived from the data obtained at 3000 rpm and the known loading concentration of the protein.

The excluded volume was derived from the diffusion coefficient of SAM in dilute solution in sedimentation velocity experiments. The diffusion coefficient permits us to calculate the Stoke’s radius (\( R_s \)):

\[ R_s = \frac{kT}{6\pi \eta D} \]

Here \( k \) denotes the Boltzmann constant and \( \eta \) the dynamic viscosity of the solvent. \( B_{20} \), here the excluded volume contribution, was determined according to Wills and Winzor (1992) from the particle radius (\( R_p \)), the molecular mass (\( M \)), and the Avogadro number (\( N_A \)) using Eq. (6):

\[ B_{20} = \frac{16\pi \eta N_A}{3M} \]

A value of \( B_{20} = 0.0034 \) L/g was inserted in the program and held constant during the fitting procedure.

Based on the temperature dependence of the molar association constant (\( K \)), the thermodynamic parameters \( \Delta H, \Delta G, \) and \( \Delta S \) were determined by the following equations:

\[ \Delta H = |d(lnK)/dT|RT^2 \]  

\[ \Delta G = RT \ln K \]  

\[ \Delta S = (\Delta H - \Delta G)/T \]  

The association constant allows us to calculate the partial concentrations (weight) of monomers (m) and dimers (d):

\[ \frac{c_m}{c} = \frac{2}{1 + \sqrt{1 + 4Kc}} \]  

\[ \frac{c_d}{c} = 1 - \frac{2}{1 + \sqrt{1 + 4Kc}} \]

The weight average molecular mass values were determined using Eq. (11):