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ARTICLE

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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 signaling 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.

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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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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-I, 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 solute 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 ($\bar{v}$), the molecular mass of SAM was determined by the Svedberg equation. Furthermore, these parameters allow us to calculate the frictional ratio (f/f_0):

$$f/f_0 = 10^{-8}\left(\frac{1-\rho\bar{v}}{sD\bar{v}}\right)^{1/3} \tag{1}$$

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 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:

$$\begin{aligned} c = {} & z + (K_2 - 2B_{20})z^2 + (K_3 + 6B_{20}^2 - \frac{3}{2}B_{30} - \frac{3}{2}B_{11}K_2)z^3 \\ & + \left(K_4 + 12B_{20}B_{30} - \frac{64}{3}B_{20}^3 - \frac{4B_{40}}{3}\right. \\ & \left. + K_2(4B_{11}B_{20} + B_{11}^2 - B_{21}) - K_2^2B_{02} - \frac{4}{3}K_3B_{101}\right)z^4 \ldots \end{aligned} \tag{2}$$

with:

$$z = z_0 \exp\left(\frac{M(1-\bar{v}\rho_0)\omega^2(r^2 - r_0^2)}{2RT}\right) \tag{3}$$

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 ψ function (Winzor et al. 1999). B_{20} is the known second virial coefficient. It is not possible to estimate the total number of virial coefficients, so the higher virial coefficients were calculated according to Boublik 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:

$$c_L = \frac{2}{r_b^2 - r_m^2}\int_{r_m}^{r_b} cr\,dr \tag{4}$$

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):

$$Rs = \frac{kT}{6\pi\eta D} \tag{5}$$

Here k denotes the Boltzmann constant and η 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_s), the molecular mass (M), and the Avogadro number (N_A) using Eq. (6):

$$B_{20} = \frac{16\pi R_s^3 N_A}{3M} \tag{6}$$

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 ΔH, ΔG, and ΔS were determined by the following equations:

$$\Delta H = [d(\ln K)/dT]RT^2 \tag{7}$$

$$\Delta G = RT \ln K \tag{8}$$

$$\Delta S = (\Delta H - \Delta G)/T \tag{9}$$

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

$$\frac{c_m}{c} = \frac{2}{1+\sqrt{1+4Kc}} \quad \frac{c_d}{c} = 1 - \frac{2}{1+\sqrt{1+4Kc}} \tag{10}$$

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

$$M_w = \frac{c_m M_m + c_d M_d}{c_m + c_d} \tag{11}$$

Results

Sedimentation velocity experiments on the EphB2-SAM domain

In order to characterize the SAM domain by sedimentation and diffusion coefficients, sedimentation velocity runs were carried out at protein concentrations of 1 mg/mL. Figure 1 shows time-dependent records of radial concentration distributions which were fitted globally by the program lamm (Behlke and Ristau 1997, 1998). From these experiments a sedimentation coefficient $s_{20,w} = 1.46 \pm 0.02$ S and a diffusion coefficient $D_{20,w} = (14.85 \pm 0.10) \times 10^{-7}$ cm^2/s were determined. By means of these parameters and the partial specific volume $\bar{v} = 0.737$ mL/g, obtained from the amino acid composition (Smalla et al. 1999) and the density increments, a molecular mass of 9.2 ± 0.1 kDa for the dissolved SAM domain was calculated. This result exceeds the theoretical value for the monomeric molecule insignificantly. Furthermore, the determined sedimentation and diffusion coefficients allow us to estimate the gross conformation by the frictional ratio $f/f_0 = 1.16 \pm 0.02$, indicating the SAM domain should have a nearly sphere-like shape in dilute solution. Therefore, from the diffusion coefficient the Stoke's radius $R_s = 1.44$ nm was determined. This value allows us to estimate the co-volume of the monomeric EphB2-SAM domain as the contribution to $B_{20} = 0.0034$ L/g.

Determination of association constants

Seven standard double-sector cells filled with 300 µL protein solution (loading concentration 2.2–4.0 mg/mL) dissolved in PBS buffer, pH 7.4, were centrifuged for 2 h at 30,000 rpm (overspeed) followed by an equilibrium speed at 26,000 rpm for about 48 h using an eight-hole rotor. Because of the higher loading concentration the radial concentration distributions were recorded at 301 nm and transferred into weight concentrations based on an absorbance of 0.0896 for 1 mg/mL at 301 nm (see Fig. 2). The curves, including a concentration range of a few µg/mL at the meniscus up to about 12 mg/mL near the cell base, were fitted globally using Eq. (2) and taking into account a molecular mass of 9073.2 Da for the monomeric protein. When considering only the second virial coefficient, this parameter adopts negative values, indicating that association occurs. Since the influence of higher virial coefficients on the solution structure is unknown, these were considered in the context as discussed in Materials and methods. The association constant for dimerization was found to be $K_a = (3.59 \pm 0.46) \times 10^{-2}$ g/L or 163 M^{-1} for 10 °C. This parameter is not significantly altered at higher neutral salt concentrations, indicating that the influence of net charge on the virial coefficient can be neglected. When fitting the experimental curves and considering the trimer and or tetramer association constant also, only negligible values smaller than the error bar were found. Therefore, we can assume only a monomer-dimer equilibrium to be relevant under the experimental conditions chosen. The dimer association constants are 20% smaller when the fit is made without consideration of the virial coefficients.

Temperature dependence of association constants

The association constant obtained for 10 °C is extremely small and only evident for concentrations in the mg/mL

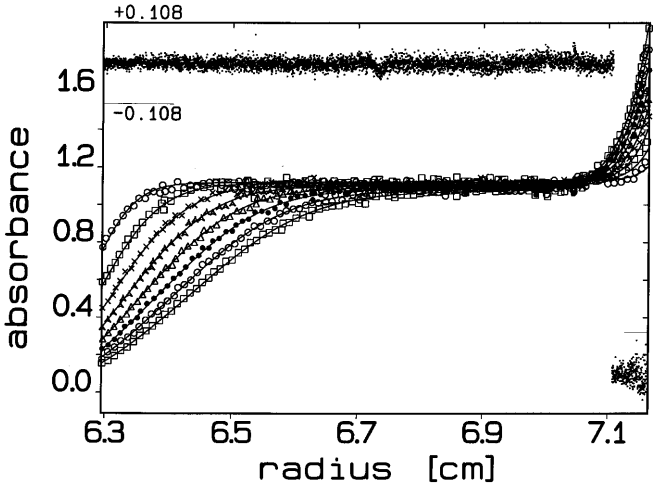

Fig. 1 Radial absorbance distributions (*symbols*) and fitted data (*curves*) of 1 mg/mL SAM domain dissolved in PBS buffer, pH 7.4 (containing 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl). The profiles were recorded at 280 nm with time differences of 15 min. The temperature was 20 °C and speed 50,000 rpm. Residuals are of all datasets and given in twofold amplification and scaled additionally

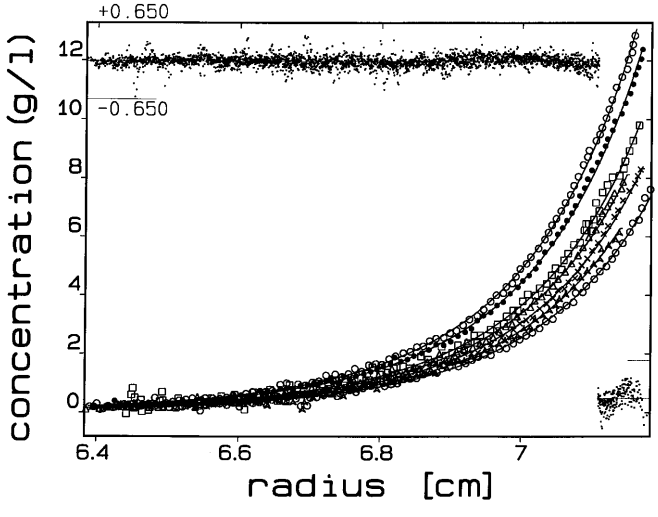

Fig. 2 Radial concentration distribution curves of SAM dissolved in PBS buffer, pH 7.4 (containing 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl). The profiles were recorded after 48 h equilibrium speed at 26,000 rpm at a wavelength of $\lambda = 301$ nm. The curves were fitted using Eq. (2). The temperature was 10 °C. Residuals calculated from all of the datasets and given in two-fold amplification related to the concentration profiles

range. When carrying out the sedimentation equilibrium experiments at higher temperatures up to 32 °C, the association constants increase somewhat up to about 395 M^{-1} (Fig. 3), indicating that weak attractive forces of hydrophobic amino acids seem to be involved in the dimerization event. From the temperature dependence of the association constant, the following thermodynamic parameters were calculated: $\Delta G_{20} = 3.2$ kcal mol^{-1}, $\Delta H_{20} = 6.6$ kcal mol^{-1}, $\Delta S_{20} = 11.6$ cal mol^{-1} K^{-1}.

Concentration dependence of partial concentrations

Because numerous experiments on the Eph-SAM domains were carried out at room or somewhat higher temperature, we have determined the concentration dependence of molecular mass or the partial concentration of monomers and dimers based on the association constant $K_a = 327$ M^{-1} obtained at 27 °C (300 K). This seems to be important since NMR studies were carried out very often at this temperature. The data are presented in Fig. 4. For these conditions the weight average molecular mass of the SAM domain does not deviate strongly from that of the monomer value. Therefore the content of monomers drops only moderately to about 65% on increasing the concentration to 12 mg/mL. A significant part of the higher oligomers can be neglected.

Discussion

The tendency of dissolved Eph-SAM domains to form weak self-associates was proposed in the literature by several groups (Smalla et al. 1999; Stapleton et al. 1999; Thanos et al. 1999). This was concluded from molecular mass studies analyzed by size exclusion chromatography and analytical centrifugation at different loading concentrations or derived from the increased overall correlation time for enhanced protein concentrations estimated by NMR spectroscopy. Exact data describing the solution structure of SAM domains with respect to their association behavior are missing. In this communication we present in thermodynamic terms the equilibrium constants which describe exactly the association behavior of the EphB2-SAM domain in solution over a large concentration range, including the moderate ionic strength that corresponds to physiological conditions. The association constants for a monomer-dimer equilibrium communicated here are extremely small and explain self-association under these non-ligated conditions only at very high protein concentrations. Association constants earlier estimated without consideration of the thermodynamic non-ideality for the related EphA4-SAM domain were more than one order of magnitude higher (Stapleton et al. 1999) in comparison to the parameters obtained by us. Furthermore, in contrast to Smalla et al. (1999), we could not recognize higher oligomers than dimers for the dissolved EphB2-SAM domain. This is due to our improved technique, which is based on a global fit of seven radial concentration profiles of the same sedimentation equilibrium experiment and a consideration of the thermodynamic non-ideality. When neglecting the influence of virial coefficients, distinct deviations in the estimated association constants and partial concentrations of monomers and oligomers were observed.

From X-ray crystallographic data on EphB2-SAM monomers, two types of interactions on the surface area were proposed (Thanos et al. 1999). The first interaction area concerns the N-terminus including Tyr8, which partly inserts into a hydrophobic cavity of the adjacent monomer or forms a hydrogen bond to other amino acids. The second interface is formed by packing helix 5

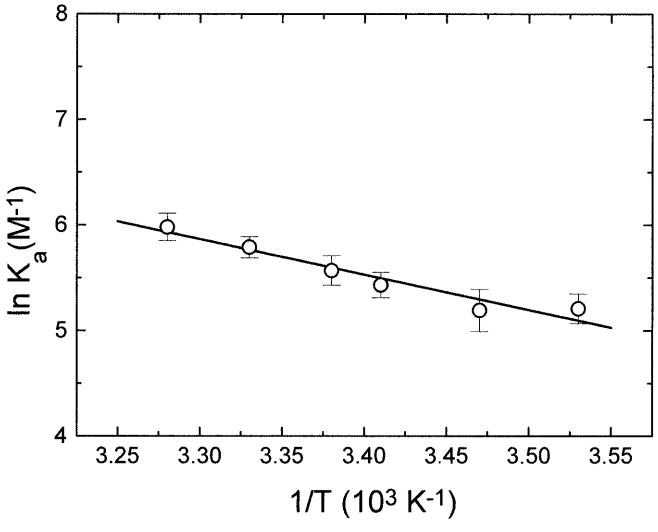

Fig. 3 Temperature dependence of association constants for the dimerization of the SAM domain (van't Hoff plot). Error bars (s.d.) are from multiple experiments

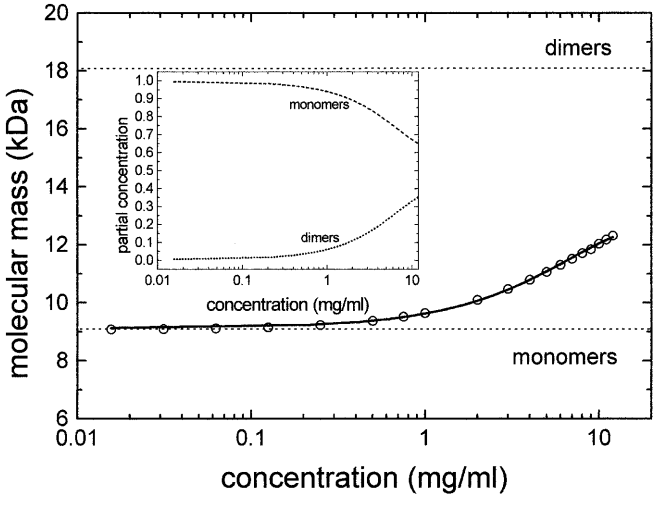

Fig. 4 Concentration dependence of molecular masses of the SAM domain derived from the association constant $K_a = 327$ M^{-1} ($K_d = 3.05$ mM) obtained for $T = 300$ °K. The *solid line* represents the simulation of the calculated molecular mass data using Eq. (11). The *insert* contains the partial concentrations of monomers and dimers, depending on the total concentration

and loop 3 involving Met45, Arg71, and Asn75. The two distinct intermonomer binding surfaces suggest that SAM domains can form extended polymer structures with highly asymmetric charge distribution. According to Thanos et al. (1999) the second interface area contains the more hydrophobic contacts. These should be dominant under the solution conditions used in our experiments. The slightly increased association constants at enhanced temperature confirm the involvement of hydrophobic interactions in the dimerization reaction of SAM domains.

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