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ARTICLE

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Analysis of interacting biopolymer systems by analytical ultracentrifugation

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Abstract Many of the functions of biological macromolecules are based on specific interactions. Extended concentration dependent studies of sedimentation coefficients or molecular masses of biopolymers are highly useful for describing the different kinds of association phenomena. These studies allow one to determine the partial concentrations of monomers and associates or reactants and complexes in self-associating systems or heterologous associations, respectively. Furthermore, in combination with corresponding measurements of biological activity these data allow one to estimate the individual activity parameters of components involved in equilibrium processes. The study of self-association and heterologous association using analytical ultracentrifugation, some recent developments therein, and its application to different examples are outlined here.

Key words Analytical ultracentrifugation · Self-association · Heterologous association · Protein protein interactions · Protein nucleic acid interaction

Introduction

The properties of biopolymers – in particular proteins – can depend significantly on their structure or molecular mass. Particularly those enzymes possessing quaternary structure can frequently vary in their ability to bind or modify substrates (Durrie et al. 1987; White et al. 1993). Qualitative differences in properties between protein monomers and associates can also be observed (see e.g. Benndorf et al. 1994). Because monomers are often in equilibrium with their associates, in such mixtures we observe average properties which reflect the composition of partial concentrations of the different components. To understand these events we have to analyze the corresponding equilibria with respect to the self-association and to determine the number of subunits or the stoichiometry and the equilibrium constant(s). Biological activity can also be adjusted by heterologous association with other compounds. This principle plays an important role in the regulation of cellular as well as biotechnological processes (Gaertner 1978). Such reacting systems can be described by the stoichiometry and binding constant(s). The method of choice for the study of association equilibria is the sedimentation equilibrium technique. However, valuable data can also be derived from sedimentation velocity experiments. In this communication we will give some basic equations and a selection of applications for the analysis of different interacting biopolymer systems.

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Materials and methods

The proteins and nucleic acids used for the studies were purified substances isolated as described elsewhere or were generous gifts e.g. carbonic anhydrase from Dr. H. Schindelin (Cal Tech Pasadena) and HSP25 from Dr. M. Gaestel (MDC-Berlin).

To analyze the association behavior of biopolymers the Spinco Model E and the XL-A analytical ultracentrifuges (Beckman Instruments, USA) were used. Depending on the problem either Schlieren, Rayleigh interference or UV absorbance optics were employed. Six-channel cells were used in the case of sedimentation equilibrium runs and double sector cells in the case of sedimentation velocity studies. Sedimentation coefficients were determined from the time dependent moving boundary measured according to Eq. (1)

$$s = \ln(r/r_m)/\omega^2(t - t_0) \quad (1)$$

with r being the radial position at time t, r_m the meniscus position (time t_0) and ω the angular velocity. When using the Schlieren optical system we obtain the derivative

r^{-1} (dc/dr) as a Gaussian curve for the concentration distribution. The area below the curve is proportional to the initial concentration. For a monodisperse solution with only one solute from the time dependent movement of the peak fraction we can obtain directly the sedimentation coefficient by Eq. (1). For a solution also containing oligomers such as dimers, trimers, tetramers etc., the Gaussian curve becomes more asymmetric because of the somewhat faster sedimentation of the associates. Their sedimentation coefficients s_n (n = 2, 3, 4…) can be estimated by means of the sedimentation coefficient s_1 of the monomers by Eq. (2) which assumes all components are spherical.

$$s_n = s_1 \cdot s^{2/3} \tag{2}$$

If the sedimentation coefficients are relatively small the total distance travelled by the moving boundary and the speed used in the experiment are not sufficient to separate the single oligomer fractions, for which we would also expect Gaussian curves with areas proportional to their partial concentrations. Therefore, we have to analyze an asymmetric concentration distribution curve. By rearrangement of Eq. (1) we obtain a function

$$r_n = r_m \exp(s_n \cdot \omega^2 \cdot t) \tag{3}$$

which permits us to calculate the radial positions of the hypothetical Gaussian maxima of the oligomers according to their partial concentrations at time t. If the resolution of the asymmetric Gaussian curve with several species is insufficient we have to use the dc/dr values at r_n which approximate the partial concentration in the total weight concentration at a given radial position. Considering the molar mass of the species we obtain the molar partial concentrations from which we can calculate the association constants according to Eq. (4)

$$K_n = (c_n)/(c_{n-1}) \cdot (c_1) \tag{4}$$

with n = 2, 3, 4… for the dimerization (K_2), the association of one monomer with one dimer (K_3) etc. If desirable, by means of K_n we can calculate the free energy (ΔG) for the different association steps using Eq. (5)

$$\Delta G_n = RT \ln (K_n) \tag{5}$$

The molecular mass of biopolymers was determined from the radial concentration distribution (c_r) at sedimentation equilibrium by Eq. (6)

$$c_r = c_{rm} \exp[M(1-\bar{v}\rho)\omega^2(r^2 - r_m^2)/2RT] \tag{6}$$

with c_{rm} the concentration at the meniscus, $1-\bar{v}\rho$ the buoyancy term, ω the angular velocity, R the gas constant and T the absolute temperature. If there are two or more species in solution the concentration and the stoichiometry must be considered. This also has to be done for experiments of heterologous association. Assuming a reaction: $R + n\,L \leftrightarrow RL_n$ we have to fit a sum of at least three exponentials to the experimental curve according to Eq. (7).

$$c_r = c_R \cdot \exp(M_r \cdot F) + \cdot c_L \cdot \exp(M_L \cdot F) + c_R \cdot \sum \frac{1}{n^j}\left(\frac{n}{j}\right) \cdot (c_L \cdot K_1)^j \cdot \exp(M_R + j \cdot M_L)\, F \tag{7}$$

This is the simplified equation to describe the radial concentration distribution curve at sedimentation equilibrium for such a reacting system with the reactants (R = receptor and L = ligand molecule) and the complex(es) formed, assuming equal binding sites. In this equation F equals $[(1-\bar{v}\rho)\omega^2(r^2 - r_0^2)]/2RT$. Using the absorbance optical system to describe the concentration distribution curves we need the extinction coefficients ε_R and ε_L to analyze the remaining partial concentrations c_R and c_L, or those of the complexes. If there is a difference in buoyancy between receptor and ligand molecule we also have to consider this in Eq. (7). To reduce the number of parameters to be fit we have to determine the molecular masses M_R and M_L separately. Further details of the analysis and the fitting procedure using our program "POLYMOL" have been described earlier (Behlke et al. 1994, 1995).

The amount of complex (c_{RL}) can be calculated from association constant determined from the initial concentrations of reactants by Eq. (8).

$$c_{RL} = [0.5(c_R + c_L + 1/K_a)] - \{[0.5(c_R + c_L + 1/K_a)]^2 - c_R c_L\}^{1/2} \tag{8}$$

This equation can also be used for the calculation of simple self-associating systems. If such equilibria (e.g. a monomer dimer system) are disturbed by a competition reaction by an inhibitor (I) which binds in the interface region we have to calculate the overall association constant K_{oa} by Eq. (9).

$$K_{oa} = K_a/(1 + K_I \cdot c_I) \tag{9}$$

In this equation c_I is the free inhibitor concentration and K_a and K_I the association and inhibitor constants, respectively.

Results and discussion

1. Self-associating systems

Self-association can be considered as a closed process to form a quaternary structure of a protein as well as an open association with infinite reaction steps to take up a large number of monomers in the associates. In contrast to the latter, during the closed association event the monomers are mostly in a simple equilibrium with the association products containing a defined number of subunits or monomers. Both forms of self-association can be discriminated by concentration dependent measurements of the weight average molecular masses. In special cases we can also describe these events by weight average sedimentation coefficients. First we will consider the closed association and discuss some problems involved in these processes.

Carbonic anhydrase

A pre-requisite for the analysis of self-associating biopolymers is the concentration dependent determination of sed-

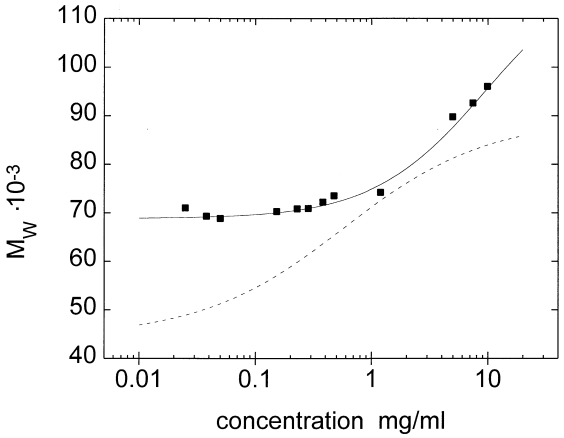

Fig. 1 Concentration dependence of M_w for carbonic anhydrase from *Methanosarcina thermophila* in 20 mM K-phosphate buffer, pH 7.0, containing 1 µM $ZnSO_4$. The solid line describes a trimer-hexamer equilibrium. When considering this as a dimerization of trimers the association constant is $K_a = 1.4 \cdot 10^4\ M^{-1}$. A dimer-tetramer equilibrium (*dotted line*) can be excluded

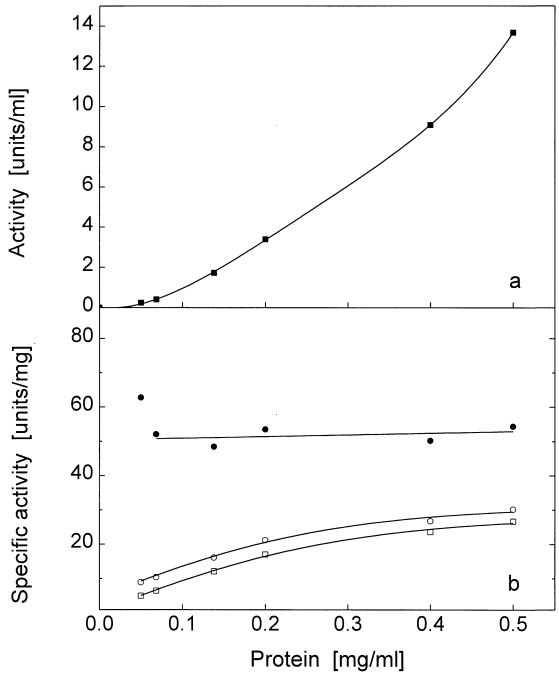

Fig. 2 **a** Activity of a specifically modified yeast phosphofructokinase (Bär et al. 1988) in 0.1 M K-phosphate buffer, pH 7.0. **b** Specific enzyme activity plotted against either the total protein concentration containing tetramers, dimers and monomers (□), or against the tetramers and dimers (○) or only against the partial concentrations of tetramers (●)

imentation coefficients or the molecular masses. This has to be done over a wide range of biopolymer concentration (at least two orders of magnitude) to recognize the alterations involved. If the size of monomers M_1 is known (often derived from the amino acid sequence) we have to divide the molecular mass determined by the corresponding value for monomers. An example for a weak association reaction is the oligomerization of carbonic anhydrase. In contrast to many other carbonic anhydrases (McKinlay and Whitney 1976; Komarova and Doman 1981) the extracellular enzyme produced by *Methanosarcina thermophila* growing on acetate and supporting a H^+/CH_3COO^- symport mechanism and/or converting CO_2 to HCO_3^- facilitating the removal of excess CO_2, forms trimers, as recently shown by X-ray crystal structure analysis (Kisker et al. 1996). This is in agreement with our molecular mass studies at very low concentration. Therefore, we have to assume a trimer is the smallest dissociation unit in solution under non-denaturating conditions. As pointed out by Kisker et al. (1996), in the crystal two trimers dimerize to form a hexamer with 32-symmetry by association of the N-termini of six monomers into a 6-stranded β-structure with a hydrophobic core. We can clearly recognize this relatively weak association process in solution by increasing molecular mass in the higher concentration range up to 10 mg/ml (see Fig. 1). If we reduce the association reaction of two trimers forming one hexamer to a monomer dimer equilibrium the concentration dependence of molecular masses can be described by $K_a = 1.4 \cdot 10^4\ M^{-1}$. A possible dimer tetramer equilibrium, based on the substructure of carbonic anhydrase of bean leaves with 45000 Da and considering the M_w data only near 1 mg/ml, can be excluded from the extended concentration dependent molecular mass studies (Fig. 2). In crystal and in solution carbonic anhydrase from the archeaon *Methanosarcina thermophila* exists as trimer and hexamer.

Phosphofructokinase

Extended concentration dependent molecular mass determinations on enzymes with a quaternary structure can be helpful for recognizing whether only the associates or also the subunits possess biological activity. As we were able to demonstrate earlier (Bär et al. 1988) slightly modified yeast phosphofructokinase exists as a tetrameric molecule in solution. Upon dilution its activity becomes unexpectedly smaller; however, this occurs in a non-linear manner (Fig. 2a). Molecular mass determinations as a function of the protein concentration also yield non-linear behavior, indicating a dissociation of tetramers (Fig. 3). Interpreting the molecular mass data as weight average values according to Eq. (6) we can estimate the dependence of the partial concentrations of tetramers (c_4), dimers (c_2) and monomers (c_1) on the total protein concentration (Fig. 3 insert). When plotting the enzymatic activity of phosphofructokinase as a function of the total protein concentration or the amount of tetramer plus dimer we observe a non-linear dependence. However, when plotting the same data for the activity only against the amount of tetramer a concentration independent behavior is observed (Fig. 2b), indicating that only the tetramers are active in solution. During dilution these species disappear quickly and cause a non-linear diminution of the enzyme activity.

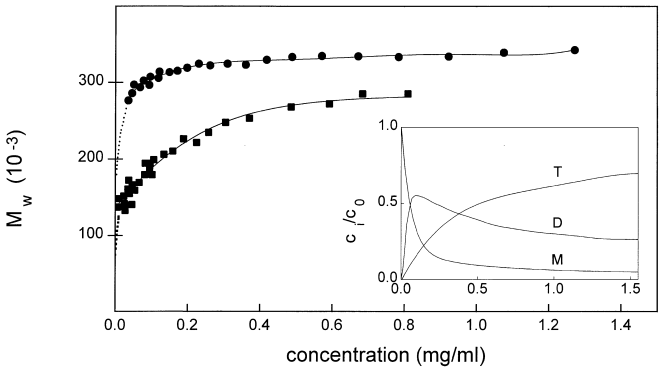

Fig. 3 Presentation of concentration dependent molecular mass of yeast phosphofructokinase in 0.1 M K-phosphate, pH 7.0 (■) including the partial concentrations of tetramers (*T*), dimers (*D*) and monomers (*M*) given in the *insert*. The molecular mass data in the same buffer with additionally 0.5 M $(NH_4)_2SO_4$ (●) are clearly higher

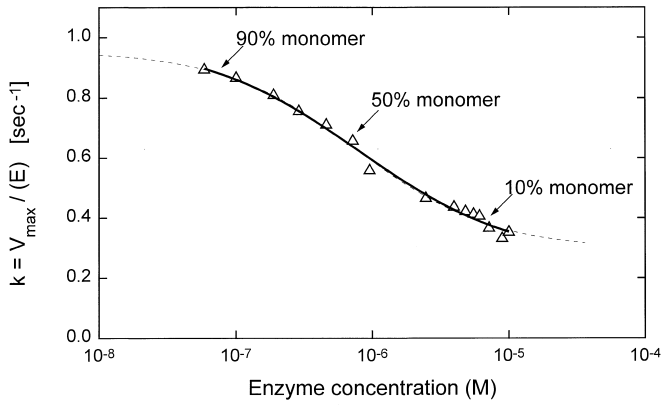

Fig. 4 Kinetic constants k given as V_{max} per monomeric enzyme concentration (E) for D-amino acid oxidase in dependence on the total concentration. According to the association constant of 10^6 M^{-1} the percentage of monomers at different initial concentrations are indicated. On the average k values of 0.95 (monomer) or 0.32 s^{-1} (dimer) were estimated

D-amino acid oxidase

Enzymes can possesses activity in the associated as well as the monomeric state. This is observed, for example, in the D-amino acid oxidase which forms an equilibrium between monomers and dimers. The association constant is pH dependent and is about 10^6 M^{-1} at pH 8. Likewise, the ability to metabolize substrates varies with the pH, indicating that the dimers possesses two independent binding sites. Accordingly the kinetic constants for monomers and dimers are different (Fig. 4). With respect to the K_a value of 10^6 M^{-1}, micromolar enzyme solutions containing a mixture of monomers and dimers demonstrate an average activity corresponding to their partial concentrations, which can be calculated by Eq. (7). To get conditions under which only monomers or only dimers exist in solution we have to reduce or increase the protein concentration by at least two orders of magnitude. However, this is difficult to realize. Nevertheless, the kinetic constants for monomers or dimers can be easily estimated from at least two data pairs of the activity and the partial concentrations of both components (Fig. 4).

Hairpin duplex equilibria

In addition to proteins, single stranded antiparallel oriented oligonucleotides are also able to undergo a self-association to form a duplex structure. Such equilibria are strongly influenced by the base composition of DNA oligonucleotides, forming an equilibrium mixture of ordered antiparallel hairpin and double stranded helical structures in solution containing 0.1 or 0.5 M NaCl between 5 and 40 °C. The dimeric duplex is favored at low temperature and high salt concentrations (Ross et al. 1991). According to our studies on parallel stranded oligomers, duplex formation at 5 °C requires high salt concentrations of at least 1.0 M NaCl (unpublished results).

Nuclei formation

As a special case of self-association with characteristics of an open association we can consider the nucleation process of biological molecules to form crystals. The initial association events in the process of nucleation are of critical importance for the crystallization. Usually we start from a highly concentrated protein solution containing only monomers. Some collisions among the molecules lead to the temporary formation of dimers. These are unstable and most of them dissociate after a short period. However, a few of them survive and can take up another monomer forming a trimer or an additional monomer to become a tetramer etc. (see Fig. 5). These small oligomers are of low stability and therefore are in equilibrium with monomers. The process continues up to higher associates which occur in only very small amount. From sedimentation velocity studies on protein solutions carried out under crystallization conditions we can determine the partial concentrations of the different components involved in the association process as described in the Methods section. The results demonstrate the highest concentration for monomers and steadily decreasing amounts of oligomers from dimers up to n-mers. By calculation of the equilibrium constants for the various association reactions and comparing the free energy values it can be recognized that the formation of the small associates from dimers up to hexamers is unfavorable (positive free energy). This behavior explains the low stability of the small oligomers. Beyond a certain critical size of the associates the free energy change upon adding a monomer to the associates becomes less unfavorable or even slightly favorable. Such stable associates or nuclei in solution are a pre-requisite for successful crystal growth. Two or three days after their appearance protein crystals can be observed. Because these association processes can

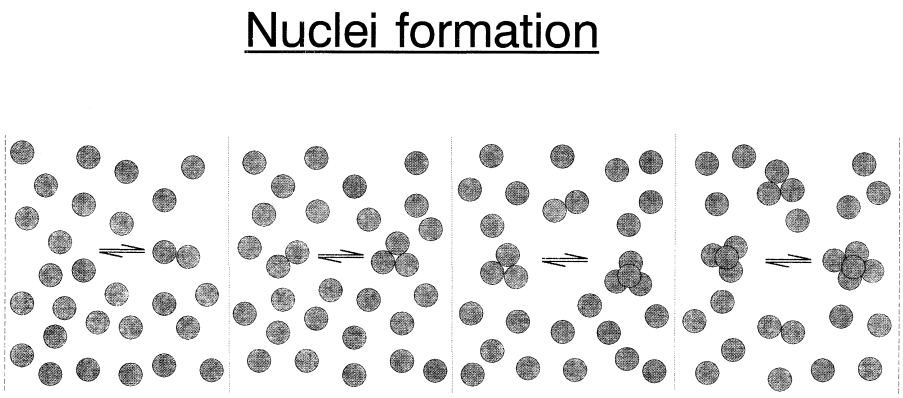

Fig. 5 Model proposed for the different steps of oligomer formation (open association) during a nucleation process

be analyzed by analytical ultracentrifugation after only one hour we have proposed this technique as an early recognition method for the successful crystallization of proteins (Behlke and Knespel 1996).

Peptide competition mapping

In proteins forming a simple monomer dimer equilibrium only few amino acids are involved in the contact region between the two polypeptide chains. The peptide competition mapping method offers a possibility to identify peptide binding regions. Beside knowledge about the primary structure of the protein this method requires peptide fragments of the total amino acid sequence. Peptides which are able to disturb the association equilibrium are candidates for the interface region in the dimers. This effect is based on the fact that the peptides with identical sequence can bind at the site where the two monomers join together (see Fig. 6). Peptides from other protein regions are not able to provoke such an effect. By this method we are able to predict the peptide which is responsible for the dimerization reaction in the HIV core protein p24, including its influence on the overall association constant according to Eq. (9), (unpublished results).

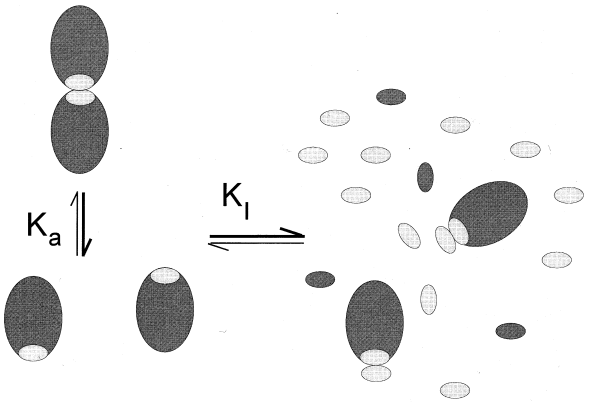

Fig. 6 Scheme for the peptide competition mapping. The monomer dimer equilibrium is shifted to the monomers if peptides containing the binding region (small light gray ellipses) are added to associate with free monomers and compete with the dimerization reaction. Peptides (small dark gray ellipses) of other regions from the polypeptide chain are ineffective in reducing the protein molecular mass

2. Heterologous association

Interactions between different macromolecules under in vitro as well as in vivo conditions are significant for molecular and cell biology. Complex formation of different proteins or proteins and nucleic acids or proteins and polysaccharides is a central feature of life processes involving metabolic pathways, gene regulation, transcription, replication and supramolecular structure. The interactions are of different binding strength and require various approaches to analyze the stoichiometry and equilibrium constant(s). The sedimentation equilibrium technique allows one to study strong as well as moderate or weak association reactions (see Fig. 7). When analyzing strong interactions with association constants $K_a > 10^8\ M^{-1}$ in micromolar solutions we need only a small amount of ligand (L here also means a macromolecule) to occupy the binding site on the receptor molecule (R) and result in a high degree of complex formation. In sedimentation equilibrium experiments we get molecular mass values corresponding only to the stable complex, which allow us to calculate the stoichiometry very simply as the sum of the reactants. In contrast, when analyzing moderate or weak interactions we have to add a larger molar excess of ligand to the receptor to attain complex formation. When using separation methods to remove the free ligand molecules or the complex dissociation into the reactants is possible. In sedimentation equilibrium experiments the reactants and complexes formed exist side by side. Therefore, equilibria of weak interacting components are also not disturbed and we can readily determine the partial concentrations of all components involved in the reacting system using e-function analysis according to Eq. (7). In the following some examples of heterologous interaction will be discussed in more detail.

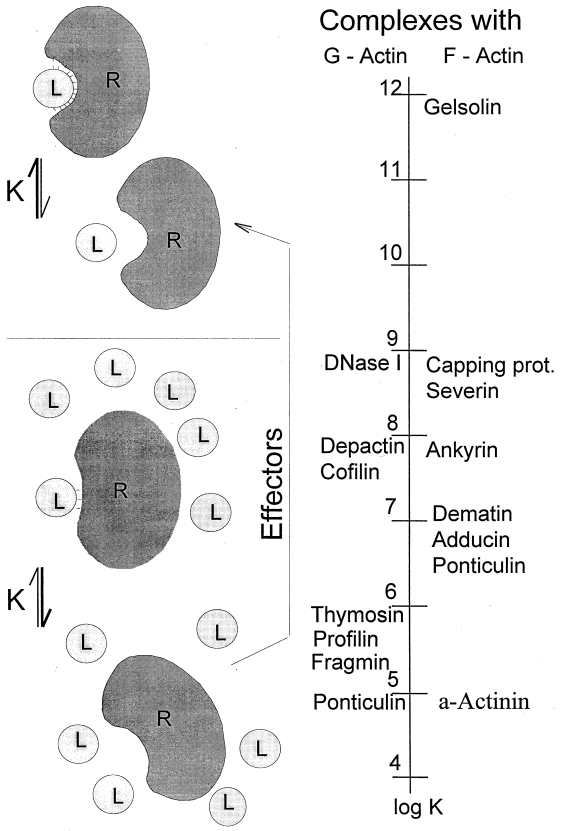

Fig. 7 *Left side:* Two schemes for strong (*top*) intermolecular and moderate or weak intermolecular interaction (*bottom*) between two different kinds of macromolecules. *R*: receptor molecule, *L*: ligand. Effectors (electrolytes, protons or other small molecules) can influence the readiness for stronger interaction. *Right side:* Scale of different actin binding proteins with their association constants. The data are taken from Kreis and Vale (1993)

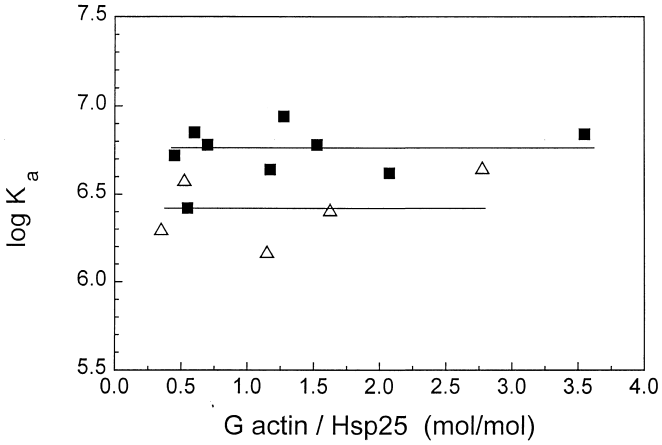

Fig. 8 Association constants for the interaction of G actin with murine (■) or human HSP25 (△). Experimental conditions. 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol; temperature 10 °C. The *solid lines* represent average values for the different species

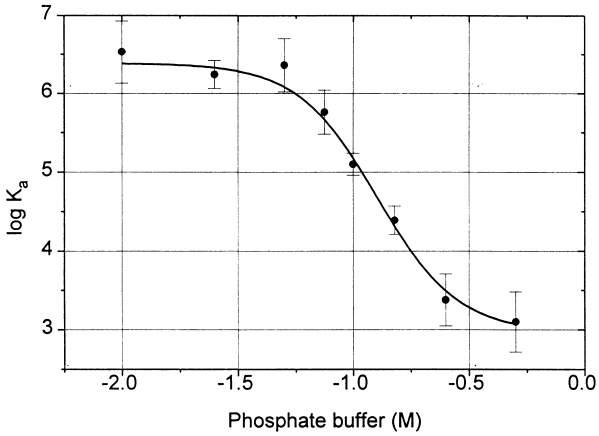

Fig. 9 Plot of molar association constants for the adrenodoxin-reductase adrenodoxin interaction in dependence of the phosphate concentration. Buffer: K-phosphate, pH 7.4. Temperature 10 °C. The error bars are given in sd

G actin-HSP25 interaction

The small heat shock protein HSP25, a cytosolic protein, is known to inhibit the polymerization of G actin (Miron et al. 1988, 1991; Benndorf et al. 1994). It is expressed in all cells under physical stress conditions, mainly at higher temperature. This behavior raises the question, is HSP25 a real actin binding protein and can we consider this property as a real function? To clarify this problem we need to know more about the binding parameters of both proteins. Therefore, sedimentation equilibrium experiments on both proteins in the isolated state and in different mixtures have been carried out. To prevent a salt-induced polymerization of G actin the electrolyte concentrations of buffer used in the experiments were kept very small. These conditions have led to a strong dissociation of the supramolecular structure of HSP25. Analyzing the radial concentration distribution curves using Eq. (7) we obtained binding constants between 10^6 and 10^7 M^{-1}, which are nearly independent of the ratio of the proteins involved in the reaction (Fig. 8). Small differences in the affinity between murine and human HSP25 are recognizable. From these results we can conclude that the moderate binding strength of the small heat shock protein to actin can only lead to a minor alteration of the G actin-F actin equilibrium.

Adrenodoxin-reductase interaction with adrenodoxin

Both proteins belong to the cytochrome P450-dependent monooxygenase system transferring electrons to metabolize exogenous drugs and toxins or endogenously produced steroids, vitamin D, prostaglandins etc. To analyze the structure of both proteins, including the interface region where both components come together during the electron exchange, a co-crystallization of both proteins was necessary. However, this project requires knowledge of the binding properties of both proteins in solution. Sedimentation

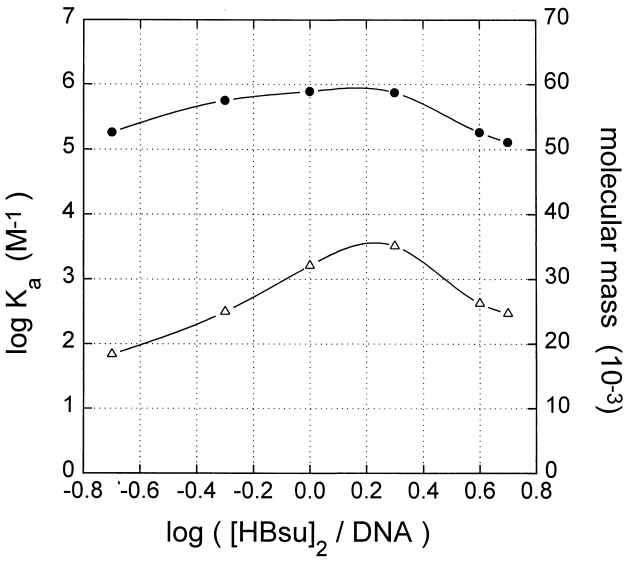

Fig. 10 Association constants (●) and weight average molecular masses (△) for the interaction of HBsu dimers with the double stranded 10 basepair DNA oligonucleotide CGCACACACG/CGTGTGTGCG depending on different molar ratios. Buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, Temperature 14.3 °C

equilibrium experiments carried out with different ratios of the proteins resulted in a 1:1 complex in every case with affinities depending on the ionic strength. As can be seen in Fig. 9 only in low buffer concentrations of 20–50 mM phosphate are the association constants high and they drop by about three orders of magnitude at the high salt concentration of 0.5 M phosphate. This behavior requires a complex stabilization by cross-linking before crystallization under high salt concentrations. To obtain a high yield of the hetero-complex, suitable conditions with respect to optimal concentrations of the reactants are necessary. These can be derived using Eq. (8). The hetero-complex between adrenodoxin-reductase and adrenodoxin obtained in this way could be crystallized successfully (Behlke et al. 1995).

Protein-nucleic acid interactions

In cellular systems nucleic acids are usually complexed with different kinds of binding proteins. The latter can have either specific functions or only protect nucleic acids against nucleolyic attack. Depending on the protein function the binding constants can differ by several orders of magnitude (see also Record et al. 1991). To explain the DNA-condensing activity of histones or histone-like proteins several models have been suggested where DNA is wrapped around a core of proteins (Tanaka et al. 1984, White et al. 1989). These models are based on the assumption that each molecule covers a length of about 10 basepairs of DNA. From spectrocopic experiments using such a histone-like protein, HBsu, a binding site size of only 3.5 basepairs was determined. To clarify this contradiction we have investigated the heterologous association between the dimeric HBsu protein (M = 19 800) and a 10 basepair oligonucleotide (M = 6233) by means of the sedimentation equilibrium technique. We analysed different mixtures from a fivefold excess of DNA over protein to a reversed ratio. In all experiments complexes between the reactants are formed. The concentration of the 1:1 complex exceeds that of the 2:1 complex (two protein dimers per DNA) at high oligonucleotide concentration but the opposite behavior is observed at high protein concentration. From the partial concentrations the association constants and the M_w values representing all components in the mixtures have been determined (Fig. 10). The highest molecular mass obtained for the 2:1 stoichiometry reflects the strongest interaction between both reactants. Because other protein-DNA ratios contain a higher content of free reactants a reduction of M_w was observed. The association constant for the binding of the first protein dimer on the oligonucleotide is somewhat smaller than 10^6 M^{-1} (see Timmermann et al. 1995). The maximal number of HBsu dimers bound by the DNA decamer amounts to two and confirms the value of (3.5 ± 0.5) basepairs of DNA per HBsu dimer obtained from fluorescence spectroscopic measurements (Groch et al. 1992).

Concluding remarks

The different examples presented here as a small subset of the large number of possibilities for studying the association behavior of biopolymer systems. The methods applied are suitable to analyze the stoichiometry, partial concentrations and association constants of self-associating systems as well as heterologous associations in solution. The low speed sedimentation equilibrium technique also allows one to determine moderate and weak interactions with association constants up to 10^3 M^{-1}. At present, association events of up to 20 mg/ml reactant down to a few µg/ml can be studied. In order to analyse biopolymers at lower concentrations new optical systems – e.g. highly sensitive fluorescence optics – are necessary to record the concentration distribution curves with sufficient accuracy.

In combination with the biological activity data insight into structure-function relationships can be derived. These kind of measurements will also play an important role in future work.

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