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Equilibria of oligomeric proteins under high pressure – a theoretical description.

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Abstract

High pressure methods have become a useful tool for studying protein structure and stability. Using them, various physico-chemical processes including protein unfolding, aggregation, oligomer dissociation or enzyme-activity decrease were studied on many different proteins. Oligomeric protein dissociation is a process that can perfectly utilize the potential of high-pressure techniques, as the high pressure shifts the equilibria to higher concentrations making them better observable by spectroscopic methods. This can be especially useful when the oligomeric form is highly stable at atmospheric pressure. These applications may be, however, hindered by less intensive experimental response as well as interference of the oligomerization equilibria with unfolding or aggregation of the subunits, but also by more complex theoretical description. In this study we develop mathematical models describing different kinds of oligomerization equilibria, both closed (equilibrium of monomer and the highest possible oligomer without any intermediates) and consecutive. Closed homooligomer

equilibria are discussed for any oligomerization degree, while the more complex heterooligomer equilibria and the consecutive equilibria in both homo- and heterooligomers are taken into account only for dimers and trimers. In all the cases, fractions of all the relevant forms are evaluated as functions of pressure and concentration. Significant points (inflection points and extremes) of the resulting transition curves, that can be determined experimentally, are evaluated as functions of pressure and/or concentration. These functions can be further used in order to evaluate the thermodynamic parameters of the system, i.e. atmospheric-pressure equilibrium constants and volume changes of the individual steps of the oligomer-dissociation processes.

Keywords: oligomeric protein, high pressure, theory, equilibrium, inflection point

1. Introduction

High-pressure methods became a common tool of investigation of structure and function of proteins during the last two decades (Gross and Jaenicke, 1994; Royer, 1995; Mozhaev et al., 1996; Silva et al., 2001; Marchal et al., 2005; Rivalain et al., 2010; Silva et al., 2014). In some cases they are used to study the properties of proteins from marine organisms living deeply under the sea level (Shrestha et al., 2015), but vast majority of these studies is aimed at elucidation of the structure-function relationships of proteins from common organisms extrapolating their high-pressure behavior to the atmospheric pressure. High-pressure methods are used to investigate protein denaturation, unfolding, conformational changes, enzyme kinetics, etc., but they also have valuable application in studying quaternary structure and equilibria of oligomeric proteins. Many oligomeric proteins have been investigated by high-pressure methods, including those of low number of subunits, mainly dimers (Paladini and Weber, 1981; Silva et al., 1986; Ruan and Weber, 1988; Erijman et al., 1993; Kornblatt et al., 2004; Marchal et al., 2012; Ingr et al., 2015) and tetramers (Jaenicke and Koberstein, 1971; Royer et al., 1986; Ruan and Weber, 1989; Pin et al., 1990; Devillebonne and Else, 1991; Ruan and Weber, 1993; Girard et al., 2010), hexamers (Foguel and Weber, 1995), higher oligomers and viral capsids (Silva and Weber, 1988; Silva et al., 1989, 1992; Da Poian et al., 1993; Silva et al., 1996; Weber et al., 1996) or prion oligomers (Torrent et al., 2015), protein aggregates with less organized structure like casein micelles (Gebhardt et al., 2005, 2006, 2011) and even polymeric structures, e.g. TMV-virus (Bonafe et al., 1998) or microtubules and microfilaments (Messier and Seguin, 1978; Kobori et al., 1996; Nishiyama et al., 2010). Structural changes of oligomeric proteins prior to subunit dissociation were studied, too (Cioni and Strambini, 1996). These studies were concerned with different structural and functional features and in some cases the key thermodynamic parameters, especially the volume change of the oligomer dissociation ΔV and the atmospheric pressure

equilibrium constant K_{atm}, were determined (Silva et al., 1986; Ruan and Weber, 1988, 1989; Pin et al., 1990; Dapoian et al., 1993; Erijman et al., 1993; Ruan and Weber, 1993; Foguel and Weber, 1995; Kornblatt et al., 2004; Ingr et al., 2015). These studies exploit the fact that the high pressure favors a process accompanied with a negative change of the total volume of the system. They show that the oligomeric form is destabilized by high pressure, i.e. the total volume of the monomers is lower than that of the oligomer, which is considered as a general rule for the oligomer dissociation processes supported by whole the experimental evidence. This fact allows us to study the dissociation equilibrium even for oligomers highly stable at atmospheric pressure and dissociating only at very low concentration where the signal of the detection methods is insufficient (Royer, 1995). Application of high pressure is most often coupled with different spectroscopic and fluorometric detection techniques, but methods of light (Meier and Kriegs, 2008) or neutron (Shrestha et al., 2015) scattering, as well as optical microscopy (Nishiyama et al., 2006, 2010) or gel electrophoresis (Paladini et al., 1987, 1994), can be used, too. In addition, properties of the monomeric forms of highly stable oligomers as well as intermediate structures of protein unfolding can be studied by high-pressure X-ray crystallography and NMR (Collins et al., 2011).

Application of high pressure can induce various structural changes from oligomer dissociation via reversible unfolding to an irreversible aggregation, sometimes observable at a single protein (Dumay et al., 1994; Seefeldt et al., 2005; Ingr et al., 2015). It is, therefore, necessary to be able to distinguish among these processes, especially unfolding and oligomer dissociation. The processes can be identified according to the concentration dependence of their transition curves. As was shown in numerous experimental studies (Lange et al., 1996; Mozhaev et al., 1996; Ruan et al., 2001; Royer, 2002; Rouget et al., 2010, 2011; Cioni et al., 2014), the transition curve of a reversible folding-unfolding equilibrium, i.e. the dependence

of the fraction of one of the forms on pressure, is a concentration independent sigmoid with the inflection point pressure

$$p_{\inf} = \frac{RT}{\Delta V} \ln K_{atm} \tag{1}$$

and the fraction of the unfolded form α_u is ½ (for the proof follow the derivation given by eqns 3-14 below for n=1). The slope of the transition curve in the inflection point is

$$\frac{d\alpha_u}{dp} = -\frac{\Delta V}{4RT}. \tag{2}$$

These two quantities allow us to determine the thermodynamic characteristics ΔV and K_{atm} of the process. On the contrary, the transition curve of the oligomer-monomer equilibrium moves towards higher pressures when the concentration grows. This shift can be used to determine the volume change of the process ΔV and the equilibrium constant K for any pressure, including the atmospheric pressure equilibrium constant K_{atm}, as was previously shown for several oligomeric proteins (Ruan and Weber, 1988, 1989, 1993; Foguel and Weber, 1995; Kornblatt et al., 2004; Ingr et al., 2015). In addition, processes with negative ΔV in the direction of association of monomeric subunits are also known. They are usually aggregations with high and not precisely defined number of monomeric units, as was demonstrated on the case of myoglobin (Gebhardt et al., 2003).

Besides many experimental studies, some theoretical works dealing with the thermodynamics of oligomeric-proteins dissociation under high pressure were published as well (Weber, 1986, 1993). In this paper we provide a contribution to the theoretical analysis of some of these equilibria with the stress on the detailed description of the transition curves, especially their significant points, i.e. inflection points and extremes, which may be used as a versatile tool for evaluation of eventual future experiments with oligomeric proteins.

2. Results and discussion

2.1. General assumptions.

In this work we describe closed equilibria (i.e. equilibria between the highest oligomer and the monomer without any intermediate states) of homooligomers of any degree, heterodimer and heterotrimers, and consecutive equilibria (containing intermediate oligomers of lower degree than the highest one) of homo- and heterotrimer. In all the cases only pressure-independent negative volume changes of oligomer dissociations will be considered as it seems to be a good approximation supported by the overall experimental evidence as well as our recent theoretical simulation (Kutalkova et al., 2014).

The parameters that should be determined using the proposed theoretical background are especially the volume changes ΔV accompanying the individual oligomer-dissociation steps and the atmospheric-pressure equilibrium constants of these processes K_{atm}. Their determination is based on the analysis of the transition curves – i.e. responses of the experimental device to the system under changing pressure at a given concentration or, in a reciprocal approach, changing concentration at a given pressure.

For simplicity, the concentrations of individual chemical entities are denoted by simple capital letters with intuitive meaning (*M* – monomer, *A* – subunit A, *D* – dimer, etc.) equal with those used in the respective chemical equations. All the concentrations are considered as dimensionless relative quantities related to the standard concentration of 1 mol dm^{-3}. Accordingly, the equilibrium constants are dimensionless, too. As many of the mathematical derivations are rather lengthy, they are presented in Electronic Supplementary Information, hereafter referred to as ESI.

2.2. Closed equilibria systems.

2.2.1. Homooligomers

Consider the equilibrium between a homooligomeric protein M_n, consisting of n monomeric subunits, and its subunits M described by a chemical equation

$$M_n \rightleftarrows nM. \tag{3}$$

The equilibrium constant of this process for a given pressure p is

$$K(p) = \frac{(M)^n}{M_n}. \tag{4}$$

Denoting the total protein concentration related to the monomeric form M_0 and considering the balance equation

$$M_0 = M + nM_n, \tag{5}$$

the relation between M_0 and the monomer fraction $\alpha = M / M_0$ is given by the equation

$$n\alpha^n + \frac{K(p)}{M_0^{n-1}}\alpha - \frac{K(p)}{M_0^{n-1}} = 0. \tag{6}$$

The reaction change of the Gibbs energy is

$$\Delta G = \Delta G_{atm} + \Delta p \Delta V \tag{7}$$

where ΔG_{atm} is the same quantity at atmospheric pressure and $\Delta p = p - p_{atm}$ is the difference between the current and atmospheric pressures. For simplicity, we approximate Δp by p because p_{atm} is negligible in comparison with p, which is in the order of tens to hundreds MPa in all relevant experiments. As

$$K(p) = \exp\left(-\frac{\Delta G}{RT}\right) = \exp\left(-\frac{\Delta G_{atm} + p\Delta V}{RT}\right) = K_{atm}\exp\left(-\frac{p\Delta V}{RT}\right) \tag{8}$$

where R is the molar gas constant and T is the thermodynamic temperature, eqn (6) becomes

$$n\,\alpha^{n} + \frac{K_{atm}\exp\left(-\dfrac{p\Delta V}{RT}\right)}{M_0^{\,n-1}}(\alpha - 1) = 0. \qquad (9)$$

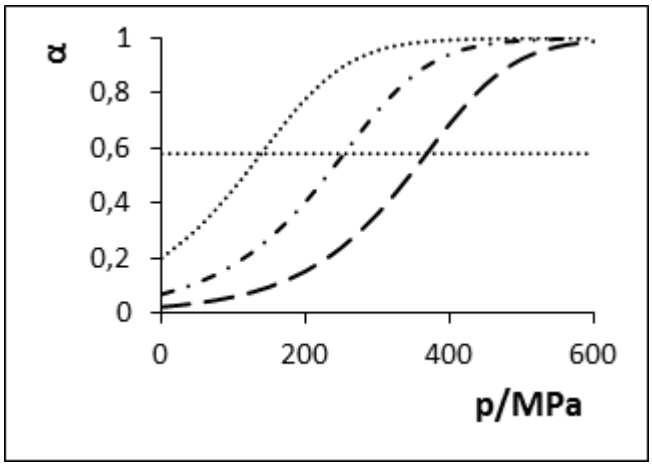

Figure 1. Monomer-dimer equilibrium at high pressure. Fraction of monomer α is plotted as a function of pressure for different total concentrations of monomer. $K_{atm} = 10^{-6}$, $\Delta V = -50$ ml mol^{-1}, R = 8.314 J mol^{-1}K^{-1}, T=300 K; ----- $M_0 = 10^{-3}$; ·-·-·$M_0 = 10^{-4}$; ·····$M_0 = 10^{-5}$. Inflection points of all the curves lie on the horizontal dotted line – eqn (15). Concentrations are given in mol l^{-1}.

This equation can be solved analytically for α as a function of pressure only for $n \le 4$ and only for $n = 2$ it is relatively simple. In this case

$$\alpha(p) = \frac{K_{atm}e^{\frac{-p\Delta V}{RT}}}{4M_0}\left(-1 + \sqrt{1 + \frac{8M_0}{K_{atm}e^{\frac{-p\Delta V}{RT}}}}\right) \qquad (10)$$

(Figure 1). However, it is generally possible to express K as a function of α

$$K(p) = K_{atm} \exp\left(-\frac{p\Delta V}{RT}\right) = \frac{n\,\alpha(p)^n}{(1-\alpha(p))} M_0^{n-1}. \quad (11)$$

Based on this equation K_{atm} and ΔV can be determined provided that $\alpha(p)$ is known for a series of pressure points and $K(p)$ can be determined for each of them, as was shown previously (Ruan and Weber, 1988, 1989, 1993; Foguel and Weber, 1995; Kornblatt et al., 2004; Ingr et al., 2015). In this case

$$\ln K(p) = \ln K_{atm} - \frac{p\Delta V}{RT}. \quad (12)$$

The expression $-\Delta V / RT$ is thus the slope of the linear dependence of $\ln K$ on pressure while $\ln K_{atm}$ is its intercept on y-axis. A drawback of the experimental method based on eqn (12) may arise from the necessity to measure the signal for the whole pressure range in order to reach the extreme cases, i.e. practically pure monomer on one side and oligomer on the other side. This may be difficult for several reasons. First, the "pure" oligomer region may be experimentally inaccessible if the oligomer is not highly stable. Second, the "pure" monomer region may be biased by interference of the oligomer dissociation with other structural transitions, unfolding or aggregation, that often take place at high pressure. Finally, the spectral response can drift with pressure in a manner independent of structural transitions, often differently for the monomer and oligomer cases.

A more robust evaluation method based on the inflection points of the transition curves can be derived from eqn (9) expressing p as a function of α:

$$p(\alpha) = \frac{RT}{\Delta V} \ln \frac{(1-\alpha)}{n\,\alpha^n} + \frac{RT}{\Delta V} \ln K_{atm} - (n-1)\frac{RT}{\Delta V} \ln M_0. \quad (13)$$

It can be shown (see ESI, section S-1) that its inflection point and the corresponding value of α are given by the expressions

$$p_{\mathrm{inf}} = \frac{RT}{\Delta V}\left[(n-1)\ln\left(1+\sqrt{n}\right)-\left(\frac{n}{2}+1\right)\ln n\right]+\frac{RT}{\Delta V}\ln K_{atm}-(n-1)\frac{RT}{\Delta V}\ln M_0 \qquad (14)$$

$$\alpha_{\mathrm{inf}} = \frac{n-\sqrt{n}}{n-1}. \qquad (15)$$

Obviously, for a given n the inflection point occurs at constant value of α independently of other parameters – this value moves towards 1 with growing number of subunit (Figure S-2). This effect can be observed e.g. in (Bonafe et al., 1994) – see figure 4 and solid circles in figure 5 thereof – where a protein consisting of 20 identical subunits is studied.

Eqn (14) represents the inflection point pressure as a linear function of the logarithm of the total protein concentration (expressed as monomer). The thermodynamic parameters ΔV and K_{atm}, eventually $\Delta G_{atm} = -RT\ln K_{atm}$, can be obtained by a linear regression of this function. Using previously published works it can be shown that this approach gives results in a good agreement with those reported by the authors (Paladini and Weber, 1981; Ruan and Weber, 1988; Da Poian et al., 1993; Foguel and Weber, 1995) in spite of the fact that the published transition curves were usually measured only at two different concentrations. Certainly, if the transition curves can be reliably detected in the whole range of α, then a single curve measured at any concentration is, in principle, sufficient to evaluate ΔV and K_{atm} in accord with eqn (12). However, if the limits of the transition curve for α tending to 0 and 1 cannot be well detected, but the central part containing the inflection point is still observable, then the evaluation method based on eqn (14) is an optimal choice, although the curve should be measured for more concentrations. As an example, consider the data by Ruan and Weber (Ruan and Weber, 1988) who determined, according to their figure 2, the thermodynamic

parameters for dissociation of hexokinase dimer of $\Delta V = -150\ ml\,mol^{-1}$ and $K_{atm} = 6.1\times10^{-10}$. Using the same plot, we can estimate the inflection-point pressures as 140 MPa for 1.7 μM dimer and 100 MPa for 0.17 μM dimer. Linear regression according to eqn (14) then gives $\Delta V = -131\ ml\,mol^{-1}$ and $K_{atm} = 8.9\times10^{-10}$ showing a good agreement of both the methods. (For more detailed analysis including the reproduced original plot see ESI, section S-15.) However, this comparison must be taken with caution since only two points were taken into account.

Actually, when ΔG_{atm} is not too small in absolute value, i.e. K_{atm} is substantially different from 1, the first term in eqn (14) is negligible since the expression in brackets is within the range (-1; 1) for all values of n up to 59 (for $n = 8$ it is 1.00003) and even further it does not grow dramatically. Thus, in these cases the y-axis intercept can be in a good approximation identified with $RT \ln K_{atm} / \Delta V$. (For instance, when $K_{atm} = 10^{-3}$, i.e. $\Delta G_{atm} = 17{,}1 kJ\,mol^{-1}$ at 25 °C, the value of $\ln K_{atm} = -6.9$ and thus the error of this quantity is at maximum 17% and is decreasing when the oligomer is more stable at atmospheric pressure.) It may be useful, especially for oligomers with higher number of subunits n, to define a volume change related to one monomer as

$$\Delta V_{mon} = \Delta V / n. \qquad (16)$$

The slope of eqn (14) then takes the form $[-(n-1)/n]RT / \Delta V_{mon}$, which tends to $-RT / \Delta V_{mon}$ for higher n. Hence, it is possible to determine the volume change per monomer unit for equilibria of higher oligomers even when the number of subunits n is not known. It should, however, be known in order to determine ΔV eqn (16) and K_{atm}.

Although for most of proteins n is usually known, its determination can be desirable for many-subunits, e.g. micelles or viral capsids. It can be estimated from eqn (15), but it is

necessary to measure the transition curve in a wide pressure range in order to see both the limit cases, complete oligomerization and complete monomerization of the system. The former state can be usually assumed at the atmospheric pressure. The latter state may be approximated by the point of maximum curvature of the transition curve which is always very close to $\alpha = 1$, especially for high values of n (see Figure S-2). The region behind this point can be used to identify the biasing trend of the curve. This determination should be, however, considered only as a rough estimate, due to the eventual limited sensitivity of the experimental methods.

2.2.2. Heterodimer

Heterodimer is the only system consisting of non-equal subunits that necessarily undergoes only one-step oligomerization equilibrium

$$D \rightleftarrows A + B. \tag{17}$$

It is, therefore, interesting to compare it with homodimer. Considering the dissociation constant definition

$$K_d = \frac{AB}{D} \tag{18}$$

and balance equation

$$A_0 = A + D \tag{19}$$

(in case that the total concentrations of subunits A and B are different, we consider, without the loss of generality, A to be the lower concentrated one), the fraction of monomeric form of A defined as

$$\alpha_A = \frac{A}{A_0} \tag{20}$$

is obtained as a solution of the quadratic equation

$$A_0\alpha_A^2 + \alpha_A\left(B_0 - A_0 + K_d(p)\right) - K_d(p) = 0. \tag{21}$$

This equation gives, after introducing the pressure dependence of K_d given by eqn (8), a solution

$$\alpha_A(p) = \frac{A_0 - B_0 - K_{d,atm}e^{\frac{-p\Delta V}{RT}}}{2A_0}\left(1 + \sqrt{1 + \frac{4K_{d,atm}e^{\frac{-p\Delta V}{RT}}A_0}{\left(A_0 - B_0 - K_{d,atm}e^{\frac{-p\Delta V}{RT}}\right)^2}}\right) \tag{22}$$

which is plotted in Figure 2.

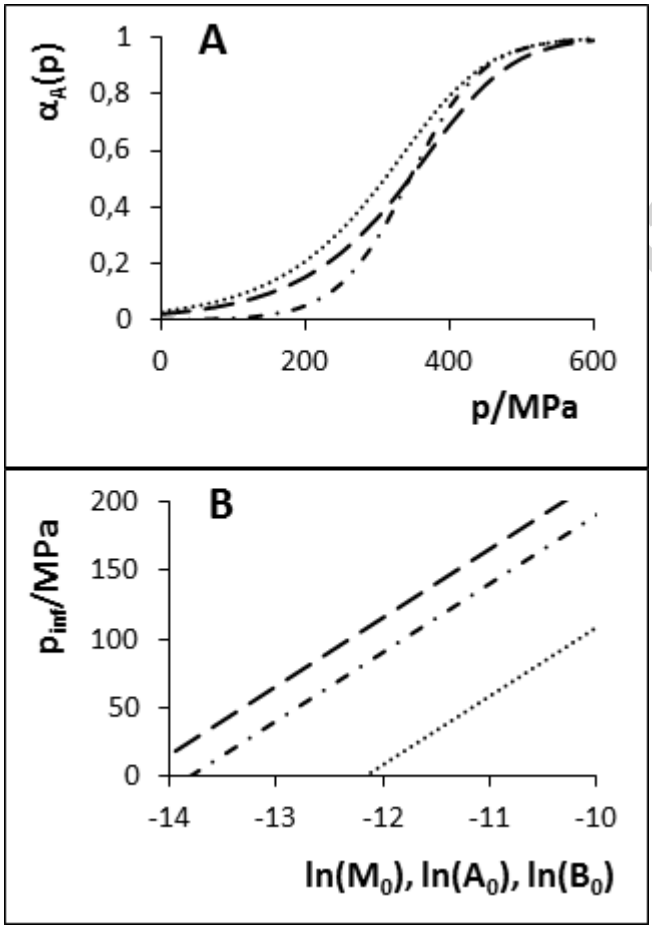

Figure 2. A: Fraction of the monomeric subunit A as a function of pressure for heterodimer at stoichiometric (·····) and non-stoichiometric (·-·-·) ratio of concentrations of subunits A and B and for a homodimer (-----). $K_{d,atm} = 10^{-6}$; ΔV = -50 ml mol^{-1}; R = 8,314 J mol^{-1}K^{-1}; T = 300 K; $B_0 = 10^{-3}$ mol l^{-1}; $M_0 = 10^{-3}$ mol l^{-1}. In case of non-stoichiometric ration of A and B the concentration $A_0 = 10^{-8}$ mol l^{-1}. B: Linear dependences of inflection points of transition curves plotted in panel A on the monomer concentration.

In analogy with the previous section, an equation can be derived that gives the value of the inflection point α_A as a function of A_0 and B_0:

$$\alpha_A^4 - 4\alpha_A^3 + 2\alpha_A^2\left(3 - 2\frac{B_0}{A_0}\right) - 2\alpha_A\left(+\frac{B_0^2}{A_0^2} - 3\frac{B_0}{A_0} + 2\right) + \frac{B_0^2}{A_0^2} - 2\frac{B_0}{A_0} + 1 = 0. \qquad (23)$$

This equation is of the fourth order and thus difficult to solve in general. However, the equlibrium can be studied in two significant special cases, for $A_0 = B_0$ and for $B_0 >> A_0$. In the former case, which corresponds e.g. to the work with isolated natural heterodimeric protein, the linear and absolute terms in α_A vanish and the equation gets, after some algebra, the form

$$\alpha_A^2 - 4\alpha_A + 2 = 0. \qquad (24)$$

with the solution $\alpha_{A,\mathrm{inf}} = 2 - \sqrt{2} = 0.586$, equally as in the case of a homodimer. The corresponding inflection point pressure is

$$p_{\mathrm{inf}} = \frac{RT}{\Delta V}\ln\left(\frac{1+\sqrt{2}}{2}\right) + \frac{RT}{\Delta V}\ln K_{d,atm} - \frac{RT}{\Delta V}\ln A_0. \qquad (25)$$

Thus, this expression is completely analogous with eqn (14) and can be analyzed in the same way in order to obtain $K_{d,atm}$ and ΔV. Its use can be verified e.g. on the published data by Foguel and Weber (Foguel and Weber, 1995), see ESI, section S-15. The only difference is in the logarithmic expression in the first term which has the value of ln(1.207) = 0.188 for heterodimer, while for homodimer it is equal to $\ln\left[\left(1+\sqrt{2}\right)/4\right] = -0.504$.

The latter case, in which $B_0 >> A_0$, can be helpful especially at systems of less stable heterodimers where the individual subunits can be either purified or prepared by recombinant expression as independent proteins. In this case the concentration of subunit B can be considered as a constant of the value B_0. Then

$$\alpha_A = \frac{1}{\frac{B_0}{K_{d,atm}} e^{\frac{p\Delta V}{RT}} + 1} \tag{26}$$

and the inflection point pressure of this transition curve is

$$p_{\mathrm{inf}} = \frac{RT}{\Delta V} \ln K_{d,atm} - \frac{RT}{\Delta V} \ln B_0 \tag{27}$$

(for derivation see ESI, section S-2) which allows us to determine ΔV and $K_{d,atm}$ by means of a linear regression. Thus, the analysis of a heterodimer is formally equal to that of a homodimer, but the subunit difference gives us two ways how to easily determine the parameters $K_{d,atm}$ and ΔV, one suitable especially for highly stable heterodimers and the other one for heterodimers of lower stability.

2.2.3. Closed equilibria of heterotrimers.

As the subunits of heterotrimers are not equal, it is generally reasonable to consider the equilibria in heterotrimers as consecutive. For instance, the dissociation of a trimer consisting of two subunits A and one subunit B (A_2B heterotrimer) may consist of the trimer dissociation to A_2 and B followed by the dissociation of the A_2 dimer. However, the consecutive equilibrium changes to the closed equilibrium when the dimer is unstable in comparison with the trimer, and therefore the dimeric subsystem A_2 exists in substantial amount only in a complex with the subunit B. In this case the following equilibrium can be expected

$$T \rightleftarrows 2A + B, \tag{28}$$

which is characterized by the dissociation constant

$$K = \frac{A^2 B}{T} \tag{29}$$

and the balance equations

$$A_0 = A + 2T; \qquad B_0 = B + T. \tag{30}$$

Let us define the fractions of monomer and trimer as fractions of the subunit A present in one or another state, i.e.

$$\alpha_M = \frac{A}{A_0}; \qquad \alpha_T = \frac{2T}{A_0}. \tag{31}$$

At the stoichiometric ratio of the subunits A and B the position of the inflection point of the trimer-monomer transition curve is given by a linear function

$$p_{\inf} = \frac{RT}{\Delta V} \ln\left(\frac{6 + 4\sqrt{3}}{9} K_{atm} \right) - 2 \frac{RT}{\Delta V} \ln A_0 \tag{32}$$

which can be used to determine the thermodynamic parameters K_{atm} and ΔV.

Analogously, a similar derivation can be carried out for the heterotrimer composed of three different subunits A, B and C (ABC heterotrimer). Considering the stoichiometric ratios of all three subunits, we arrive at a solution identical to the A_2B heterotrimer, the inflection point pressure is thus given by eqn (32).

2.3.Consecutive-equilibria systems.

2.3.1. Homotrimer.

If an oligomer consists of more than two subunits, its association or dissociation can run consecutively, i.e. via one or several intermediate states. The situation was partially described by Ruan and Weber (Ruan and Weber, 1989) for the system homotetramer-dimer-monomer

and later even studied experimentally by Foguel and Weber on a hexamer-dimer-monomer system (Foguel and Weber, 1995). In both the cases, however, the dimer was very unstable and thus the equilibrium was reduced to the closed one, as discussed below. Even simpler example is a protein consisting of three identical subunits (homotrimer) which can consecutively dissociate from trimer (T) to dimer (D) plus monomer (M) and the dimer can further dissociate to monomers:

$$T \rightleftarrows D + M; \qquad D \rightleftarrows 2M. \tag{33}$$

At a given pressure the equilibrium of the system is described by the trimer and dimer dissociation constants

$$K_T = \frac{DM}{T}; \qquad K_D = \frac{M^2}{D}. \tag{34}$$

If the total concentration of the protein expressed in terms of the monomeric units is M_0, the balance equation

$$M_0 = M + 2D + 3T \tag{35}$$

is valid. The mathematical model derivation is complicated by the presence of the two pressure-dependent equilibrium constants. It is, therefore, convenient to solve the problem for a fixed pressure and then introduce the pressure dependence to the obtained solutions.

Let us define the fractions of monomeric units present in the forms of monomer, dimer and trimer

$$\alpha_M = \frac{M}{M_0}; \qquad \alpha_D = \frac{2D}{M_0}; \qquad \alpha_T = \frac{3T}{M_0}, \tag{36}$$

respectively. Considering this, the following equation is obtained (for derivation see ESI, section S-4):

$$\sqrt{\frac{K_D\alpha_D}{2M_0}}+\alpha_D+3\sqrt{\frac{K_D\alpha_D^3M_0}{8K_T^2}}=1. \qquad (37)$$

This equation can be solved for α_D as a function of M_0 and, consequently, α_M and α_T can be determined, too. However, as the equation is of the third degree in α_D, the solution is rather cumbersome and complicated to understand. Nevertheless, the equation was solved by the Wolfram Mathematica 9 package and the result is shown in Figure 3 for several sets of the thermodynamic parameters. The complete expression is given in ESI, appendix S-1.

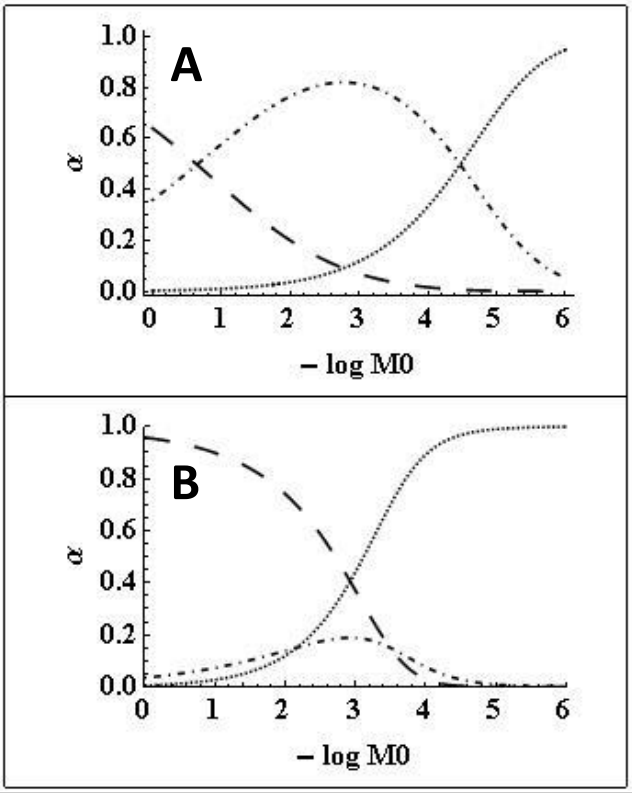

Figure 3. Consecutive equilibrium of homotrimer – concentration dependence. Fractions of trimer, dimer and monomer as functions of protein concentration (expressed as a monomer) for different sets of thermodynamic parameters. A: $K_{D,atm}$ = 10^{-5}; $K_{T,atm}$ = 10^{-4}; ΔV_D = -20 ml mol^{-1}; ΔV_T = -50 ml mol^{-1}; B: $K_{D,atm} = K_{T,atm}$ = 10^{-4}; ΔV_D = -50 ml mol^{-1}; ΔV_T = -20 ml mol^{-1}. In both cases T = 298,15 K; p = 150 MPa; ····· monomer; ·-·-· dimer; ----- trimer.

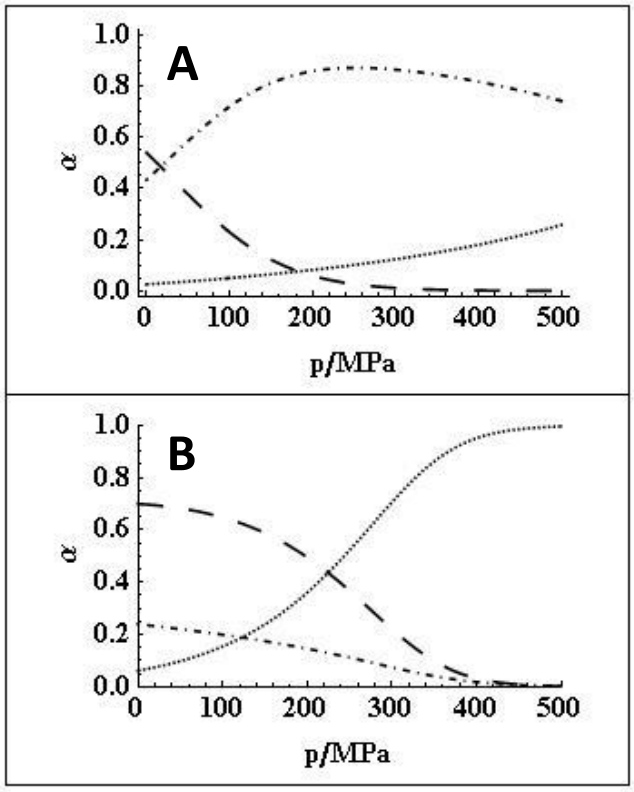

Figure 4. Consecutive equilibrium of homotrimer – pressure dependence. Fraction of trimer, dimer and monomer as functions of pressure for different sets of thermodynamic parameters. A: $K_{D,atm}$ = 10^{-5}; $K_{T,atm}$ = 10^{-4}; ΔV_D = -20 ml mol^{-1}; ΔV_T = -50 ml mol^{-1}; B: $K_{D,atm} = K_{T,atm}$ = 10^{-4}; ΔV_D = -50 ml mol^{-1}; ΔV_T = -20 ml mol^{-1}; In both cases T = 298,15 K; -log M_0 = 2.5; ····· monomer; ·-·-· dimer: ----- trimer.

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If the pressure dependence of K_D and K_T is introduced in analogy with eqn (8) with $K_{D,atm}$ and $K_{T,atm}$ being their atmospheric-pressure values and V_D and V_T the respective volume changes, fractions of the individual states can be plotted also as functions of pressure. Figure 4 presents this plot for two different sets of parameters.

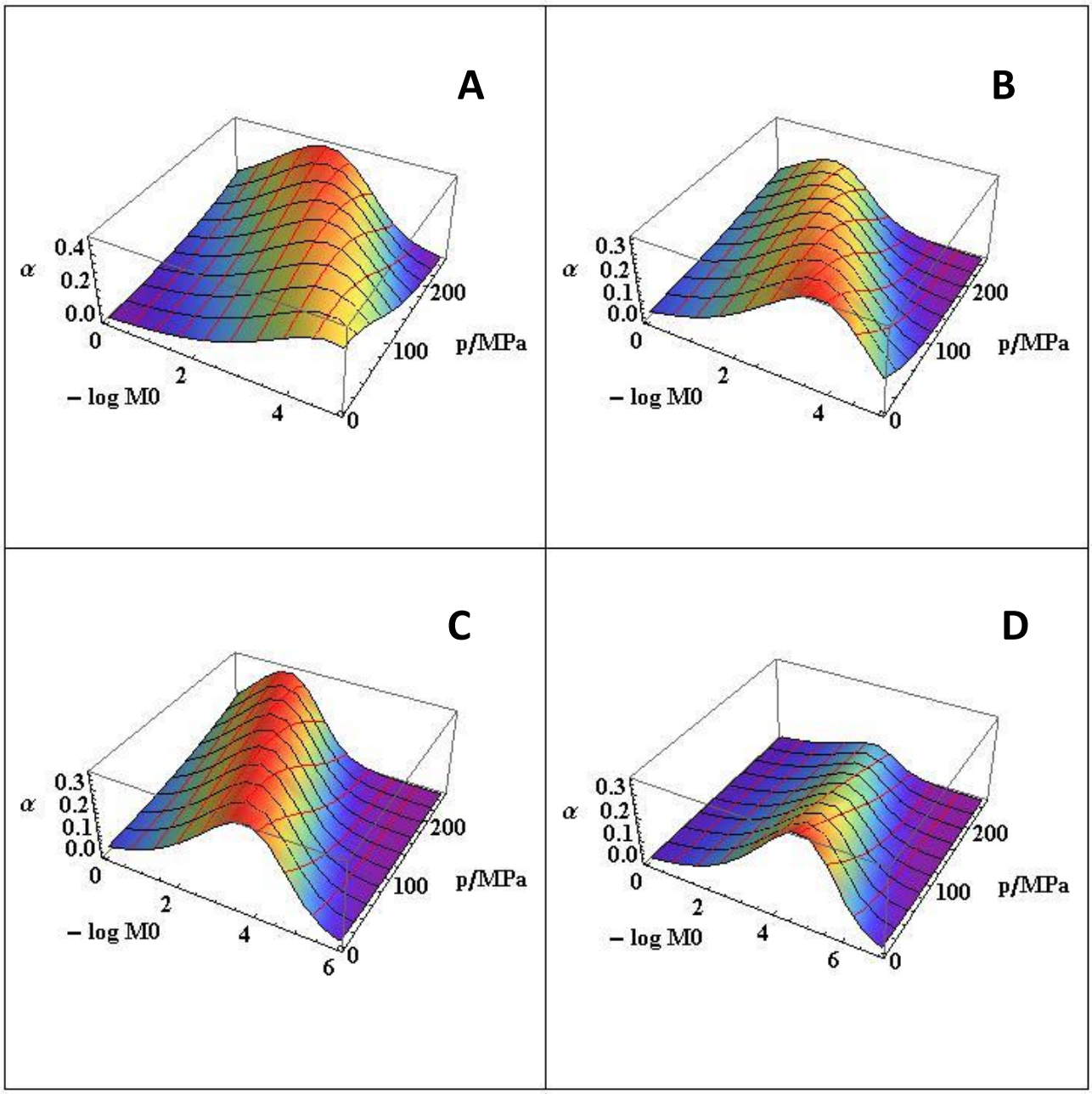

Figure 5. Dimer fraction as a function of pressure and concentration for different sets of thermodynamic parameters. A: Volume change (in absolute value) of the trimer-dimer (TD) transition is bigger than that of the dimer monomer (DM) transition – the separation of the TD and DM transitions is growing with increasing pressure. $K_{D,atm} = K_{T,atm} = 10^{-5}$; ΔV_D = -50 ml mol^{-1}; ΔV_T = -60 ml mol^{-1}; B: Opposite situation than in panel A – the separation of the transitions is decreasing with growing pressure $K_{D,atm} = K_{T,atm} = 10^{-4}$; ΔV_D = -60 ml mol^{-1}; ΔV_T = -50 ml mol^{-1}; C: Volume changes of both the transitions are equal, the separation of TD and DM transitions is pressure-independent. $K_{D,atm} = K_{T,atm} = 10^{-4}$; $\Delta V_D = \Delta V_T$ = -50 ml mol^{-1}; D: Absolute value of ΔV_D is more than twice bigger than absolute value of ΔV_T – no cross sections of the 3D plot of α_D at constant concentration M_0 has maximum – it only decreases with growing pressure. $K_{D,atm} = K_{T,atm} = 10^{-5}$; ΔV_D = -50 ml mol^{-1}; ΔV_T = -20 ml mol^{-1}. In all cases T = 298,15 K.

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A more complex view of this situation can be obtained if a 3D plot is constructed of the fraction of a dimer (or, eventually, the other states) as 2D functions of concentration and pressure (Figure 5). Here, it can be seen that the shape of the function differs considerably depending on the thermodynamic parameters and the positions of the significant points of the standard 1D functions can be deduced from it.

As can be seen in Figure 4, the nature of the equilibrium at given pressure differs in dependence on the values of equilibrium constants. If $K_D << K_T$, i.e. if the dimer is remarkably more stable than the trimer, there is a wide range of dimer prevalence and practically no region of co-existence of all three forms. On the other hand, when the trimer's stability highly exceeds that of the dimer, i.e. $K_T << K_D$, the dimer is almost absent at any concentration and the trimer directly decays to the monomer – the equilibrium thus changes to the closed one and can be described by eqn (6). Depending on the pressure, the system can change its nature between these two cases in several different ways, as is shown in Figure 4A and B. Regarding the experimental approach, it is, therefore, necessary to measure the experimental response of the system as a function of concentration and pressure on a 2D area given by a product of suitably long intervals of both these quantities. Depending on the method of choice, the experimental response can be measured either as a function of

concentration at a fixed pressure, repeating the measurement for different pressure values, or vice versa.

It should be noted that at the consecutive equilibria of trimers or higher oligomers the sensitivity of the experimental methods to the individual oligomerization states is a crucial issue. Contrary to the closed equilibria, where only two states have to be distinguished, consecutive systems require at least three-state resolution. Depending on the mutual ratios of the detected signals of the individual states, the overall transition curve of a given system may look differently for different detection methods. Determination of the relative contributions of each state to the total signal may be possible in case that the states can be isolated chemically (Foguel and Weber, 1995). Nevertheless, it is especially valuable if a method sensitive exclusively to one of the states can be used. Possible suggestions of such techniques based on the Förster resonant energy transfer (FRET) (Sun et al., 2010) and neutron scattering (Jacrot, 1976; Svergun and Nierhaus, 2000) are given in ESI, section S-7.

As the nature of the equilibrium may change with the varying pressure and concentration, it is worthwhile to identify regions where the complex model can be approximated by linear equations. The simplest way is to choose a range of pressure (or concentration in a complementary approach) with a significant area of dimer prevalence when the concentration (pressure) changes from the lower to the upper border of its interval. If this range exists and has a sufficient extent, the two inflection points corresponding to the transitions from trimer to dimer and from dimer to monomer are well separated and are characterized by a practical absence of the third form of the system, i.e. monomer in the former and trimer in the latter case. In this case the last or the first term in eqn (37) can be neglected for the trimer-dimer (TD) and the monomer-dimer (DM) transitions, respectively. The solutions of the resulting equations lead to linear dependences of the inflection point pressures on $\ln(M_0)$ (for derivation see ESI, section S-6)

$$p_{\mathrm{inf},TD} = \frac{RT}{2\Delta V_T - \Delta V_D} \ln\left(\frac{K_{T,atm}^2}{K_{D,atm}} \frac{8\left(6+2\sqrt{6}\right)}{81} \right) - \frac{RT}{2\Delta V_T - \Delta V_D} \ln M_0 \qquad (38)$$

$$p_{\mathrm{inf},DM} = \frac{RT}{\Delta V_D} \ln\left(K_{D,atm} \frac{1+\sqrt{2}}{4} \right) - \frac{RT}{\Delta V_D} \ln M_0 \qquad (39)$$

which allows us to determine the thermodynamic parameters $K_{D,atm}$, $K_{T,atm}$, V_D, and V_T by means of a linear regression.

Similarly, the thermodynamic parameters can be estimated from the inflection points of α_D as a function of pressure provided that both the inflection points exist and are well separated in the experimentally accessible region. As can be proved (see ESI, section S-6), the inflection points are identical with those identified in the previous way, therefore eqns (38) and (39) remain unchanged. It is, therefore, only necessary to decide which way is more convenient for the specific experimental data.

In case that no region of dimer prevalence exists in the experimentally accessible region of pressure and concentration, it is probable that an area exists where, on the contrary, $K_T << K_D$. In this situation the dimer is practically absent from the system and the equilibrium can be considered as closed and analyzed in accord with eqn (14). The equilibrium constant K of eqn (14) is then equal to the product $K_T K_D$, and, accordingly, $K_{atm} \to K_{T,atm} K_{D,atm}$ and $\Delta V \to \Delta V_T + \Delta V_D$. In case that $K_T << K_D$ everywhere in the experimentally accessible region, the individual equilibrium constants and volume changes for the processes of dimerization and trimerization cannot be determined and their separation has no meaning.

In some cases, however, none of the discussed simplified cases takes place in a sufficiently large region. For instance, if $\Delta V_D = \Delta V_T$ (Figure 5C), the inflection points run along parallel

lines and the pressure changes have no effect on their separation. If their separation corresponds with none of the previously discussed cases, determination of the thermodynamic parameters from simple linear functions is impossible. In such cases it may be convenient to start from an estimate based on the distance between the inflection points of the experimentally determined transition curve. These points do not have to correspond with the inflection points of α_D, but rather of α_M (for the DM transition – low concentration side) and α_T (for the TD transition – high concentration side). Solving eqn (37) for α_M and determining the inflection point of α_M (as a function of concentration) numerically in Wolfram Mathematica 9 it can be demonstrated that this inflection point occurs within the interval $\alpha_{M,\mathrm{inf}} \in (0.56; 0.64)$ for any values of K_D and K_T. The corresponding variation of $\ln M_0$ is small, therefore an arbitrary value from this interval can be taken to estimate the thermodynamic parameters. Analogously, the inflection point of $\alpha_{T,\mathrm{inf}}$ occurs within the interval (0.33; 0.45). If it is possible to estimate the nature of the equilibrium, both the values can be chosen with higher accuracy as they can be determined exactly for some special cases (Table 1).

Table 1: Inflection-point values of monomer and trimer fractions of a homotrimer for different natures of the equilibrium.

Nature of equilibrium	$\alpha_{M,\mathrm{inf}}$	$\alpha_{T,\mathrm{inf}}$
General	0.56 – 0.64	0.33 – 0.45
Closed	0.634	0.366
Consecutive – well separated transitions	0.586	0.450
Consecutive – $K_T = K_D$	0.562	0.334

The inflection point concentrations (in the logarithmic form) are then given by

$$\ln M_{T,\mathrm{inf}} = \ln 3 - \ln \alpha_{T,\mathrm{inf}} + 2\ln K_D - \ln K_T - 3\ln\left(\sqrt{1+3\frac{K_D\left(1-\alpha_{T,\mathrm{inf}}\right)}{K_T\alpha_{T,\mathrm{inf}}}}-1\right) \tag{40}$$

$$\ln M_{M,\mathrm{inf}} = -\ln 3 - \ln \alpha_{M,\mathrm{inf}} + \ln K_T + \ln\left(\sqrt{1+3\frac{K_D\left(1-\alpha_{M,\mathrm{inf}}\right)}{K_T\alpha_{M,\mathrm{inf}}}}-1\right). \tag{41}$$

These lines, together with other significant-point dependences on concentration and pressure, are depicted in Figure 6.

It should be pointed out that the difference of these expressions, i.e. the approximate distance between the inflection points, is only a function of the ratio K_D / K_T:

$$\begin{aligned}\ln M_{M,1/2} - \ln M_{T,1/2} = -\ln 9 + \ln\frac{\alpha_{T,\mathrm{inf}}}{\alpha_{M,\mathrm{inf}}} - 2\ln\frac{K_D}{K_T} + \\ + \ln\left(\sqrt{1+3\frac{K_D\left(1-\alpha_{M,\mathrm{inf}}\right)}{K_T\alpha_{M,\mathrm{inf}}}}-1\right) + 3\ln\left(\sqrt{1+3\frac{K_D\left(1-\alpha_{T,\mathrm{inf}}\right)}{K_T\alpha_{T,\mathrm{inf}}}}-1\right).\end{aligned} \tag{42}$$

This equation, together with eqns (40) and (41), can thus be used to estimate the desired thermodynamic parameters using methods based on approximate model functions (see ESI,

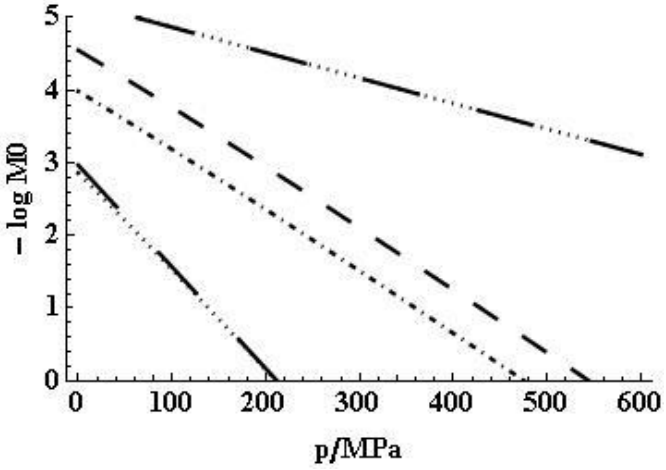

Figure 6. Significant points of the homotrimer equilibrium. Individual significant points as functions of pressure and concentration lie on curves not much different from linear functions. Inflection point of TD transition is represented by the two lowest lines, dotted and dashed, inflection point of DM transition by the two uppermost lines (dotted – eqn (40) with $\alpha_{T,\mathrm{inf}} = 0.450$ and eqn (41) with $\alpha_{M,\mathrm{inf}} = 0.586$, respectively; dashed – estimate according to eqns (38)(38 and (39), respectively). The curves of maxima in the concentration (dot-dashed – eqn (43)) and pressure (short-dashed – eqn (44)) domains are close to parallel lines. $K_{D,atm} = 10^{-5}$, $K_{T,atm} = 10^{-4}$, $\Delta V_D = -20$ ml mol^{-1} and $\Delta V_T = -50$ ml mol^{-1}.

sections S-8, S-9). If the experimental methods enable reliable determination of the functions α_M and α_T, an iterative method can be used to determine the parameters with higher accuracy (see ESI, section S-10).

In some cases the experimental methods may allow us to determine also the maximum of α_D, either as a function of concentration at a given pressure, or vice versa. If α_D is considered as a function of concentration, its maximum also corresponds with the crossing point of α_M and α_T and occurs at the concentration given by the formula

$$\ln\left[M_{0,\max}(p)\right] = \ln\left(\frac{2}{3}K_{T,atm}\right) - \frac{p\Delta V_T}{RT} + \ln\left(1 + \sqrt{\frac{3K_{D,atm}}{K_{T,atm}}\exp\left[\frac{p\left(\Delta V_T - \Delta V_D\right)}{RT}\right]}\right). \qquad (43)$$

Its derivation is given in ESI, section S-4, together with its application in an iterative method of determination of the thermodynamic parameters (section S-10).

In many cases it can be more convenient to measure the signal at a given concentration as a function of pressure. The inflection points of the transition curve can be again approximated by the inflection points of α_M and α_T. Using all the previous approximations, these points are given by eqns (40), (41). A set of linear equations can be derived that allow us to estimate

the thermodynamic parameters, and, if the functions α_M and α_T can be determined reliably, an iterative procedure can be used to determine the parameters accurately (see ESI, section S-9, S-10). It is also possible to find the maximum of α_D at a given concentration (as a function of p) which is, in general, a different point than the maximum at constant pressure (see Figure 6). As can be seen in ESI, section S-5, its position is given by the formula

$$\ln M_{0,}(p_{max}) = \ln\left(\frac{2}{3}K_{T,atm}\right) + \ln\left(\frac{\Delta V_D}{2\Delta V_T - \Delta V_D}\right) - \frac{p_{max}\Delta V_T}{RT} + \\ + \ln\left(1 - \exp\left[\frac{p_{max}(\Delta V_T - \Delta V_D)}{2RT}\right]\Delta V_T \sqrt{\frac{3K_{D,atm}}{K_{T,atm}\Delta V_D(2\Delta V_T - \Delta V_D)}}\right). \quad (44)$$

This equation is rather complicated for direct determination of the thermodynamic parameters, but can be used in an iterative way analogously to eqn (43) (see ESI, section S-10). It should be pointed out that this maximum does not exist for all possible values of ΔV_D and ΔV_T. As follows from the derivation given in ESI, section S-5, the condition $|\Delta V_T| > |\Delta V_D|/2$ has to be fulfilled. If it is not, the fraction of dimer α_D decreases with growing pressure within the whole range irrespective of the concentration, because the dimer is destabilized much more rapidly than trimer and the DM transition has always larger extent than the TD transition (Figure 5D). In this case the analysis based on eqn (44) cannot be carried out. However, the thermodynamic parameters can be evaluated by analyzing the pressure-dependent transition curve at two different concentrations, the first one at which the system at atmospheric pressure is predominantly trimeric and the second one at which it is mostly dimeric. In the first situation the system reduces to the closed trimer-monomer equilibrium, in the second one to the dimer-monomer equilibrium, both described by eqn (13).

It is, unfortunately, impossible to demonstrate the validity of the theory on real experimental data, since, to our knowledge, no experiment directly coherent with this model was published

so far. However, it was reported several times that for equilibria considered as closed there was an incoherence between the ΔV values determined from the individual transition curves (in accord with eqn (12), often denoted as ΔV_P in the literature) and from the shift of transition curves (in analogy with eqn (14), denoted as ΔV_C), the latter method providing higher values (King and Weber, 1986; Silva et al., 1986; Ruan and Weber, 1993). This discrepancy was always observed only for higher oligomers than dimer, even for oligomers with high number of subunits like viral capsids (Weber et al., 1996). It was explained by the heterogeneity of the oligomer population and the deterministic equilibrium (Erijman and Weber, 1991). However, another plausible explanation, at least in some cases, can be that the equilibria might behave as consecutive. If the TD and DM transitions are separated, but the separation is not very large, the resulting transition curve may still appear as that of the closed equilibrium due to the low sensitivity and noise of the experimental methods. However, the value of ΔV determined by the two methods is inconsistent. Consider an example (in detail discussed in ESI, section S-11) of a homotrimer equilibrium of the parameters ΔV_T, ΔV_D, $K_{T,atm}$, and $K_{D,atm}$. Depending on their values the equilibrium can behave either as closed or as consecutive with more or less separated TD and DM transitions. For determination of the apparent ΔV (i.e. for the putatively closed equilibrium) often the pressure difference between the points of $\alpha = 0.1$ and $\alpha = 0.9$ is used. These points can be, in a good approximation, identified with those of $\alpha_T = 0.9$ and $\alpha_M = 0.9$, respectively. Using eqn (37) the pressure difference between these points can be determined. Considering, for simplicity of the example, that $\Delta V_T = \Delta V_D$, the difference is

$$\Delta p = p_{T90} - p_{M90} = \frac{RT}{\Delta V_D + \Delta V_T}\left(4\ln\frac{K_{T,atm}}{K_{D,atm}} + 18.72\right). \qquad (45)$$

For the genuine closed equilibrium, where $\Delta V = \Delta V_T + \Delta V_D$, the corresponding difference determind by eqn (14) is

$$\Delta p = p(\alpha = 0.1) - p(\alpha = 0.9) = 8.79 \frac{RT}{\Delta V_D + \Delta V_T}. \quad (46)$$

As the dimer has to be rather stable in case of a consecutive equilibrium, it is probable that $K_{D,atm}$ is not considerably higher than $K_{T,atm}$ (ten or more times). In this case, however, the expression in eqn (45) is higher than that in eqn (46). Thus, when the experimental quantity corresponding with eqn (45) is substituted to eqn (46) and the apparent volume change ΔV_P, is determined, the resulting value is lower than in the case of authentic closed equilibrium. On the other hand, the other method based on the concentration shift of the transition curves provides rather different results. Here, the apparent inflection point of the curve can be identified with the maximum of α_D and determined with the aid of eqn (44). If we consider, for simplicity, only the limit case of the consecutive equilibrium with well separated transitions, i.e. when the expression in the parenthesis of eqn (44) reduces to 1, the maximum of α_D occurs at

$$p_{max} = \frac{RT}{\Delta V_T} \ln\left(\frac{K_{T,atm}}{3} \frac{2\Delta V_D}{2\Delta V_D - \Delta V_T} \right) - \frac{RT}{\Delta V_T} \ln M_0. \quad (47)$$

Comparing this expression with eqn (14) describing the closed equilibrium, it is obvious that the apparent volume change $\Delta V_C / 2$ is equal to ΔV_T. Thus, if $\Delta V_T \approx \Delta V_D$, then $\Delta V_C \approx \Delta V_D + \Delta V_T$, which is a result expected for the closed equilibrium. Hence, although some approximations were taken into account, it can be seen that the apparent inflection points move with the concentration changes in a similar way for both closed and consecutive equilibrium and the apparent volume change ΔV_C is not much dependent on the character of

the equilibrium. On the contrary, the apparent volume change ΔV_P depends on it considerably, which might be the reason of the observed incoherence between the two quantities. Thus, although it is not possible to reevaluate the previously published data, they may indicate that the studied equilibria were in fact consecutive but the used experimental methods did not enable uncovering this feature.

It should be, however, noted that in some cases the inconsistence of ΔV_P and ΔV_C might have still another reason. The dissociation process can be irreversible or only partially reversible, especially due to the simultaneously running unfolding of the monomers. This phenomenon, which then governs the observed dissociation process, is of the first order and thus concentration independent. In case of partial reversibility the concentration dependence of the transition curve can be seen, but smaller than in the fully reversible case. This can be demonstrated on the published examples of higher oligomers in (Silva et al., 1989; Bonafe et al., 1994) – especially in the latter case the difference between the irreversible and partially reversible system is apparent. Therefore, the mutual agreement of ΔV_P and ΔV_C can be used as an indicator of reversibility of the dissociation-association process.

2.3.2. Heterotrimer A_2B.

Many oligmeric proteins form a heterotrimer consisting of two identical subunits A and a different subunit B. The most common mechanism of its association – dissociation equilibrium is the following:

$$T \rightleftarrows D + B \tag{48}$$

$$D \rightleftarrows 2A \tag{49}$$

Here, T denotes the trimer A_2B and D stands for the dimer A_2. The principal difference with respect to the homotrimer is that, using recombinant subunits A and B, the dimerization

process (eqn (49)) can be studied separately in accord with eqn (14). Therefore, the dimerization parameters $K_{D,atm}$ and ΔV_D can be determined prior to studying trimer formation. In addition, the difference in the subunits allows us to use non-stoichiometric ratios of A and B which can further help the experimental procedures.

Heterotrimer equilibrium is described by the dissociation constants of dimer

$$K_D = \frac{A^2}{D}; \qquad K_T = \frac{BD}{T}. \tag{50}$$

In addition, the following balance equations have to be obeyed:

$$A_0 = A + 2D + 2T; \qquad B_0 = B + T, \tag{51}$$

where A_0 and B_0 are the total concentrations of the subunits A and B. As the subunit A is present in all three forms of the system, it is useful to define the fractions of monomer, dimer and trimer, respectively, as follows:

$$\alpha_M = \frac{A}{A_0}; \qquad \alpha_D = \frac{2D}{A_0}; \qquad \alpha_T = \frac{2T}{A_0}. \tag{52}$$

Restricting our interest immediately on the stoichiometric case, in which

$$T_0 \equiv B_0 = \frac{A_0}{2}, \tag{53}$$

where T_0 denotes the total concentration of trimer, i.e. the concentration of trimer provided that all the subunits are present in the trimeric form, we arrive at the following equation

$$\sqrt{\frac{K_D \alpha_D}{4T_0}} + \alpha_D + \frac{\alpha_D T_0}{K_T + \alpha_D T_0} = 1. \tag{54}$$

This equation can be rearranged to the form of an algebraic equation of the fourth degree in α_D. More detailed discussion is given in ESI, section S-12, together with its analytical solution carried out in Wolfram Mathematica 9 (appendix S-2).

It is again useful to discuss the individual special cases of eqn (54). Let us start with the case presuming a highly stable dimer and less stable trimer which is characterized by the relation $K_T >> K_D$. As in the homotrimer case, a region of prevailing dimer exists and the two transitions, TD and DM, can be analyzed separately. Eqn (54) can thus be simplified by the neglect of the first or the last term, respectively. Both these simplified equations depend on only one dissociation constant. The TD transition can be considered as a heterodimer equilibrium of subunits A_2 and B, while the DM transition as a homodimer equilibrium of two A subunits. These transitions are, therefore, described by eqns (25) and (14), respectively. (For the justification see ESI, section S-12.) Hence, the two transitions can be considered separately and the parameters $K_{D,atm}$, ΔV_D, $K_{T,atm}$, and ΔV_T can then be determined straightforwardly.

If, on the other hand, the trimer is substantially more stable than the dimer, i.e. $K_T << K_D$, the system undergoes the closed equilibrium described by eqn (32) with the constants K_{atm}, and ΔV given by the expressions $K_{atm} \to K_{T,atm}K_{D,atm}$ and $\Delta V \to \Delta V_T + \Delta V_D$, analogously with the homotrimer case.

If the separation of the two transitions is not so striking and it does not allow us to use these approximations at any pressure, it is valuable when an experimental method sensitive exclusively to either the trimer or the free subunit B is available. If this is the case, the fraction of free B denoted α_B obeys the fourth-degree equation

$$4T_0^2\alpha_B^4 + 8K_T T_0 \alpha_B^3 + K_T \alpha_B^2 \left(K_T + 4K_D - T_0\right) - K_T \alpha_B \left(8K_T + K_D\right) + 4K_T^2 = 0. \qquad (55)$$

However, eqn (55) is only quadratic equation in T_0 and can be solved for T_0 as a function of α_B:

$$T_0 = \frac{K_T(1-\alpha_B)}{\alpha_B^2}\left(1+\sqrt{\frac{K_D}{4K_T}\frac{\alpha_B}{1-\alpha_B}}\right). \quad (56)$$

Although the inflection point of $\ln T_0$ cannot be determined analytically, the numerical solution in Wolfram Mathematica 9 shows that the inflection point value of $\alpha_{B,\mathrm{inf}}$ is in a very narrow interval (0.58; 0.64) for any values of K_D and K_T. For K_D / K_T ratio between 0.1 and 10, i.e. when none of the previously discussed special cases applies, the interval reduces to (0.60; 0.64). Thus, the inflection point value of T_0 can be obtained from eqn (56) substituting $\alpha_{B,\mathrm{inf}} \approx 0.62$ for α_B. Introducing the pressure dependence of K_T and K_D and exploiting the fact that $K_{D,atm}$ and ΔV_D can be determined independently, eqn (56) becomes (in the logarithmic form)

$$\ln T_0 = \ln K_{T,atm} + \ln\frac{1-\alpha_{B,\mathrm{inf}}}{\alpha_{B,\mathrm{inf}}^2} - \frac{p\Delta V_T}{RT} + \ln\left[1+\sqrt{\frac{K_{D,atm}}{4K_{T,atm}}\frac{\alpha_B}{1-\alpha_B}\exp\left(\frac{p}{RT}\left(\Delta V_T - \Delta V_D\right)\right)}\right]. \quad (57)$$

This equation can be solved iteratively in a similar manner as in the case of homotrimer (see ESI, section S-13).

2.3.3. Heterotrimer ABC.

Another kind of a heterotrimer is composed of three different subunits A, B and C. As the solution of this system is formally analogous to the A_2B heterotrimer, it is given in ESI, section S-14.

3. Conclusions

A theoretical description of the equilibria among the subunits of oligomeric proteins under high pressure is provided in this work. The dependences of significant points of the transition curves – inflection points and extremes – on pressure and concentration have been derived, in most cases in the form of linear functions. Analysis of these functions allows us to determine the thermodynamic characteristics of the systems, i.e. the volume changes and atmospheric-pressure equilibrium constants of the individual transitions. The theory can be developed for the closed equilibrium of homooligomers of any number of subunits in a close analytical form. For these systems a rough estimate of the number of subunits is possible in case that it is not known a priori. In addition, an analogous description was developed also for the closed equilibria of a heterodimer and different forms of heterotrimers. Systems undergoing the consecutive equilibria represent far more complex problem. A homotrimer, which was chosen as the simplest example, was analyzed in detail. Here, it is difficult to develop a universal theory valid in the whole pressure and concentration ranges because the description is based on algebraic equations of higher than the second degree. Although these solutions were developed using Wolfram Mathematica 9 software and are shown in several figures, they are too complicated for the detailed analysis. Therefore, special cases that can be reached by a specific choice of pressure and concentration ranges were studied in which the solution is simplified and can be turned to linear functions. Finally, a heterotrimeric system undergoing consecutive equilibria was analyzed in a similar way.

Although we have recently verified the theory on the model of dimerization of HIV-1 protease (Ingr et al., 2015), more extensive experimental studies including also higher oligomers are needed to demonstrate its utility. In spite of possible experimental difficulties we hope that this work may encourage further high-pressure experimental studies with oligomeric proteins.

Footnotes

Electronic Supplementary Information available: Detailed mathematical derivations, analytical solutions of the third-and fourth-degree algebraic equations, illustrative figures.

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Highlights

- A theory of oligomeric-protein equilibria under high pressure was developed.
- A generalized model of closed equilibria was proposed.
- Detailed description of consecutive high-pressure equilibria in trimeric proteins is provided.
- Solutions of models not-solvable analytically were elaborated in Wolfram Mathematica.