Concentration Dependence of the Subunit Association of Oligomers and Viruses and the Modification of the Latter by Urea Binding

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ABSTRACT   A theoretical model is presented that accounts for the facilitation of the pressure dissociation of R17 phage, and for the partial restoration of the concentration dependence of the dissociation, by the presence of subdenaturing concentrations of urea. As an indifferent osmolyte urea should promote the stability of the protein aggregates under pressure, and the decrease in pressure stability with urea concentration demonstrates that such indirect solvent effects are not significant for this case, and that the progressive destabilization is the result of direct protein-urea interactions. By acting as a "homogenizer" of the properties of the phage particles, urea addition converts the pressure-induced deterministic dissociation of the phage into a limited stochastic equilibrium. The model establishes the origin of the uniform progression from the stochastic equilibrium of dimers, to the temperature-dependent and partially concentration-dependent association of tetramers, to the fully deterministic equilibrium observed in many multimers and in the virus capsids.

THE DEPENDENCE OF MONOMER-OLIGOMER EQUILIBRIA UNDER HYDROSTATIC PRESSURE UPON THE NUMBER OF SUBUNITS

The dissociation of oligomeric proteins into subunits by hydrostatic pressure indicates that, according to the principle of Le Chatelier, the separated solvated subunits occupy a smaller volume than the original aggregate (for a review see Silva and Weber, 1993). This rule applies to many aggregates irrespective of the number of subunits, from dimer or tetramer proteins, to erythrocruorin and hemocyanins, and the capsids of viruses and phages. Experiment shows that in all of these cases the pressures that induce the dissociation of the aggregates into subunits are in the range of 0.5–3 kbar. On examining the experimental observations thus far collected on the pressure dissociation of aggregates of increasing complexity, from dimers to multimers of more than 100 subunits, and to virus capsids, two phenomena appear to have particular significance:

1. Studies of the effects of hydrostatic pressure on oligomer dissociation indicate that the law of mass action is followed within experimental error in monomer-dimer equilibria, but it progressively fails as the number of subunits in the aggregate increases. In tetramer-monomer equilibria, dilution produces much smaller changes in the pressure of mid-dissociation than expected from the law of mass action (Ruan and Weber, 1989, 1993), and the multimer aggregates of snail hemocyanin (Bonafe et al., 1994) and erythrocruorin (Silva et al., 1988, 1989; Bonafe et al., 1991) as well as the virus capsids (Silva and Weber, 1988; Da Poian

et al., 1993) exhibit pressure-dissociation profiles that are virtually independent of the particle concentration of the solutions. The failure of the law of mass action in the pH-dependent equilibria of snail hemocyanin with its subunits, at atmospheric pressure, was one of the earliest observations of a macromolecular equilibrium employing the ultracentrifuge (Svedberg and Brohult, 1938). More recently a number of authors have confirmed and extended these observations, reviewed by van Holde and Miller (1982). The use of hydrostatic pressure to ensure the partial dissociation of oligomers has made it possible to demonstrate the prevalent character of these earlier observations. However, ultracentrifugation studies of a hexamer hemocyanin (van Holde and Brenowitz, 1982; Herskovits, 1992) and of decameric octopus hemocyanin (van Holde and Miller, 1985) show a pronounced effect of the concentration on the degree of dissociation consistent with the high order of dependence expected in these cases, and it would be of great interest to study the effects of hydrostatic pressure in these very same cases.

2. Differences in the character of the equilibrium with the complexity of the aggregate are demonstrated more directly by a study of the exchange of labeled subunits among oligomers in solution. Erijman and Weber (1991, 1992) have observed that in lactate dehydrogenase the time required for the dissociation of a fraction of the tetramers by hydrostatic pressure at 0°C is shorter, by more than one order of magnitude, than the subsequent rate of exchange of the free subunits and the remaining aggregates, and entirely similar observations have been made in muscle glycogen phosphorylase (Ruan and Weber, 1993). The difference between the times for particle dissociation and subunit exchange decrease readily with increase in temperature in both cases. On the other hand, in dimers, the times for dissociation and subunit exchange are very similar at all temperatures, whereas in the multimer proteins and virus capsids it is not possible to demonstrate the existence of an

actual dynamic equilibrium between the aggregates and the subunits.

These phenomena cannot be explained other than by the potential existence of a heterogeneous molecular population as regards the free energy of association. The component fractions of the population exchange into each other in times that in the dimers are very short in comparison with the times for attainment of the equilibrium between subunits and aggregate. In the multimers and virus capsids there is an absence of concentration dependence, and the apparent effect of pressure is that of selecting a fixed fraction of the population for dissociation, while leaving the remainder intact (Brauch et al., 1990). The tetramers are particularly interesting in affording a system in which the transition from the behavior of dimers to that of large multimers may be observed: lowering the temperature favors the increasing permanence of the individual molecular conformations to the extent that the subunit association, which could be considered a chemical reaction of a homogeneous molecular population at the higher temperatures, must be viewed at the lower temperatures as a set of independent reactions, each with its own free energy and volume of association. In the aggregates of many more subunits, and in the virus capsids, the heterogeneity of the population is already apparent at all temperatures. The underlying explanation of all these observations is that the degree of persistence of a plurality of conformations of the aggregates depends upon the relative rates of the opposing processes of conformational drift of the free monomers and the regeneration of the most stable forms in the aggregate (King and Weber, 1986; Silva et al., 1986; Weber, 1986, 1992; Erijman et al., 1993). The large predominance of the latter rate relative to the former creates the conditions for the observation of a stochastic equilibrium in the dimers. On the other hand, the inversion of these conditions, namely the relative slowness of the rate of regeneration of the most stable forms of the aggregate, is responsible for the deterministic character of the equilibrium of tetramer and subunits at low temperature (Erijman and Weber, 1991). At this stage of our knowledge of the virus capsids it is not possible to go beyond the statement that the heterogeneity of the preparations appears to be the result of the circumstances at the time of the original subunit association.

The adherence to the law of mass action and the similarity of the times for subunit exchange and dissociation in dimers, and the large difference with the tetramers as regards these properties, suggests an easy explanation for the slow rate of regeneration of the most stable forms of the aggregates, and the existence of different fractions as regards their free energy of association: they arise from the restricted exchange between those conformations that require displacements of more than two subunits relative to each other.

In the R17 phage we observed (Da Poian et al., 1993) a behavior similar to that already seen in other multimers and virus capsids, namely the absence of any dependence of the pressure dissociation profile upon the concentration of the

particles, and no indication of the existence of a dynamic equilibrium between associated and dissociated fractions. However, a partial restoration of the dependence of the pressure dissociation profile upon the particle concentration is observed when urea is present in the range of concentration of 2.5 to 5 M. At higher concentrations of urea, dissociation, and eventually denaturation of the subunits, is observed already at atmospheric pressure. R17 phage shows excellent reversibility of the biological and physicochemical properties when the pressure is removed, both in the absence and in the presence of urea at concentrations below 5 M, and this circumstance permits us to derive a consistent phenomenological theory of the interplay of the effects of urea and hydrostatic pressure that may be extended to other cases—like the tetramers—in which there is a partial dependence of the pressure effects upon the particle concentrations.

THEORY OF THE UREA EFFECT

The observations made by Da Poian et al. (1993) offer us three different yet connected problems:

1. In spite of the large number of oligomers and the independence of the pressure effects upon the concentration, R17, and Brome mosaic virus as well, (Silva and Weber, 1988) dissociates in the range of pressures (0.5 to 3 kbar) found in simpler aggregates like dimers, trimers, and tetramers.

2. The lack of concentration dependence upon the intact virus particles at urea concentrations of <2.5 M indicates that the progressive dissociation of the virus under increasing pressures is a deterministic equilibrium that requires the presence of a heterogeneous population of particles with different characteristic dissociation pressures (Erijman and Weber, 1991). Observation of such an effect requires the persistence of the characteristic dissociation pressures over times that are long in comparison with the duration of the experiments.

3. Addition of urea in the range of 2.5 to 5.0 M gives rise to concentration dependence, apparently converting the deterministic equilibrium of capsid and subunits into a limited stochastic equilibrium.

The first of these questions is perhaps the easiest to answer: the dependence of the thermodynamic dissociation constant of an n-mer with its n constitutive monomers upon the hydrostatic pressure is given by the equation

$$K(p) = n^n \alpha^n C^{n-1}/(1 - \alpha) = K(0)\exp(p\Delta V/RT) \quad (1)$$

where $K(p)$ and $K(0)$ are the respective dissociation constants at pressure p and atmospheric pressure, respectively, α is the degree of dissociation of the aggregate, C is the concentration of protein as aggregate, and ΔV is the standard volume change on association. If p_{90} and p_{10} designate, respectively, the pressures required to attain 90% and 10% dissociations, then $dp = p_{90}-p_{10}$ is the characteristic "pressure span" of the dissociation, and introducing successively

p_{90} and p_{10} in Eq. 1 and subtracting the second from the first equation we derive:

$$(n + 1)\ln 9 = dp\ \Delta V/RT \qquad (2)$$

Expressing ΔV as the sum of equal contributions δV from each dissociated subunit we have $n\delta V = \Delta V$ and

$$dp = [(n + 1)/n]\ln 9 \cdot RT/\delta V \qquad (3)$$

At 25°C, $RT = 24.8\ 10^2$ J, and for small oligomers $dV = 50-75$ ml/subunit. Therefore setting $(n + 1)/n = 1$, Eq. 3 gives dp equal to 0.73–1.1 kbar for any multimer in which the subunits contribute volume changes on association similar to those seen in dimers or tetramers. Although Eq. 3 gives the pressure span of the dissociation, it does not fix the range of pressures in which the dissociation will occur. However, the explanation of the effects of pressure in terms of the differential compressibility of the intermolecular bonds that are exchanged in the equilibria of protein and subunits (Weber, 1993) predicts that the dissociation will occur in the range of atmospheric pressure to about 4 kbar. Addition of a sufficient concentration of any osmolyte (e.g., sucrose, xylose) produces a stabilization of protein oligomers against pressure dissociation as their concentration is increased and in general acts to reverse the effects of hydrostatic pressure (Robinson and Sligar, 1994, 1995). The osmolyte acts by decreasing the active concentration of solvent, and the effects are particularly noticeable in those reactions that proceed with solvation of a very large protein surface like oligomer dissociation. Urea must also be expected to act in that way, but the observation of the opposite effect, a destabilization of the protein association with increasing urea concentration, indicates that the nonspecific osmotic effect of the stabilization of the protein aggregates through urea-solvent interactions is unimportant in comparison with the specific effects owing to direct urea-protein interactions.

The virtually complete absence of concentration dependence observed in many protein multimers, Brome mosaic virus, and R17 phage in the absence of urea indicates directly that reassociation of subunits that result from dissociation at a fixed pressure does not take place to any appreciable extent during the time of the pressurization experiments (hours). Similarly, during such a time period there can be no appreciable conversion of the undissociated particles into forms that are sensitive to lower pressures than those used to achieve equilibrium (Erijman and Weber, 1991). We are then justified in postulating a distribution of pressure sensitivity of the particles that depends upon both differences in the intrinsic free energies of association (ΔG_i) and association volume (ΔV_i) so that the characteristic dissociation pressure of the particle is given by $p_i = \Delta G_i/\delta V_i$. The distribution of characteristic pressures, which exists in the absence of urea or any other ligand, we designate as the primary distribution of characteristic pressures. The shape of the distribution is in principle unknown. However, we expect it to depend on a very large number of interactions of

atoms or groups of atoms at the subunit interfaces, each interaction introducing small differences in p_i in comparison with the width of the distribution. Then, in agreement with the Lyapunov theorem (e.g., Gnedenko, 1975) it will be a normal (Gaussian) distribution, for the description of which we need to specify the characteristic center pressure, the standard deviation, and the number of members of the distribution. The characteristic center pressure must correspond to $p_{1/2}$, the pressure of half-dissociation, and the standard deviation of the Gaussian is fixed by the experimental pressure span. The number of distinct fractions in the distribution is immaterial, as long as the interval in characteristic pressure between neighboring fractions remains smaller than the experimentally resolvable effects of pressure. A distribution of this type that generally fits the dissociation curves observed in R17 in the presence of urea at concentrations below 2.5 M is shown in the bottom of Fig. 1.

The appearance of increasing concentration dependence in the presence of urea requires an increase in the rates of association of monomers and dissociation of whole particles so that now these take place within times that are short
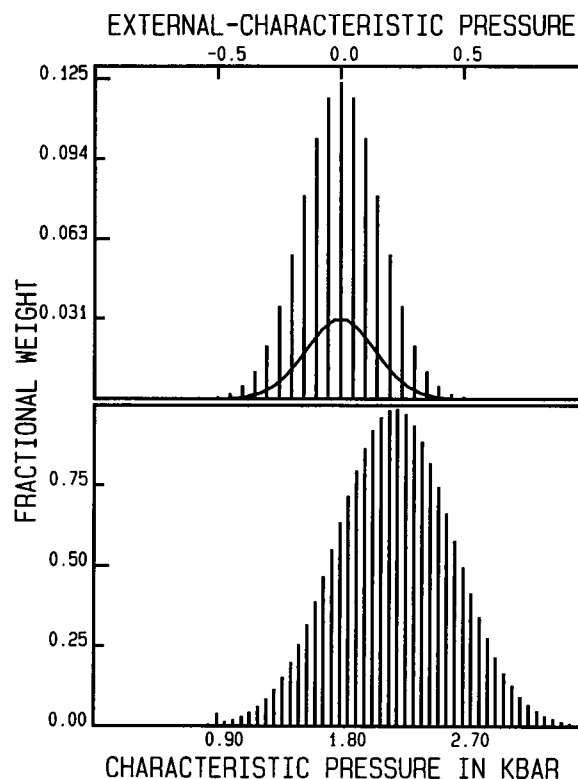

FIGURE 1   (Bottom) Primary distribution of characteristic pressures: Gaussian distribution with a maximum at 2.2 kbar and an amplitude span (10–90%) of 2 kbar, that grossly corresponds to the observations on R17 phage. (Top) The splitting of each member of the primary distribution according to the numbers of urea molecules bound to 40 sites. [Urea]/K_{urea} = 1. Characteristic pressure shift per urea bound = −0.05 kbar. The continuous curve gives the extent of the rapid exchange between associated and dissociated particles that results from variation of the number of bound urea molecules.

compared to the experimental times. The model that we propose to explain the acceleration of these rates is as follows:

1. Each urea molecule bound decreases the absolute value of the free energy of association of the virus particle to which it is attached by a fixed (average) amount dG. A particular fraction, which in the absence of urea had a free energy of association ΔG_i, or equivalently a characteristic dissociation pressure $p_i = \Delta G_i/\Delta V$, has, after binding n molecules of urea, a free energy of association $\Delta G_1 + n dG$, or a characteristic dissociation pressure $p_i - n dp$.

2. Assuming that all sites for the binding of urea have similar a dissociation constant K_{urea}, the probability of binding a single urea molecule is $[U]/(K_{urea} + [U])$, where $[U]$ is the molar concentration of free urea. If all the fractions of the distribution in the absence of urea (primary distribution) have identical properties as regards urea binding, then the random binding of urea at a fixed number M of sites splits each member of the primary distribution into a secondary normal distribution of characteristic dissociation pressures, as shown in the top of Fig. 1 for the case $[U]/K_{urea} = 1$. For the relation between ligand binding at multiple equal sites and the ensuing normal distribution see Weber (1992).

3. The final distribution of free energies of association results from the superposition of the primary and secondary distributions. The secondary distribution is the same for any member i of the primary distribution. It is formed by M members with lowest free energy of association ΔG_{io}, or characteristic pressure p_{io}, corresponding to zero urea molecules bound, and increases to values $\Delta G_{io} + j dG$ ($0 < j \le M$), or $p_i - j dp$ for j urea molecules bound. Then a description of the appearance of the secondary distribution by urea addition requires providing three parameters to define the secondary distribution: a) the number M of binding sites for urea, b) the ratio $[U]/K_{urea}$, and c) the pressure shift $-dp$ caused by the binding of each urea molecule. As the molarity of urea binding sites in the virus population is negligible in comparison with the molarity of urea ($2.5 < [U] < 5$), the ratio $[U]/K_{urea}$ increases linearly with urea concentration.

4. Whereas the primary distribution may be expected to remain unchanged during any experiment, the secondary distribution has no such permanency. From the small free energies of urea association, which follow from the large urea concentration necessary to produce appreciable effects, we expect that a cycle of association and dissociation of a molecule of urea will take only a small fraction of a second. Application of an external pressure p_{ext} will affect the members of the primary distribution as follows:

a. If $p_{ext} > p_{io} = \Delta G_{io}/\Delta V$, then all members of the fraction will dissociate and will remain so regardless of changes in the number of bound urea molecules at any time.

b. If $p_{ext} < p_{iM} = \Delta G_{iM}/\Delta V$, then all members of the fraction will remain associated, regardless of the number of urea molecules bound by it or the changes that these may undergo in time.

c. If p_{ext} is between the values $p_{io} = \Delta G_{io}/\Delta V$ and $p_{iM} = \Delta G_{iM}/\Delta V$, the distribution within this ith fraction is made of two parts separated by the characteristic pressure $p_{ik} = \Delta G_{ik}/\Delta V$, the highest characteristic pressure just inferior to p_{ext}. The part with characteristic pressures $p_{ij} \le p_{ik}$ will be split, whereas those with $p_{ij} > p_{ext}$ will remain undissociated. The former will make up the dissociated subfraction α_i, and the latter the undissociated subfraction $1 - \alpha_i$. However, the monomers and aggregates that respectively form the subfractions have no permanent existence: the monomers will find themselves in the category of those that can reassociate if they lose urea molecules to the point that the number bound becomes less than k. Similarly, the undissociated aggregates will dissociate if the number of urea molecules that they bind increases above k. A state of rapid equilibrium will be reached by the exchange of the monomers and aggregates thus generated by the changes in bound urea molecules, which will be proportional to the product $\alpha_i(1 - \alpha_i)$. This exchanging fraction of each member of the distribution is shown as a function of the difference between external pressure and characteristic pressure, by the solid curve in the upper panel of Fig. 1, for the case $[U]/K_{urea} = 1$. The absolute concentration of monomers and aggregates that undergo such transitions at any given pressure will depend upon the total protein concentration and will thus generate the dependence of the degree of dissociation of the particles on the protein concentration. Fig. 2 shows the broadening and shifting to lower dissociation pressures of two composite distributions with $[U]/K_{urea}$ respectively equal to 0.005 and 2.0, and the bottom of Fig. 3 shows the virus dissociation curves and total exchanging fractions as a function of applied pressure. It is therefore clear that as a result of the rapidly varying number of urea molecules bound by aggregate and subunits, both of these species lose
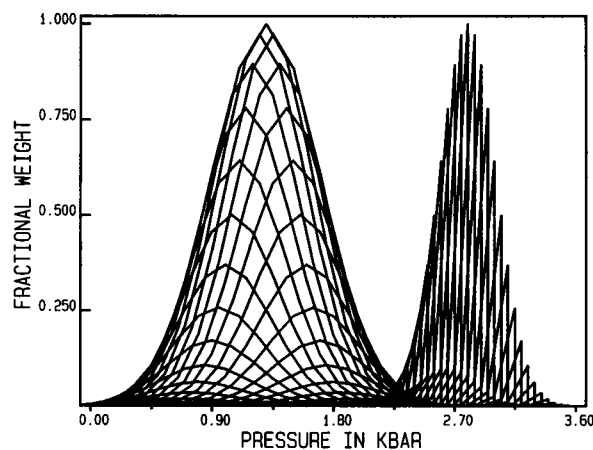

FIGURE 2 The composite distribution of pressure sensitivities brought about by the combination of the primary and secondary distributions for two cases: $[Urea]/K_{urea} = 0.005$ at right, and $[Urea]/K_{urea} = 1$ at left. The parameters used: primary midpressure = 2.7 kbar, Gaussian spread 1 kbar, urea sites = 20, pressure shift per urea molecule bound = -0.15 were chosen to separate the distributions along the pressure axis to clarify the spread of the individual members by the secondary distribution.
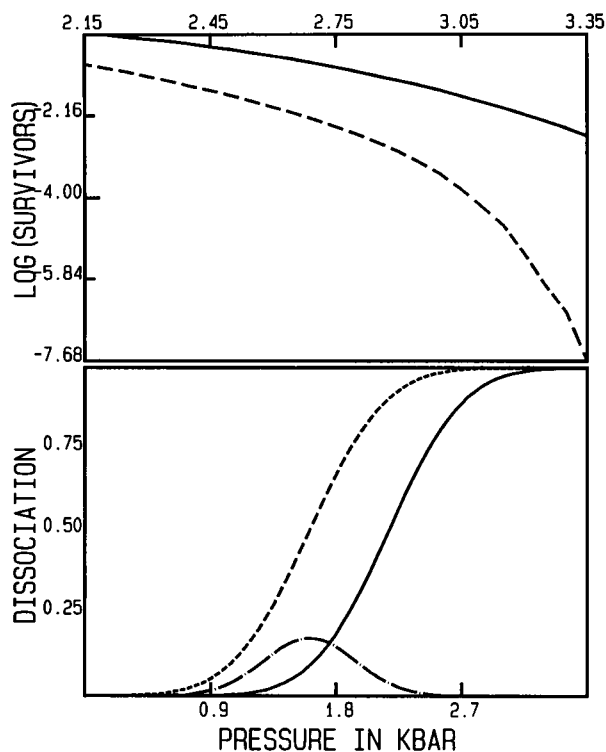
FIGURE 3 (*Bottom*) Pressure-dissociation curves in absence and presence of urea. ——, no urea; – – –, 40 sites, [Urea]/K_{urea} = 1, dp = -30 bars; – · – · –, exchanging fraction. (*Top*) Logarithm of the number of virus survivors corresponding to the pressure dependences of the dissociation shown in the lower panel.

the permanence that characterizes them in the absence of urea. The result is that dissociation and reassociation take place at every degree of dissociation attained by the application of pressure, but involving only a limited fraction of both dissociated and undissociated species, and that this equilibrium is concentration dependent.

ESTIMATION OF THE PARAMETERS, AND A PRACTICAL APPLICATION TO VIRUS SURVIVAL

The primary distribution of the virus population is specified by the pressure of mid-dissociation of the virus at concentrations of urea less than 2.5 M and the width of the pressure dissociation curve. The parameters governing the secondary distribution are independent of those describing the primary distribution: Setting $z = [U]/K_{urea}$, the average number of molecules of urea bound per virus particle is $\langle n \rangle = Mz/(1 + z)$, and the shift of the pressure dissociation curve toward the lower pressures is $\Delta p_{1/2} = \langle n \rangle dp$. The two factors, $\langle n \rangle$ and dp, that determine $\Delta p_{1/2}$ cannot be independently determined, but because this latter quantity is experimentally known, $\langle n \rangle$ and dp are not independent of each other. Also,

$$d\Delta p_{1/2}/dz = Mdp/(1 + z)^2 \qquad (4)$$

and from figure 12 of Da Poian et al. (1993) $d\Delta p_{1/2}/dz$, in R17, is virtually independent of z and equals 300 bar mol^{-1}

for the urea concentration range of 2.5 to 5 M. $z = [U]/K_{urea}$ depends on the value of K_{urea}, but as [U] varies by only a factor of 2 in that range, in the probable span of $K_{urea} = 0.2$ to $K_{urea} = 4$ the dependence of the pressure shift $\Delta p_{1/2}$ upon the urea concentraton remains linear with [U] and is normalized to the experimental value $\Delta p_{1/2}$ by a suitable choice of M. The ratio $R \equiv \Delta p_{1/2}/(d\Delta p_{1/2}/dz)$ equals

$$R = z(1 + z) \qquad (5)$$

and therefore

$$z^2 + z - R = 0 \qquad (6)$$

Taking into account the relations in Eqs. 4, 5, and 6, we can simulate the experimental effects of urea, upon both the pressure shift and the increase in the rapidly exchanging fractions responsible for the regain of the concentration dependence, by specifying only two parameters: the number M of urea binding sites responsible for the pressure dissociation shift, and $z_o = 3.5/K_{urea}$ that assigns a value of z at [U] = 3.5. Figs. 1–3 have been calculated under these assumptions, introducing the parameters shown in the figure legends.

The decrease in the pressure that produces a given degree of virus dissociation by the addition of urea suggests an important application in the pressurized preparations of virus for vaccination purposes (Silva et al., 1992; Jurkiewicz et al., 1995). This is demonstrated in the top of Fig. 3, which shows the logarithm of the number of undissociated virus particles (survivors) against the applied pressure. In constructing this plot it is assumed that a pressure of 2.15 kbar was applied for a time sufficient to reduce the survivors to one-half of the total population, in the absence of urea, and that the displacement of the pressure dissociation profile is that observed for R17 phage with 4.5 M urea, namely 600 bar. At 3 kbar the relative decrease in survivors brought about by addition of urea is nearly 5 orders of magnitude. Experiments employing vesicular stomatitis virus to test this prediction of the practical extinction of the infectivity in the presence of suitable urea concentrations are currently under way (Bonafe et al., manuscript in preparation).

We finally note that in the theory developed above to explain the appearance of concentration dependence of the virus dissociation by pressure as a result of the addition of urea, we have disregarded any modification of the solvent properties by urea, a cause that would be expected to increase the stability of the virus. Instead we have treated the urea effects by the assumption of multiple pairwise chemical equilibria between the molecular species involved. In this case, as in innumerable others, ordinary polyvalent protein-ligand equilibria make it possible to interpret quantitatively the experimentally observed effects.

THE RESTRICTED CONCENTRATION DEPENDENCE OF TETRAMER DISSOCIATION

The anomalously small concentration dependence observed in the dissociation of tetramers, in the absence of urea or

similar ligands, may be explained along lines analogous to those of the restoration of the concentration dependence by urea in the R17 phage. We can safely assume, from the small energies involved in the elementary interactions between protein parts, that in any aggregate, regardless of its complexity, there exists at any fixed time a distribution of free energies of dissociation of unknown width. In dimers the interconversion between neighboring fractions is fast enough to ensure the relaxation of any fraction into the whole distribution in times that are short in comparison with the experimental times. Consequently, the experiments reveal a completely homogeneous behavior. In tetramers each component of the primary distribution may be expected to have a secondary distribution generated by changes in conformation that are similar to those responsible for the dynamics of the dimers, but which in the tetramer case results in only partial overlap with other members of the distribution, as in the case of the phage in the presence of urea. We can also think, in the case of tetramers and higher order oligomers, in terms of two distributions. The primary distribution is determined by conformations that require displacement of subunits with respect to each other, whereas the secondary distribution involves changes in free energy owing to conformation changes within each member of the primary distribution that do not involve relative displacements of the subunits. The secondary distribution results in an overlap of the free energies corresponding to adjacent members of the primary distribution. It is this partial overlap between close members of the primary distribution that is responsible for the establishment of a concentration-dependent equilibrium. Then, unlike the case of the dimers, the interconversion of the members of the primary distribution of tetramers involves only that fraction of it that overlaps with the near-by fractions. A sufficiently large overlap results in a population that approaches a homogeneous one as regards the dissociation by hydrostatic pressure, and a decreasing overlap generates progressively more heterogeneous populations. The pressure dissociation dependence on the concentration of an ideal tetramer-monomer stochastic equilibrium should be governed by the equation

$$4^4\alpha^4 C^3/(1 - \alpha) = K(0)\exp(p\Delta V/RT) \qquad (7)$$

The relative overlap of nearby fractions determines the effective exponent of the concentration appearing in Eq. 7. This exponent, m, can vary from $m = 0$, in the case of virus and very high-order multimers to $m = n - 1$, where n is the number of particles in the aggregate. The upper limit is approached in dimers. Determination of m requires observations of the change in degree of dissociation with dilution under a pressure sufficiently high to produce measurable dissociation at the highest concentrations employed, but this experiment presents some technical difficulties. A simpler but no less reliable determination of m can be made from the pressure dissociation profile at two concentrations of protein sufficiently different to produce appreciable displacement of the dissociation profile along the pressure axis. This

displacement is best characterized by that of the pressure of half-dissociation, as this pressure is determinable with least error. For two concentrations C_1 and C_2 and corresponding pressures of half-dissociation p_1 and p_2 Eq. 7 gives

$$m \log(C_1/C_2) = (p_1 - p_2)RT/(2.302 \, \Delta V) \qquad (8)$$

An exponent $m = n - 1$ characterizes the stochastic case typical of dimer-monomer equilibria (Silva and Weber, 1993), and in general $m/(n - 1)$ represents the fraction of the primary distribution that exchanges in those times. In glyceraldehyde phosphate dehydrogenase (Ruan and Weber, 1989) for $C_1/C_2 = 10$, $p_1 - p_2 = 160$ bar, as against 784 bar expected for the cubic concentration dependence. Therefore $m = 0.204(n - 1) = 0.612$. In muscle glycogen phosphorylase (Ruan and Weber, 1993) the observed and expected pressure displacements were, respectively, 193 and 818 bar, giving $m = 0.236(n - 1)$ or $m = 0.708$. Thus we anticipate that in tetramers at 0°C the secondary distribution has a width equal to 1/4 or 1/5 of the total distribution of characteristic pressures. From the free energy differences that correspond to the pressure span of the dissociation profile for a tetramer we can estimate the width of the primary distribution of free energies in tetramers to be 4-6 kcal at 0°C (Ruan and Weber, 1989), thus making the width of the secondary distribution approximately 1-1.5 kcal.

Virus capsids, like R17 in the absence of urea, show total independence of the degree of dissociation from the concentration, an independence that cannot be achieved unless each particle and its subunits form an independent system as regards their free energy of association. In the viruses the biological necessity of this independence is obvious: any interdependence of the particle stability must arise from reversible dissociation of subunits that will expose the particle to the action of proteases and nucleases and defeat the infectivity. The cases of partial concentration dependence require that the particle independence no longer be complete, a situation achieved in the case of R17 by the addition of urea, and existing as a temperature-dependent property in tetramers. In these latter cases experiment shows that the isolated subunits resulting from dissociation by dilution at atmospheric pressure, or by application of hydrostatic pressure at constant concentration, undergo a "conformational drift" (Weber, 1986), and on reassociation form oligomers with transiently altered properties (Silva et al., 1986; Ruan and Weber, 1989). The lack of reversibility thus implied should facilitate the recognition and removal of the free monomers.

CONCLUSIONS

Although application of pressure in the range of atmospheric pressure to 2.5 kbar results in only a small dissociation of R17 phage, a more complete, reversible dissociation, independent of the phage particle concentration can be obtained in the presence of urea concentrations between 1.0

and 2.5 M (Da Poian et al., 1993). The pressure-dissociation equilibria in this urea concentration range result from a deterministic equilibrium in which each particle responds to pressure independently of the others and, at any given pressure, is in one of two states, whole or dissociated, which persists for times that are long compared with the duration of the experimental procedure. At urea concentrations between 2.5 and 5 M the pressure necessary for the dissociation of each fraction depends upon the number of the urea molecules bound, and as this fluctuates rapidly during experimental times, it results in the appearance of a further stochastic equilibrium that partially restores the dependence of the pressure sensitivity upon the particle concentration. These two factors, general increase in pressure sensitivity of the virus upon the binding of specific ligands and rapid structural fluctuations, may be expected to help in attaining complete extinction of the infectivity and greater homogeneity of the virus population, conditions of importance in the design of effective vaccination procedures.

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