Phenomenological Description of the Association of Protein Subunits Subjected to Conformational Drift. Effects of Dilution and of Hydrostatic Pressure

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ABSTRACT: The native conformation of oligomers may be expected to undergo reversible changes when they separate upon dissociation of the original aggregate. When these changes are slow in comparison with the time of an association–dissociation (AD) cycle, they give rise to characteristic effects in the dependence of the dissociation: upon dilution, at constant pressure, and upon the applied pressure, at constant concentration. The phenomenological description of these effects is examined by comparing two possible models: The first model assumes a continuous loss in free energy of association with the extent of dissociation; the second supposes the existence of two or more distinct aggregates differing in subunit affinity and present in proportions that vary with the extent of dissociation. The latter model fits better the experimental data available, with regard to both the concentration and the pressure dependence of the association, and gives a particularly simple explanation of the hysteresis phenomena observed in several oligomeric proteins after application of pressure. The validity of the principle of detailed balance, often assumed in dealing with complex equilibria, is discussed in detail as it does not appear possible to reconcile it with some of the experimental observations or with the proposed model.

Experimental evidence presented in two accompanying papers (King & Weber, 1986a,b) demonstrates the loss of affinity of the protein subunits of an oligomeric aggregate when they become serparated from each other. This loss of affinity is apparent even in those situations in which a dynamic equilibrium between the aggregate and its constitutive subunits is clearly established. We attribute the loss of affinity to a progressive, time-dependent disorganization of the monomers occurring when, after the dissociation of the aggregate, they cease to exert influence upon each other and refer to this phenomenon in the following by its probable cause: a "conformational drift".

Dilution Effects in Dimer–Monomer Equilibria

The implications of the loss of association free energy in a partially dissociated aggregate are best appreciated by considering the simplest case: a dimer formed by two identical subunits. In a study of enolase Xu and Weber (1982) have shown that at very low degrees of dissociation ($a \rightarrow 0$) the free energy of association of the monomers, DG(0), has a larger absolute value than DG(1), the association free energy in a dilute solution in which a is close to unity. The variation of DG with a implies that neither dimer nor monomer can have constant, association-independent, chemical potentials. Instead, these depend upon the extent of reaction. This dependence is one that, though not forbidden by the principles of thermodynamics, is not explicitly considered in classical chemical thermodynamics. It is, however, an assumption that is rendered inevitable in the light of repeated experimental findings that we examine in detail in the accompanying papers. Accordingly we set

$$\mathrm{DG}(1) - \mathrm{DG}(0) = \mathrm{d}G \qquad (1)$$

As DG(1) and DG(0) are, ordinarily, negative quantities and DG(0) < DG(1), it follows that dG is always positive.

Continuous Variation of the Association Free Energy. The description of the conformational drift proposed by Xu and Weber assumes the continuous variation of the free energy of association of the monomers with the degree of dissociation. The simplest formulation involves loss of association free energy proportional to the degree of dissociation in which case the dimer *dissociation* constant is given by

$$K(a) = K(0)\exp(a\mathrm{d}G/RT) \qquad (2)$$

with $K(0)$ as the dissociation constant operative for negligibly small dissociation. If C stands for the concentration of the protein as dimer

$$4a^2C/(1-a) = K(0)\exp(a\mathrm{d}G/RT) \qquad (3)$$

Taking a as the independent variable in eq 3 and solving it for $C/K(0)$, with dG/RT as parameter, the plots of a vs. log $[C/K(0)]$ (dilution curves) have the form shown in Figure 1. The span of the dilution curve may be specified as the change in concentration, in decimal log units, when a varies from 0.1 to 0.9. The normal titration span (dG = 0) is 2.86 log units, and by eq 3, it decreases linearly with increasing dG/RT (Figure 3). At dG/RT = 5.45 the plot of a vs. log C include a vertical segment [d log (C)/da = 0] characteristic of a "critical" transition. For larger values of dG/RT the curve "folds" as indicated in the figure. This behavior ressembles the "van der Waals catastrophe" that is encountered in the analysis of critical phenomena employing closed-form procedures like that indicated in eq 3. The physical implication is that for the larger losses of binding free energy by the conformational drift there is a critical concentration above which the dimer is virtually the only species present and below which only monomers are observed.

Description of the Process by Several Coupled but Independent Equilibria. Instead of the description of the conformational drift in terms of a monotonous loss of free energy with degree of dissociation, another may be given in terms of discrete molecular species. For this purpose we postulate the existence of unique monomer forms: M, normal, and M*, conformationally drifted. To begin with we may disregard the possible existence of a hybrid dimer (M–M*) and assume that the dimer forms present are only two: D = M_2 and D* = M^*_2.

At protein concentrations at which $a \rightarrow 0$, the predominant forms will be D and M, but as dissociation progresses, these

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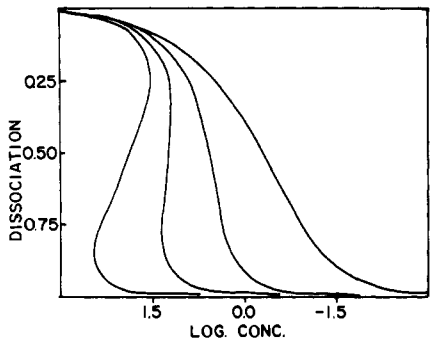

FIGURE 1: Plots of degree of dissociation vs. logarithm of the total protein molarity as dimer, in units of $K(0)$ (dilution curves). The four curves, for increasing values of dG/RT 0, 4, 7, 10, were calculated employing eq 2.

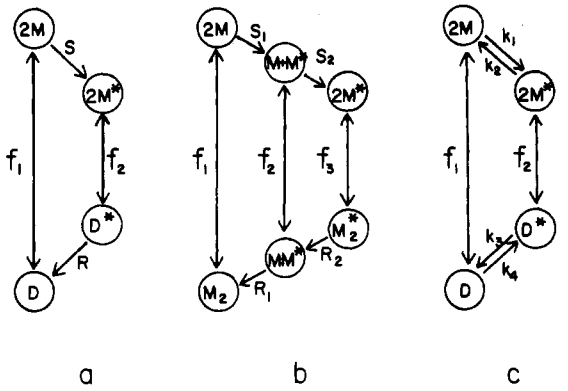

FIGURE 2: Coupled equilibria involving dimers D...D* with dissociation constants $K...K^*$. In (a) and (b) equilibrium between the forms is reached through unbalanced rates of monomer spoiling S and repair R. (a) Exchanges between two dimers. (b) Exchanges among three dimers. (c) Rates to be considered when detailed balance applies. The f's are the fractions of each form at equilibrium. In (c) they depend upon the ratio K/K^* and the concentration alone; in (a) and (b) they additionally depend upon the ratio of rates S/R.

will be replaced by increasing proportions of D* and M*. The classical assumption with regard to forms that, like M and M*, can be interconverted by first-order processes is that they exist at equilibrium in fixed proportions determined by a unique constant $k = [M^*]/[M]$, independent of a. In that case the dilution curve has a fixed logarithmic span of 2.86 units.

Dilution curves with decreased span require ratios $[M^*]/[M]$ and $[D^*]/[D]$ that vary with the degree of dissociation. This condition can exist if the conversion of M into M* takes place in the free monomer with rate constant S balanced at equilibrium, not by the reciprocal process $M^* \rightarrow M$ but by the conversion of D* into D, at rate R, also unbalanced with respect to the reciprocal process, $D \rightarrow D^*$. These assumptions are in flagrant violation of the principle of detailed balance, often used to simplify the analysis of complex equilibria but seldom if ever subjected to experimental test or detailed scrutiny. We discuss in detail the applicability of this principle to our case at the end of this paper. For the present we note that formally (Figure 2a,c)

$$\begin{aligned} S[M] &= k_1[M] - k_2[M^*] \\ R[D^*] &= k_3[D^*] - k_4[D] \end{aligned} \tag{4}$$

and that for the purpose of describing the approach to equilibrium it matters little whether we set $k_2 = k_4 = 0$ or give these rate constants values sufficiently small to make $k_2[M^*] \ll k_1[M]$ and $k_4[D] \ll k_3[D^*]$ at all relevant degrees of dissociation. Accordingly we adopt the simplified scheme shown in Figure 2a. The associated kinetic relations involve six rate constants: two rates of association, two rates of dissociation, and the rates of the unopposed processes, S and R. A further considerable simplification is introduced if we assume that the two monomer–dimer equilibria are established in times that are short in comparison with the reciprocals of S and R. It then follows that at all times the concentrations of M, M*, D, and D* are respectively equal to [M]...[D*], the equilibrium concentrations in the reaction $D \leftrightarrow 2M$ and $D^* \leftrightarrow 2M^*$. This assumption, which corresponds to that of Michaelian enzyme kinetics, reduces the number of parameters to four, namely, the dissociation constants K and K^* and the rates of spoil S and repair R of the drift process. If the fraction of the protein present as drifted forms, M* and D*, is f and the total protein concentration as dimer is C, we then have the equilibrium condition of each of the two systems as

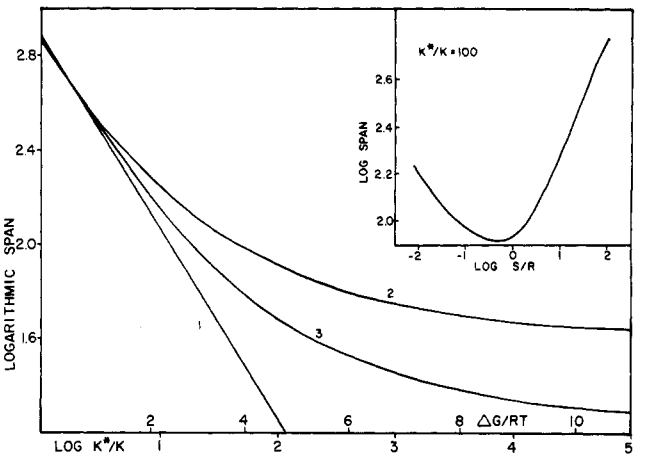

FIGURE 3: Plot of the logarithmic span of the dilution curves vs. K^*/K or dG/RT. (1) Continuous decrease in free energy of association with degree of dissociation; (2) equilibrium involving two dimer forms; (3) equilibria involving three dimer forms. (Inset) Dependence of the logarithmic span upon the ratio S/R for a typical value of $K^*/K = 100$.

$$K = 4a^2C(1-f)/(1-a) \qquad K^* = 4a^{*2}Cf/(1-a^*) \tag{5a}$$

where a and a^* are respectively the degrees of dissociation of the native and drifted forms.

When the ratios K/C and K^*/C and S and R are given, a simple iteration procedure, which actually imitates the expected kinetics of the system, may be used to solve for f, a, and a^*. With any initial arbitrary value of $f = f_0$, eq 5a are solved to give a and a^*. The change in f resulting from transfer between the two systems, native and drifted, equals

$$df = Sa(1-f)/2 + R(1-a^*)f \tag{6}$$

The dimensions of S and R are fractional transfers per iteration, and the new value of f is

$$f_1 = f_0 + df \tag{7}$$

The iteration is continued until $df < e$, where e is a preestablished small value. At this point the condition of equilibrium

$$Sa(1-f)/2 = R(1-a^*)f \tag{8}$$

will be obeyed within an error e. The corresponding average dissociation is

$$\langle a \rangle = a(1-f) + a^*f \tag{9}$$

The iteration process converges to any desired degree of accuracy and, as demanded by (8), the equilibrium f does not depend upon the choice of S and R but only upon their ratio. Moreover (Figure 3, inset), minimum logarithmic span is found when $S = R$. The procedure just described can be easily extended to the analysis of equilibria among three or more dimer forms having increasing degrees of drift. Use of three

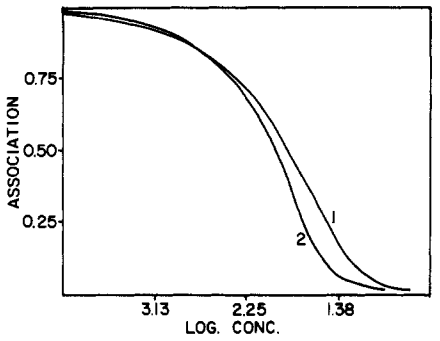

FIGURE 4: Dilution curves for equilibria of two dimer forms (1) calculated employing the scheme in Figure 2a (eq 5–9) and three dimer forms (2) calculated from the scheme in Figure 2b. $K^*/K = 1000$. The concentration in the abscissa is in units of K.

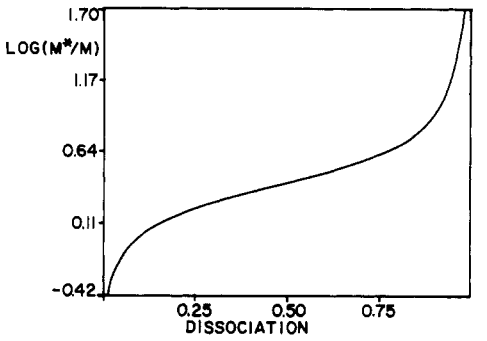

FIGURE 5: Plot of the logarithm of the ratio of drifted and native monomer concentrations vs. the average degree of dissociation, for $K^*/K = 100$.

forms (Figure 2b) may be considered a minimum for the description of equilibria in dimers, since realistically we expect that at intermediate degrees of dissociation an important fraction of dimers is of the mixed type MM*, a species with free energy of association intermediate between MM and M*M*.

Comparison of the Two Approaches. Figure 3 compares the computed logarithmic span as a function of dG/RT, or K^*/K, for three cases: (1) continuous variation of free energy of association; (2) equilibrium between two dimer forms (MM, M*M*); (3) equilibria among three dimers (MM, MM*, M*M*). In this latter case the loss in free energy of association of MM* is assumed to be half of the loss of the M*M* form; the additional dissociation constant is then $(KK^*)^{1/2}$. It is clear from the figure that the decrease in logarithmic span in the cases of coupled equilibria (2 and 3) is much more limited than on the assumption of continuous loss of free energy of association (1). At $K^*/K = 10^5$, or $dG/RT = 11.5$, the logarithmic span is 1.63 for two dimers in equilibrium and 1.28 for equilibria among three dimers. Figure 4 compares the dilution curves for these two latter cases when $K^*/K = 1000$. The curves are similar in shape with some difference in logarithmic span, 1.75 and 1.45, respectively. The critical behavior characteristic of the continuous change in free energy with average degree of dissociation (Figure 1) is not found when simulation of equilibria between two or more forms is carried out as described above. It is noticeable that the dilution curves of malate dehydrogenase (Shore & Chakrabarthy, 1976) and enolase (Xu & Weber, 1982) have logarithmic spans in the vicinity of 1.7, that this treatment predicts for values of K^*/K of the order of 10^3. Additionally the shape of the dilution curves for enolase reported by Xu and Weber agree reasonably well with those shown in Figure 4. An important characteristic of the curves is that they predict at high protein concentrations a degree of dissociation that can be much larger than that corresponding to the equilibrium of the native form. The observation by gel filtration analysis of small amounts of monomer in equilibrium with the tetramers in lactate dehydrogenase at micromolar concentration (King & Weber, 1986b) is in agreement with this prediction.

It is possible to extend the previous ideas to the equilibria of trimers and tetramers with monomers. Computations made for tetramer–monomer equilibria involving five different dimers (M_4, M_3M^*, ... M^*_4) show a further reduction in logarithmic span but still the absence of criticality. The variation of the ratio M*/M with average degree of dissociation in coupled equilibra is shown in Figure 5. The shape of this plot is independent of the magnitude of the free energy loss owing to drift and is similar for dimers, trimers, and tetramers. As shown in the figure M*/M increases rapidly for $\langle a \rangle > 0.5$ and very rapidly for $\langle a \rangle > 0.8$. As a consequence we expect that attainment of the equilibrium favoring the drifted forms will take place most readily at the largest degrees of dissociation. This prediction is verified by the comparison of the rapid enzymic inactivation of lactate dehydrogenase at a pressure at which complete dissociation can be achieved, with the very slow drift and inactivation at pressures that produce a small dissociation and the even slower efects at atmospheric pressure and low temperature (King & Weber, 1986a,b).

Time-Dependent Effects. The loss of free energy by the separated monomers is a slow process in comparison with the fast association–dissociation cycle (AD cycle). As a result the macroscopic equilibrium is reached very slowly (Xu & Weber, 1981; King & Weber, 1986c) and there may be difficulty in deciding whether one is measuring the final equilibrium value. In the simulation of the coupled equilibria described above the number of iterations required for final equilibration is expected to be proportional to the experimental equilibration time. A limited number of iterations correspond then to observations at times after dilution too short to achieve complete equilibration. When the number of iterations is thus limited, the logarithmic span of the dilution curves increases, and it approaches the value of 2.86 units as the number of iterations approaches 1. Evidently, failure to reach equilibrium cannot cause the decrease in span experimentally observed.

Pressure Effect on the Association

The dissociation and the concentration of protein dimers, C, are related by the expression

$$-\log C = f(a) - \log K(0) \qquad (10)$$

with $f(a) = \log [4a^2/(1 - a)]$, and the relation of dissociation and pressure is

$$p\,dV^0/(2.302RT) = f(a) - \log [K(0)/C] \qquad (11)$$

where dV^0 is the standard volume change upon monomer association. Thus, the dependence of the dissociation on the logarithm of the concentration at constant pressure p and the dependence on the pressure at constant concentration have equivalent forms. Corresponding to the characteristic logarithmic span of the concentration, we have a characteristic pressure span, $\langle dp \rangle$, the difference between pressures at which 10% and 90% dissociation are achieved. From eq 11

$$\langle dp \rangle = 2.302RT \log (729)/dV^0 \qquad (12)$$

If dV^0, here assumed to be independent of pressure, is given in milliliter per mole and $T = 298$ K, then $\langle dp \rangle = 163.4/dV^0$ kbars.

Pressure Effects upon Two Coupled, Independent Equilibria. The dissociation of oligomeric proteins by hydrostatic pressure permits to infer the existence of a conformational drift

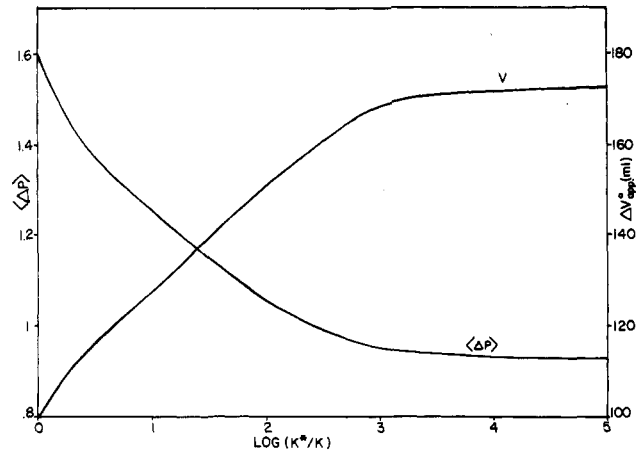

FIGURE 6: Dependence of pressure span, $\langle dp \rangle$, and computed (apparent) standard change in volume upon association, dV^0_{app}, on free energy of drift. $dV^0 = 100$ mL mol^{-1}.

because of the altered properties of the aggregates observed after decompression. The most easily recognized are the changes in enzyme activity (Mueller et al., 1981a,b; King & Weber, 1986a–c; Silva et al., 1986a,b). King and Weber (1986a,b) and Silva et al. (1986a,b) have demonstrated that reassociation and regain of enzymic activity occur in different time spans. Silva et al. (1986a,b) have found that in the β_2 dimers of tryptophan synthase several spectroscopic properties differ from each other and, from the enzymic activity, in the recovery characteristics. It is then evident that the simple model described above in which the properties follow from the change of the isolated monomer into a unique characteristic form can only be expected to account for some but not all of the complexities of the system. Indeed, the proposed name of "conformational drift" implies the kind of process that cannot be easily described as a chemical equilibrium between well-defined forms. However, the simple model of two coupled, independent equilibria developed earlier can provide a description of the persistence of the decreased subunit affinity and other properties that is observed upon the release of pressure.

Because of the equivalence of (10) and (11), we expect the conformational drift to result in a decrease in the pressure span just as it produces a decrease in the logarithmic span of the dilution curves. The simulation of pressure effects employs eq 6–8, with eq 5a replaced with

$$K(p) = K_1 \exp(p\,dV^0/RT)$$
$$K^*(p) = K_1^* \exp(p\,dV^{0*}/RT) \qquad (5b)$$

where K_1 and K_1^* are values at atmospheric pressure. In our computations we further assumed $dV^0 = dV^{0*}$, which reduces the number of parameters and appears to be in broad agreement with the small number of present observations.

The decrease in the pressure span with the ratio K^*/K is shown in Figure 6 for a dimer with $dV^0 = 100$ mL mol^{-1}. The pressure span decreases from 1.6 (at $K^*/K = 1$) to 1 at $K^*/K = 300$ and only marginally thereafter, as it reaches 0.925 at $K^*/K = 10^5$. This behavior parallels that of the logarithmic span shown in Figure 3. If dV^0 were independently known, the pressure span could be used to characterize the free energy loss by the drift. However, we do not have at present other methods for determination of dV^0. It follows that, as indicated in Figure 6, the conformational drift can result in a considerable overestimation of dV^0. Additionally the existence of a conformational drift does not cause the plots of log $a^2/(1 - a)$ vs. p to depart from linearity by amounts that would

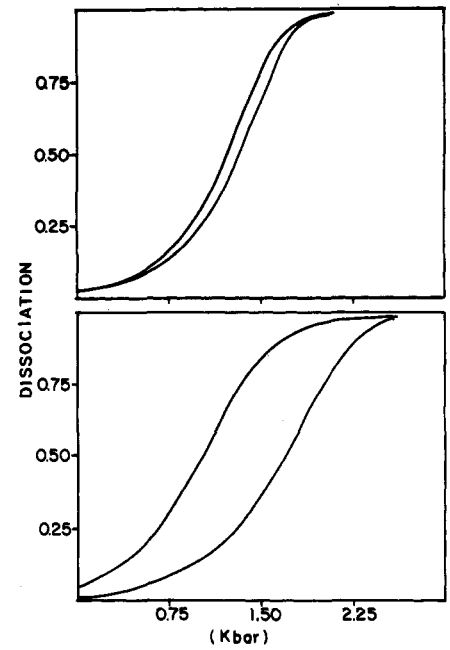

FIGURE 7: Simulation of the hysteresis effects by incomplete equilibration (reduction of the number of iterations). The curves are for increasing (→) and decreasing (←) pressures. Equilibration to $df < 10^{-6}$ required 2804 iterations. (Top) 280 iterations at each pressure. (Bottom) 28 iterations.

permit the qualitative demonstration of drift at any but the exceptionally large values of K^*/K. As a consequence of this absence of curvature the values of dV^0 calculated from the slope of these plots around $p_{1/2}$, the pressure at which $a = 0.5$, agree within 5–10% with dV^0 calculated from the pressure span (eq 12).

If two concentrations of protein C_1 and C_2 are employed, and dV^0 is pressure independent, the difference in pressures dp at which the same degree of dissociation is reached is from eq 11

$$dp = (2.302RT/dV^0) \log (C_2/C_1) \qquad (13)$$

If dV^0 is given in milliliters per mole and the temperature is 25 °C, $dp = (57.08/dV^0) \log (C2/C_1)$ kbars. The change in concentration simply shifts the curve toward higher pressures by the amount dp without changing its shape. Then, provided the volume changes on association of the native and drifted species are not too different, changes in protein concentration produce the same effects whether drift is present or absent.

Because of technical needs, the experiments of pressure dissociation have to be carried out in times that are limited and, at least in some instances, do not permit ready verification of the attainment of equilibrium. [For a more extensive discussion of this point, see King & Weber (1986a).] The simulation of coupled exchanges among systems at equilibrium that we described above permits to follow in detail the dynamics of this process and to describe the effects of incomplete equilibration. As noted above the condition of incomplete equilibration is mimicked by reducing the number of iterations below that necessary for equilibrium. If the simulation is carried out to equilibrium, the curves obtained on increasing or decreasing the pressure are coincident. Figure 7 shows plots of dissociation against pressure when this is increased by steps of 0.1 kbar until 98% dissociation is reached and then decreased to zero pressure by an equal number of steps. On reducing the number of iterations to 1/10 and 1/100 of the number required for equilibrium, the curves for ascending and descending pressures are no longer coincident. This hysteresis phenomenon arises from two distinct causes: one, easily

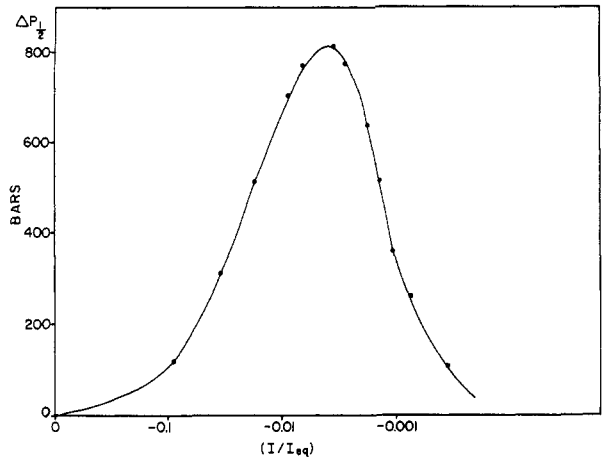

FIGURE 8: Plot of difference in mid-dissociation pressure, $dp_{1/2}$, vs. the ratio I/I_{eq} (allowed iterations/iterations necessary for equilibrium). $K^*/K = 100$; $I_{eq} = 2804$.

perceived, is the persistence of the drifted form when the pressure is rapidly reduced. The other is the large increase in the ratio M*/M at almost complete dissociation already illustrated in Figure 5. As the time allowed for interconversion of the forms (equals number of iterations) is decreased, the curves for ascending pressure increasingly retain the character of the native dimer, but at almost complete dissociation the very large value of the ratio M*/M causes the drifted form to become the predominant one and the descending pressure curve to correspond more closely to the drifted form. The difference in $p_{1/2}$ between the ascending and descending branches of the plot, $dp_{1/2}$, increases as the number of permitted iterations decreases. With further reduction of the number of iterations $dp_{1/2}$ passes through a maximum, then decreases, and finally almost vanishes when the reduced number of iterations does not permit appreciable drift, even at complete dissociation. These features are shown in Figure 8 which plots $dp_{1/2}$ vs. the logarithm of the following ratio: allowed iterations/iterations necessary for equilibration. The figure shows a maximum $dp_{1/2}$ in excess of 800 bars, corresponding to 70% of the difference in free energy of association of the native and drifted forms for the case plotted: $K^*/K = 100$, $dV^0 = 100$ mL mol^{-1}. Therefore, in experiments of rapid increase and decrease of pressure one can expect not only to demonstrate directly the phenomenon of conformational drift but also to obtain a lower bound for the change in free energy of association that the drift brings about. The rapid reversibility of the pressure application affords a method of investigation that has great advantages over the study of dilution curves when it comes to detecting the conformational drift and estimating its magnitude. Hysteresis of the pressure curves has been experimentally observed in lactate dehydrogenase (King & Weber, 1986a) and in the β_2 dimer of tryptophan synthase (Silva et al., 1986a,b). Their absence in the case of the enolase (Paladini & Weber, 1981) is to be attributed to times of drift of the monomer and recovery of the dimer that are not too long in comparison with the times that were employed in the application of pressure.

Conformational Drift and the Principle of Detailed Balance. Since the classical publications of Onsager of 1931 (Onsager, 1931a,b) [see also Denbigh (1951, 1977) and Landsberg (1961)] it has been customary to accept as generally valid that in the state of thermodynamic equilibrium "every microscopic process and its reciprocal occur with equal frequency". It does not seem easy to reconcile the description of the effects of the conformational drift offered here and the principle of detailed balance (Berg & Weber, 1983). Either there is a totally different explanation for the experimental facts that we attempt to describe by our phenomenological treatment or the principle of detailed balance has a more limited validity than hitherto assumed.

Onsager recognized that detailed balance cannot be derived from thermodynamic constraints and that it must be introduced as an independent assumption. In his publications he referred only to first-order processes of interconversion of chemical forms and did not consider the possibilities that would arise in chemical equilibria in which both first-order and second-order processes are interlocked. Changes in the conformation of the partners in the free and bound state must occur even in the simplest molecular complexes in solution. What sets them quite apart from the equilibria of oligomeric proteins is that in them the first-order changes in conformation occur in times which are negligible in comparison with the time for an AD cycle. With rare exceptions, interconversion of conformers of small molecules in solution occurs in times of picoseconds to nanoseconds while the AD cycle of a molecular complex with dissociation constant $K = 10^{-3}$–10^{-1} is determined by diffusion times, which in these cases are of the order of microseconds to milliseconds. Thus, the aggregate and the free partners can be considered to have average structures that receive negligible contributions from the forms generated in the previous state, respectively, dissociated or associated. As a result the reactants and product of the association have, within error, unique chemical potentials independent of the degree of dissociation of the complex. Quite a different situation is found in the specific association of protomers that form the oligomeric proteins. Experimentally the reversal of the conformational drift is observed to take a long time, minutes to hours, while the AD cycle may not take longer than a few minutes. The relevance of the principle of detailed balance for these complex cases may be best appraised by noting that, according to it, if we could record a moving picture of the microscopic events taking place at equilibrium, we could not distinguish whether the film is being run forward or backward. In consequence we would be unable to tell from it which are the causes and which are the effects at the molecular level. If we believe that the separation of the monomers and their association are the *specific causes* of the conformational changes that follow them, we are led to accept the common sense view that both the independence of the chemical potentials from the extent of reaction and the existence of detailed balance are limiting conditions that apply only when the rates of molecular conformation change are fast in comparison with the rates of association and dissociation.

The breakdown of detailed balance in the equilibria of oligomeric proteins with their subunits may be further clarified by examining in detail the mechanism of the dissociation. We may agree to consider that dissociation has taken place when the monomers have moved away from each other a sufficient distance apart, dx, at which they do not exert mutual influence and their motions become independent of each other. If D is the diffusion coefficient of the monomer, the time dt required for its diffusion through distance dx is

$$dt = \langle dx \rangle^2/(2D) \qquad (14)$$

At 20 °C in water a globular protein of 43 kDa (enolase monomer) has $D = 9 \times 10^{-7}$ cm^2 s^{-1}. dx must be at least as large as the diameter of a water molecule (4 Å), and therefore, $dt > 0.9$ ns. The lifetime of a purely energetic state (typically a vibrational state) cannot last much longer than some picoseconds (Lauberau et al., 1970). Thus, dissociation cannot be caused by the transient accumulation of energy in one or

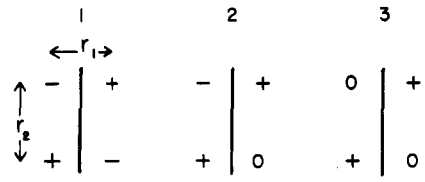

FIGURE 9: Disposition of charges (quadrupole) across subunit boundary. (1) Prevalent configuration. (2 and 3) Fluctuational states that arise from temporary protonation of carboxyl groups.

more degrees of freedom of the protein. It must follow the creation of a "long-lived" (correlation time > 1 ns) chemical species in which the attraction between the subunits is greatly diminished, or even replaced by repulsion. It is easy to envision the creation of such a state through fluctuations of charge occurring at the boundary of the subunits: Salt linkages between carboxyl and the positively charged groups of histidine or arginine have been described as occurring at the interface of the protein subunits in hemoglobin (Perutz et al., 1968; Bolton & Perutz, 1970) and lactate dehydrogenase (Holbrook et al., 1975) among other oligomeric proteins. At near neutral pH we can expect that fluctuations of charge of the carboxyl groups will often occur, leading to a decrease in subunit affinity and even, for an appropriate quadrupole disposition of charges like that shown in Figure 9, to actual electrostatic repulsion. The contribution to the energy of subunit interaction from the normal quadrupole (1) and the fluctuational states (2 and 3) may be computed after giving values to the effective dielectric constant of the surroundings, D_{eff}, and the distances between the charges of different sign: r_1, at opposite sites of the boundary, and r_2, at the same side. Setting $r_1 = 4.10^{-8}$ cm, $r_2 = 4r_1$, and $D_{eff} = 10$ we obtain as the interaction energies of dispositions 1, 2, and 3, respectively, −12.4, −4.3, and +4.1 kcal/mol. Such energies are of the order necessary to account for important contributions to the normal attractive state and for the long-lived reppulsive state that can lead to dissociation. Additionally the correlation time for the dissociation of a protonated carboxyl group is a few microseconds, ample for the diffusion of the monomers away from each other. It seems unlikely that anyone will argue that the dissociations of the dimer as a result of the electrostatic repulsion during a fluctuation in which the two negative charges are lost (state 3) will be balanced by an equal number of associations of monomers, each having a single positive charge, and that the associations between dipole monomers will be balanced by equal number of dissociations of the dimer in state 1. Yet this is what is demanded by the strict application of the principle of detailed balance.

It may be observed that the dissociation mechanism postulated as being responsible for the breakdown of detailed balance does not require that separation of the monomers be followed by their conformational drift. In this assumed mechanism the subsequent changes in conformation may provide additional cause for the failure of detailed balance but are not a necessary condition for it. Accurate observations should be able to demonstrate the failure of detailed balance in simple molecular complexes in aqueous solution subjected to dissociation by protonation. Apart from the need of finding a case in which the rates of protonation and dissociation of the complex have, or can be made to have, comparable values, there seems to be no technical difficulty in the way of this demonstration.

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