Oligomeric Protein Associations: Transition from Stochastic to Deterministic Equilibrium†

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Received August 24, 1990; Revised Manuscript Received November 5, 1990

ABSTRACT: Transfer of electronic excitation energy (sensitized fluorescence) between donor and acceptor fluorophores separately attached to dimer or tetramer proteins is used to demonstrate the exchange of subunits among the undissociated particles. In dimers subjected to a pressure that produces half-dissociation, the exchange occurs at a rate that approaches the rate of dissociation. In the tetramers of glyceraldehyde-phosphate dehydrogenase and lactate dehydrogenase at 0 °C, the times for subunit exchange are nearly 2 orders of magnitude, and at room temperature 5–10 times longer than the time required to reach the dissociation equilibrium. By application of a novel method, pressure is shown to preferentially increase the rate of dissociation in dimers and decrease the rate of association in tetramers. From these observations, we conclude that the tetramers constitute a heterogeneous population, the members of which are dissociated by pressure according to individual molecular properties that can be retained over periods of time much longer than the time for equilibration of the dissociation. The dissociation of dimers exhibits the characteristics of the classical stochastic chemical equilibria, while those of the tetramers, like the more complex protein aggregates, must already be considered similar to the deterministic mechanical equilibria of macroscopic bodies.

The dissociation of oligomeric proteins by hydrostatic pressure has permitted to study in detail the equilibria between the aggregates and their constitutive subunits. Dimers (Paladini & Weber, 1981a; Silva et al., 1986; Ruan & Weber, 1988), tetramers (King & Weber, 1986; Ruan & Weber, 1989), and higher order aggregates (Silva & Weber, 1988; Dreyfus et al., 1988; Silva et al., 1989; Gomes et al., 1990) have been examined. The dependence of the thermodynamic equilibrium between the aggregate and N equal elementary subunits is described by the relation:

$$K = N^N \alpha^N C^{N-1}/(1 - \alpha) \quad (1)$$

where K is the dissociation constant, α the degree of dissociation of the aggregate, and C the molar concentration of protein expressed as aggregate. In the cases this far studied, the effects of hydrostatic pressure are well represented by a straight line dependence between pressure (p) and the logarithm of the apparent dissociation constant [$K(p)$], thus validating the relation:

$$K(p) = K_0 \exp(p\Delta V/RT) \quad (2)$$

where K_0 is the *dissociation* constant of the aggregate at atmospheric pressure and ΔV the molar volume change upon *association*. The existence of a dynamic microscopic equilibrium between association and dissociation reflects itself in the dependence of the dissociation upon the particle concentration at a constant pressure. If eq 1 and 2 are obeyed, changing the total protein concentration from C_1 to C_2 results in

$$p_2 - p_1 = (N - 1)(RT/\Delta V) \ln (C_2/C_1) \quad (3)$$

According to eq 3, for constant volume change, the parallel displacement in the pressure profile with change in concentration must increase with the number of particles that form the aggregate. As shown in Table I, in the several dimers studied the values of ΔV calculated by the change in dissociation with pressure (ΔV_P, eq 2) and by the change in pressure of half-dissociation with concentration (ΔV_C, eq 3) are in agreement within the errors of the determinations (±15%). On the other hand, the pressure-induced dissociation of erythrocruorin from the oligochete worm *Glossoscolex paulistus* (MW = 3.1 × 10^6, 12 octameric subunits or 96 monomer subunits) is entirely concentration-independent (Silva et al., 1989). An almost complete independence from concentration is observed in the pressure dissociation of the capsid of the Brome mosaic virus (Silva & Weber, 1988) and in other icosahedral viruses (Silva and Weber, unpublished results) and in hemocyanin (Gomes et al., 1990). In these many-particle aggregates that do not show appreciable concentration effects, *the thermodynamic dependence of the degree of dissociation upon the applied pressure (eq 2) is followed just as well as in the dimers*. The conclusion must be drawn that they constitute a heterogeneous population, each member having its own characteristic dissociation pressure, rather than a population of identical polymeric aggregates in dynamic equilibrium with the interacting subunits. The designation of "deterministic equilibrium" for these cases of independence

Table I: Standard Volumes of Association (ΔV_C, ΔV_P) and Free Energies of Association (ΔG) of Dimers and Tetramers[a]

	ΔG	ΔV_P	ΔV_C
dimers			
enolase[f]	−9.5	55	47
β_2-tryptophan synthase[d]	−12.8	170	170
yeast hexokinase[c]	−12.6	120	172
tetramers			
porcine LDH[b]	−36.3	170	680
yeast GAPDH[e]	−34.9	235	783

[a] ΔG and ΔV are from fluorescence polarization observations of dansylated proteins except ΔG for enolase, derived from dilution observations. Free energies in kilocalories per mole; association volumes in milliliters per mole. [b] King & Weber (1986). [c] Ruan & Weber (1988). [d] Silva et al. (1986). [e] Ruan & Weber (1989). [f] ΔG from Xu and Weber (1982); ΔV's this study.

† Supported by USPHS Grant GM11223 to G.W.
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0006-2960/91/0430-1595$02.50/0

upon the concentration of the particles seems justified by the similarity with the behavior of macroscopic bodies. In either case, the new equilibrium that follows an introduced perturbation arises from individual characteristics of each object that are retained over periods of time much longer than those necessary for the achievement of equilibrium.

Several tetramers (King & Weber, 1986; Ruan & Weber, 1989) show changes in the pressure of middissociation with concentration that are only about one-fourth of those predicted by eq 3. This is best revealed by comparison of the values of ΔV_P calculated from the change in dissociation with pressure with those of ΔV_C, which are derived from the concentration dependence of the pressure for half-dissociation (Table I). In these tetramers, we begin to see the evolution of the typical stochastic molecular equilibrium, which applies rigorously to the dimers, toward the macroscopic-object, deterministic equilibrium of the many-particle aggregates. We have therefore undertaken a comparative study of the dynamics of the equilibrium established at various pressure-induced dissociations in dimers and tetramers. The time for achieving virtually complete equilibration following application of pressure in dimers and tetramers has been found to be of the order of 10–30 min. Typically, we establish a degree of dissociation of half by an appropriate pressure and follow the subsequent subunit exchange of the undissociated fraction. We measure the extent and rate of subunit exchange by the changes in the transfer of electronic energy between fluorophores covalently attached to the particles. We have also studied by a newly developed method the relative importance of the changes in the rates of association and dissociation owing to the application of pressure.

Methodology

Subunit Exchange. Two methods were employed, based respectively on the transfer of the excitation between fluorophores of two different kinds, respectively donor and acceptor (heterotransfer method) and between fluorophores of the same kind (homotransfer method).

(1) Heterotransfer Method. Two identical solutions of the protein—dimer or tetramer—are covalently labeled at atmospheric pressure, respectively, with the donor and the acceptor fluorophore employing conventional methods. Unreacted material is eliminated, and after equal aliquots of the solutions are mixed, the spectrum of the fluorescence emitted by excitation at a wavelength preferentially absorbed by donor is recorded. Because of the insignificant dissociation at atmospheric pressure, there cannot be appreciable subunit exchange during the short time (a few minutes) necessary to record this spectrum, which thus defines the fluorescence emission in the absence of transfer ($\beta = 0$). The fluorescence spectrum in the absence of transfer is made up of separable contributions from the donor, D_0, and the acceptor, A_0. If a fraction, β, of the donor excitations is transferred to the acceptor, the spectral contributions become $D_\beta = D_0(1 - \beta)$ and $A_\beta = A_0(1 + q\beta)$ where q is the fraction of transferred photons emitted as A fluorescence. It follows that

$$\beta = 1 - (D_\beta/D_0) = (A_\beta/A_0 - 1)/q \quad (4)$$

If the mixed solution is subjected to a pressure at which dissociation is virtually complete and then allowed to reassociate by returning to atmospheric pressure, the new fluorescence spectrum is characteristic of the maximum possible transfer ($\beta = \beta_{max}$) owing to random scrambling of the subunits. Let the solutions be mixed at zero time, then kept for time t at a pressure that results in an intermediate degree of dissociation, α, and finally allowed to reassociate by decompression. The fraction $\beta(t)$ determined by eq 4 is made up of two contributions: $\alpha\beta_{max}$ that results from complete scrambling of subunits upon reassociation of the dissociated fraction and $(1 - \alpha)\beta_x$ representative of the exchange of subunits in the undissociated tetramers. Thus

$$\beta(t) = \alpha\beta_{max} + (1 - \alpha)\beta_x \quad (5)$$

We expect β_x to be given by a function of time of the form

$$\beta_x(t) = \beta_{max}[1 - \exp(-t/\tau_x)] \quad (6)$$

where τ_x is the characteristic time for reduction of the unscrambled subunit fraction to e^{-1} of its original value. From eq 5 and 6

$$\beta(t) = \alpha\beta_{max} + (1 - \alpha)\beta_{max}[1 - \exp(-t/\tau_x)] \quad (7)$$

Equations 6 or 7 may be expected to describe the simplest type of subunit exchange. More complex cases would require a sum of exponentials with increasing values of τ_x.

(2) Homotransfer Method. In this case, a protein solution is labeled with fluorescein isothiocyanate, or a fluorophore with similar spectroscopic properties, to the extent that ideally each subunit is labeled with a single fluorophore. The fluorescence is excited at a wavelength at which the free fluorophore exhibits maximum polarization. The observed decreased emission anisotropy, r_1, is related to the emission anisotropy in the absence of transfer, r_0, by the relation:

$$r_0/r_1 = 1 + T/\lambda \quad (8)$$

where T is the transition probability of transfer of the excitation to a fluorophore not originally excited and λ the transition probability of emission (Weber & Daniel, 1966; Weber & Anderson, 1968). If the labeled protein solution is mixed at zero time with a large excess of unlabeled protein and the pressure is raised to a value that produces an intermediate degree of dissociation, α, we expect the polarization to rise owing to two distinct processes: (1) Dissociation places the subunits of the dissociated fraction at distances at which transfer is not appreciable, since for a micromolar solution the average nearest-neighbor distance between solute molecules is ca. 400 Å (Chandrasekhar, 1943). (2) There is exchange of labeled subunits of the undissociated tetramers for others belonging to the much more abundant unlabeled fraction. Completion of the two processes, dissociation and exchange, will result in an increase in polarization to a value $r_s = \alpha r_m + (1 - \alpha)r_t$ where r_m is the anisotropy of emission from an isolated monomer and r_t the anisotropy of emission from a tetramer with a single labeled subunit. For a rotational relaxation time of tetramer of 200 ns and monomer of 50 ns, and a fluorescence lifetime of 4 ns, $r_t/r_m \approx 1.15$. At $\alpha = {}^1/_2$, r_s differs little from r_m or r_t, and we shall take then a single value r_s as characterizing the emission anisotropy of both dissociation and complete exchange. Assuming the independence of these two causes of change in polarization, we set

$$r_\alpha(t) = r_1 + (r_s - r_1)[1 - \exp(-t/t_1)] \quad (9)$$

$$r_x(t) = r_1 + (r_s - r_1)[1 - \exp(-t/\tau_x)] \quad (10)$$

Equation 9 describes the change in polarization owing to dissociation of the tetramers with time constant t_1 and eq 10 the replacement of the fully labeled tetramers by others carrying a single labeled subunit. The observed emission anisotropy $r(t)$ is a weighted average of $r_\alpha(t)$ and $r_x(t)$:

$$r(t) = \alpha r_\alpha(t) + (1 - \alpha)r_x(t) \quad (11)$$

We note the similarity of eq 7 and 11. Both homo- and heterotransfer methods permit us to decide whether dissociation and subunit exchange are accomplished in comparable times ($\tau_x \approx t_1$) or otherwise. However, only the homotransfer

method enables one to make a direct quantitative estimation of both t_1 and τ_x. Because of the appreciable time necessary for decompression, the heterotransfer method does not lend itself to a direct estimation of t_1, but it is useful to characterize those cases in which t_1 is very much shorter than τ_x.

Changes in the Rates of Association and Dissociation under Pressure. The increase in dissociation following application of pressure can result from an increase in the rate of dissociation, k_-, or a decrease in the rate of association, k_+, or from both these causes. We can then set

$$k_-(p) = k_- \exp(fp\Delta V/RT) \quad (12a)$$

$$k_+(p) = k_+ \exp[(f-1)p\Delta V/RT] \quad (12b)$$

where k_- and k_+ are the corresponding rates at atmospheric pressure and f is a fraction between 1 and 0 that determines the partition of the effects of pressure upon the two rates: If $f = 1$, the increase in $K(p)$ is wholly due to the increase in the rate of dissociation, and if $f = 0$, it depends upon the decrease in the rate of association alone. We note that the relation $K(p) = K_{atm} \exp(p\Delta V/RT)$ continues to be valid if both expressions in eq 12 are multiplied by the same factor a, independent of the pressure. Physically this would correspond to an increase ($a > 1$) or decrease ($a < 1$) of both kinetic rates by the same factor, leaving f unaltered. f can be determined by a study of the relaxation to equilibrium after rapid successive pressure increases. We expect f to be a constant or perhaps a slowly varying function of pressure, and in the absence of information as to such possible variation, we shall assume its constancy. Under this assumption, the relaxation after two successive pressure increases will suffice for its determination. For ease and accuracy of operation, these two increases should be chosen to have maximum amplitude, and to that purpose, the pressure is rapidly raised from atmospheric to that of middissociation, $p_{1/2}$, and after equilibrium at this pressure is achieved, it is raised once more to the pressure of nearly complete dissociation (Silva et al., 1986). The relaxations associated with the two pressure increases are characterized by the times t_1 and t_2 at which e^{-1} of the corresponding amplitudes of the changes in dissociation is reached. The effect of an "instantaneous" increase in pressure is calculated as follows: Setting $N = 2$ in eq 1, the differential change in dissociation, $d\alpha$, of a dimer in time, dt, is given by

$$d\alpha = [k_-(p)(1-\alpha)C - 4k_+(p)\alpha^2C^2]\, dt \quad (13)$$

where $k_-(p)$ and $k_+(p)$ respectively correspond to the rates of dissociation and association at the new pressure p. Introducing the values of the rates of association and dissociation given in eq 12, $d\alpha$ takes the form

$$d\alpha = 1 - \alpha - 4\alpha^2(C/K_0) \exp(p_{1/2}\Delta V/RT) \quad (14a)$$

$$d\alpha = [k_- \exp(fp_{1/2}\Delta V/RT)C]\, dt \quad (14b)$$

Starting with a value of C/K_0 appropriate to yield a small dissociation α_0 at atmospheric pressure, the increment $d\alpha$ over a suitably small time increment dt (time units = $1/[Ck_- \exp(fp_{1/2}\Delta V/RT)]$) is calculated by eq 14b and added to α_0 to obtain a new value of the dissociation, α_1. The procedure is repeated until middissociation is asymptotically approached, and the time for e^{-1} change in dissociation is interpolated in the set of values of α versus time. The whole procedure described by eq 13 is carried out once more starting with the equilibrium value of α, and eq 14b is applied after substituting $p_{1/2}$ by the pressure at which nearly complete dissociation is calculated to occur. Figure 1 shows two successive relaxations after instantaneous application of hydrostatic pressure that produce dissociations of 0.49 and 0.98, respectively, calculated

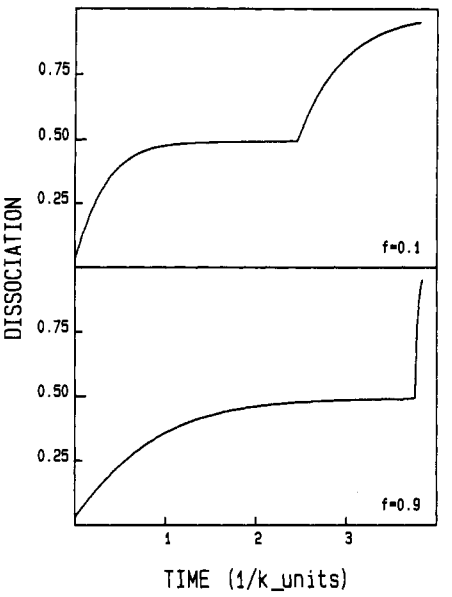

FIGURE 1: Time course of the dissociation of a homogeneous dimer population after two successive instantaneous applications of pressure, when compression affects preferentially the rate of dissociation (f = 0.9) or that of association (f = 0.1). Calculations by eq 14. The dissociation at atmospheric pressure is 0.02, reaching the equilibrium values of 0.49 and 0.98, respectively, after the two pressure increases.

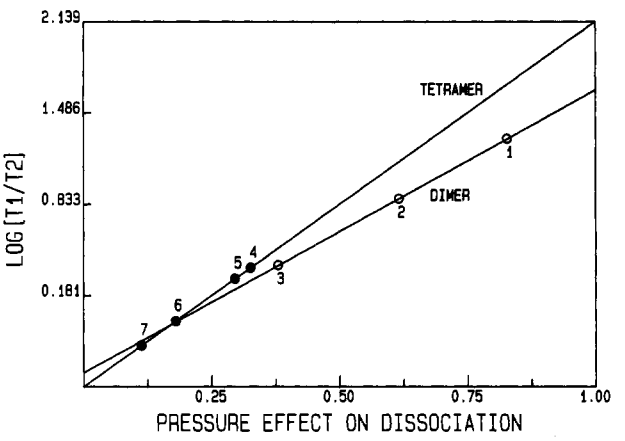

FIGURE 2: Linear dependence of f upon the logarithm of the ratio of the two successive relaxation times, t_1 and t_2 (Figure 1), for dimer and tetramers. The experimentally determined ratios of log (t_1/t_2) are placed on these lines to display the differences between dimers (open circles) and tetramers (filled circles). Numerical key: (1) enolase; (2) β_2-tryptophan synthase; (3) hexokinase; (4, 5) glyceraldehydephosphate dehydrogenase; (6, 7) porcine lactate dehydrogenase. (1, 3, 5, 7) Measurements at 0 °C; (2, 4, 6) at 25 °C.

as described. It is seen that $t_1/t_2 = 0.68$ if $f = 0.1$, and 28 if $f = 0.9$. This large difference permits a ready practical estimation of the relative effects of pressure upon the association and dissociation rates. A similar calculation can be made for a tetramer in which the dimer is a negligible fraction of the total protein at all degrees of dissociation. In that case, α^2 is replaced by α^4 and C by C^3 in eq 13 and 14. For the tetramer, $t_1/t_2 = 0.61$ if $f = 0.1$, and 75.4 if $f = 0.9$. In both cases of dimer and tetramer, log (t_1/t_2) has a strict linear dependence upon f as shown in Figure 2.

Experimental Results and Their Interpretation

Table II gives t_1 and t_2 for a series of dimers and tetramers. The relaxations were followed by the changes in the center of mass of the tryptophan fluorescence by means of an optical multichannel analyzer (OSMA, Princeton Instruments) after rapidly raising the pressure, first to 1.4 kbar and subsequently to 2.3 kbar. The time-dependent red shift of the center of mass

Table II: Relaxation Times of Dissociation after a Sudden Pressure Rise (t_1, t_2) and of Subunit Exchange (τ_x)[a]

	0 °C			25 °C		
	τ_x	t_1	t_2	τ_x	t_1	t_2
enolase		20	<1	18		
β_2-tryptophan synthase					15	<2
yeast hexokinase	40	10	4		8	<2
porcine LDH	360	4	6	40	2	<2
yeast GAPDH	>360	20	10	60	12	5

[a] Relaxation times are given in minutes. t_1 and t_2 were determined from the changes in spectral shift after two successive applications of pressure, 1.4 and 2.3 kbar, which resulted in approximately half-dissociation and nearly complete dissociation, respectively.

of the fluorescence changed monotonically with time, but it did not follow a single-exponential course, and the times t_1 and t_2 were taken as those in which 63% of the total spectral shift was realized. The results for dimers and tetramers differed consistently, as shown in Figure 2, in which log (t_1/t_2) has been used to determine the corresponding f value for tetramer or dimer. In all cases, pressure affects the opposing rates, but to different extents in the different proteins. In the dimers, f is consistently greater than 0.45, indicating that pressure acts preferentially to increase the rate of dissociation. In the two tetramers studied, f, either at the lower or at the higher temperature, is smaller than 0.3, showing that in their case the loss of subunit affinity under pressure is largely due to a decrease in the rate of association.

The time for subunit exchange τ_x was determined by one of the two methods that employ electronic energy transfer for this purpose, as described above. Figure 3 illustrates the application of the heterotransfer method: 5–10 mg of pig H_4 lactate dehydrogenase (Calbiochem) in 0.05 M carbonate buffer, pH 9.2, was reacted with 20 μL of a 5 mg/mL solution of either fluorescein isothiocyanate (Fluka) or 7-(diethylamino)-3-(4-isothiocyanatophenyl)-4-methylcoumarin (Molecular Probes) in dimethylformamide (DMF)[1] for 6–12 h. Conjugates were purified by Sephadex G-25 chromatography, dialysis against standard buffer (0.05 M Tris-HCl, 1 mM DTT, and 1 mM EDTA), and syringe filtration (Poretics) to eliminate particles >0.2 μm. The enzyme activity of the covalent conjugates was ≈90%. Concentrations and labeling ratios were determined by spectrophotometry (Beckman DU70). Pressurization was carried out at 0 °C. Fluorescence emission spectra was recorded 10 min after decompression by a Hitachi F-3010 spectrofluorometer thermostated at 0 °C. Excitation was at 385 ± 5 nm. Zero exchange is defined by the spectrum taken immediately after mixing solutions, with no previous pressurization. The progressive subunit exchange can be quantitated by the relative decline of the coumarin spectrum and the corresponding increase in the fluorescein spectrum, as shown in Figure 3. The resolution of the composite spectrum into coumarin and fluorescein contributions is easily accomplished because each spectrum is only partially overlapped by the other. Alternatively, one can measure the parameter $\langle\nu\rangle$, the center of mass of the emission of the composite spectrum, defined by the relation

$$\langle\nu\rangle = \sum\nu_i F_i / \sum F_i \qquad (15)$$

where F_i is the fluorescence emission at wavenumber ν_i and the sums in eq 15 comprise a compact spectral region over which $F_i > 0$. The inset in Figure 3 shows that $\langle\nu\rangle$ thus

[1] Abbreviations: DMF, dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; LDH, lactate dehydrogenase; GAPDH, glyceraldehydephosphate dehydrogenase.

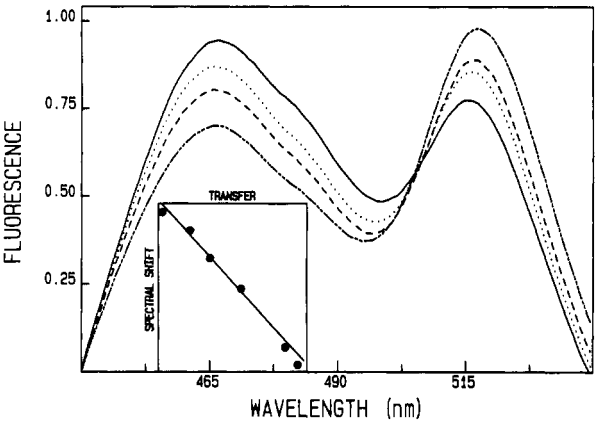

FIGURE 3: Solutions (5×10^{-7} M) of H_4 pig heart lactate dehydrogenase independently labeled were mixed and pressurized to 1.4 kbar (middissociation) at 0 °C for the following times: 0 (—); 3 h (···); 6 h (- - -); 14 h (— - - —). The spectra were determined after decompression. The inset shows the linear relation of the average wavenumber of the composite fluorescence (eq 15) with energy transfer, the latter measured by the resolved spectral contributions. The maximum spectral shift was 150 cm^{-1}, and the linearity with percent transfer was within ±7 cm^{-1}.

calculated gives a measure of the fraction transferred which agrees within a few percent with the value determined by the resolved spectral distributions.

Figure 4 shows the application of the homotransfer method by measurement of the fluorescence polarization of a conjugate of glyceraldehydephosphate dehydrogenase labeled with fluorescein isothiocyanate (three labels per tetramer) after mixing with a 10-fold excess of unlabeled protein. The enzyme activity of the conjugates was also ≈90%. Polarization of fluorescence under pressure was measured with a polarization photometer in the L-format configuration and corrected for window birefringency (Paladini & Weber, 1981b). Excitation was at 485 ± 5 nm; fluorescence filter, Corning 3-69 and 2-mm layer of 2 M $NaNO_2$. The difference in fluorescence polarization of the conjugate at atmospheric pressure ($p = 0.140$) and under complete dissociation (2.2 kbar, $p = 0.172$) characterizes the extent of energy transfer among the subunits in the initial aggregate. The points in Figure 4 are the polarizations followed over 8 h, after mixing at 0 °C labeled and unlabeled protein solutions in the ratio 1 to 10. The curve fitting the points is the sum of three exponential terms with relaxation times/amplitudes of 6 min/0.50, 98 min/0.25, and 235 min/0.25.

The exchange times τ_x for the dimers enolase and hexokinase and for the tetramers of lactate dehydrogenase and glyceraldehydephosphate dehydrogenase at two temperatures are gathered in Table II. The characteristic times for subunit exchange of the dimers of enolase and hexokinase were not conspicuously different than those for equilibration to half-dissociation. In the tetramers at room temperature, τ_x already exceeds significantly t_1, and at 0 °C, τ_x is longer than t_1 by over an order of magnitude.

The observation of this very slow monomer exchange of tetramers at 50% dissociation, in comparison with the relaxation of the dissociation, necessitates two conditions: (a) an appropriately slow rate of monomer reassociation into tetramers; (b) the tetramers remaining at 50% dissociation cannot dissociate at a rate any greater than permitted by the monomer association. Therefore, they are persistently different from those that dissociated when the pressure was initially raised to that of middissociation. Three factors must contribute to the observed very slow rate of monomer association into tetramers at low temperature: the reduction of the rate of as-

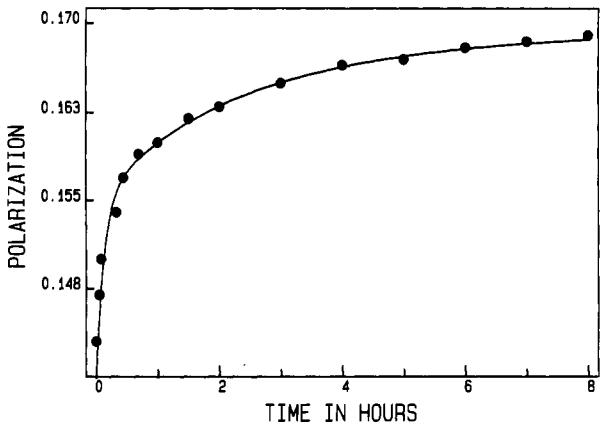

FIGURE 4: Time course of the fluorescence polarization at 1.4 kbar of a solution of yeast glyceraldehydephosphate dehydrogenase labeled with fluorescein isothiocyanate (three labels per tetramer) after mixing with a 10-fold excess of unlabeled solution and pressurization to 1.4 kbar, at 0 °C. The curve fitting the points is the sum of three exponential terms with relaxation times/amplitudes of 6 min/0.50, 98 min/0.25, and 235 min/0.25.

sociation by pressure (Figure 2); the facilitated conformational drift of the monomers by this cause (Xu & Weber, 1982; King & Weber, 1986; Silva et al., 1986; Weber, 1989); and the higher order of the association reaction that requires the formation of a reasonable stable dimer intermediate before regeneration of the tetramer. It is this high order of reaction that may be responsible for the increased importance of the effect of pressure on the rate of association in the equilibria of tetramers when compared to those of dimers. Together these factors convincingly explain the very slow rate of subunit assembly in the tetramers under pressure (condition a). We must, however, explain how, at the same time, the aggregate fails to dissociate beyond the equilibrium point prescribed by eq 2 (condition b). If the aggregate particles formed a homogeneous population as regards the free energy of association, we would expect them, in the absence of an equivalent opposing association reaction, to reach 100% dissociation at any pressure at which appreciable dissociation is achieved. In other words, the system would be unstable at any intermediate degree of dissociation, contrary to the experimental observation that intermediate degrees of dissociation under pressure are stable over many hours. We conclude that the aggregate must exist as a heterogeneous population of species with characteristic dissociation pressures, which in turn depend upon differences in the free energy and volume of association of each fraction. The existence of fractional differences in pressure stability is also clearly demonstrated by another observation employing the homotransfer method: decompression followed by return to 50% dissociation does not result in additional depolarization. Of the three relaxations shown in Figure 4, the faster one corresponds to the equilibration of tetramer and subunits with a characteristic time t_1 (eq 9) also revealed by other methods (King & Weber, 1986; Ruan & Weber, 1989). The second and third relaxations, respectively 16 and 39 times slower than the first, are to be interpreted as resulting from subunit exchange of the very heterogeneous remaining tetramer population. We surmise that at any pressure p all the fractions i of the population for which $p\Delta V_i + \Delta G_i \geq 0$ would be completely dissociated while those for which $p\Delta V_i + \Delta G_i < 0$ would remain unsplit. For further dissociation to occur at pressure p, fluctuations in ΔG must occur so that p-sensitive fractions are generated from the pool of undissociated tetramers, and τ_x must reflect the time necessary for the interconversion of these tetramer fractions with different free energies of association. We note that a broad distribution of free energies of association among the tetramers provides a valid quantitative explanation for the differences in ΔV_P and ΔV_C in tetramers (Ruan & Weber, 1989; Weber, 1989).

It is of great interest to understand how the thermodynamic relation of eq 2 comes to be followed not only in the typical stochastic and deterministic equilibria but also most importantly in the transitional case offered by the tetramer–monomer equilibria at different temperatures. A simple explanation is that the exchange between the tetramer fractions with different pressure susceptibility and the reassociation of the monomers depend upon a common cause: the motions of the contacting groups at the interfaces between subunits determine both the tetramer stability and the ease of reassociation of the monomers. At low temperature, they are sluggish and inhibit to a similar extent both the exchange between tetramer fractions of different stability and the adjustment of the monomer boundaries necessary to reconstitute the tetramer. At increasing temperatures, these motions become faster, and a typically stochastic equilibrium is approached.

Conclusions

Classically, the deterministic character of molecular processes observed on a macroscopic scale is expected to result from the statistics of a sufficiently large number of independent stochastic events, yet there is reason to expect that at some level in the biological organization determinism *at the molecular level* replaces stochastic behavior. Previous observations indicate that this is clearly the case in the pressure dissociation of many-subunit aggregates like virus capsids, hemerythrin, and hemocyanin, and the present observations show that the transition from stochastic to deterministic equilibrium is already apparent in the association of monomer subunits to form a tetramer.

The complex relations between the parts of a macromolecule appear to lead to a large restriction of the local molecular motions but only to an extent that permits what in the limit would give individual characteristics to each particle, sufficiently persistent to confer a deterministic character to some of the events in which the macromolecule participates.

Registry No. GAPDH, 9001-50-7; LDH, 9001-60-9; enolase, 9014-08-8; hexokinase, 9001-51-8; tryptophan synthase, 9014-52-2.

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