Physical Heterogeneity of Muscle Glycogen Phosphorylase Revealed by Hydrostatic Pressure Dissociation†

Kancheng Ruan‡ and Gregorio Weber*

Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: Four independent methods that employ fluorescence spectroscopy show that the tetramer of glycogen phosphorylase A (GPA) from rabbit muscle is reversibly dissociated into monomers by hydrostatic pressures under 2.5 kbar, if aggregation of the monomers is prevented by the addition of 8% glycerol. The free energy of association at 20 °C (–32 kcal mol^{-1}) depends upon a large entropy increase ($T\Delta S$ = +65 kcal mol^{-1}) that counteracts an unfavorable enthalpy of association of +33 kcal mol^{-1}. The association volumes calculated from the pressure dependence of the dissociation are nearly 4-fold smaller than those calculated from the shift in dissociation pressure with concentration. The dimer obtained by dilution of GPA at atmospheric pressure differs from the hypothesized dimer intermediate in the pressure dissociation by the much larger monomer affinity of the former. Like other tetramers, GPA shows hysteresis of the pressure profile upon decompression and conformational drift of the dissociated monomers. By use of the energy transfer method it is demonstrated that the relaxation time for half-dissociation (5 min) is over an order of magnitude shorter than that for subunit exchange (118 min). In all three tetramers studied, lactate dehydrogenase, glyceraldehyde phosphate dehydrogenase, and glycogen phosphorylase, the deterministic character of the dissociation equilibrium under pressure and the anomalous concentration dependence of the pressure dissociation demonstrate that these tetramers are heterogeneous populations with regard to their free energy and/or volumes of association.

Numerous observations on the properties of the multisubunit proteins indicate that, depending on the circumstances attending the association of the constitutive monomers, oligomeric aggregates show demonstrable differences in their energetics, physical properties, and enzymic activity. Differences in crystal structure according to the preparation of the sample (Liddington et al., 1988; Silva et al., 1992), reversible changes in enzyme activity and spectroscopic properties owing to long standing in the cold or after pressure dissociation (King & Weber, 1986a,b), and deterministic character of the monomer–aggregate equilibria (Erijman & Weber, 1991) have been described. They are indicative of the existence of heterogeneous molecular populations with members separated from each other by considerable potential barriers (Erijman & Weber, 1993). It has become increasingly clear that many of the differences in properties of a given oligomeric aggregate recorded in the literature can no longer be dismissed as artifacts of their preparation but arise from the very properties that permitted their assembly from the monomers in the first place (Weber, 1990). Previous observations employing the dissociation of aggregates by hydrostatic pressures have demonstrated that the complexity of the equilibria and the heterogeneous character of the aggregates increase with the number of monomers that associate together, from dimers to multimers like hemocyanin or erythrocruorin, and even more complex self-assembling structures like the virus capsids. Tetramers are particularly interesting in that they are the simplest aggregates in which most of the properties described above are evident. The observations on glycogen phosphorylase presented here confirm the generality of phenomena already observed in the tetramers of glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase and note also the existence of differences with these other cases.

THEORY

The methods of fluorescence spectroscopy that were used to determine the degree of dissociation under pressure have been described in previous papers: polarization of the intrinsic protein fluorescence (Silva et al., 1986; Ruan & Weber, 1988), spectral shift of the protein fluorescence (Silva et al., 1986; King & Weber, 1986a,b; Ruan & Weber, 1989), and fluorescence polarization of fluorophores of appropriate lifetime covalently attached to proteins (Paladini & Weber, 1981a, King & Weber, 1986a). In the present instance we have also determined the degree of dissociation through the increase in fluorescence polarization observed when dissociation of an aggregate labeled with fluorescein isothiocyanate suppresses the electronic energy transfer among fluorescein groups attached to different subunits (Erijman & Weber, 1991, 1993).

The theory involved in the derivation of the thermodynamic parameters of interest, the standard free energy of subunit association, ΔG, and the standard change in volume upon association, ΔV, are described in the above-mentioned papers. We need only recall here the relations between the applied pressure p, the degree of dissociation α, the concentration of protein as aggregate C, and the characteristic concentration $C_{1/2}$ at which $\alpha = {}^1/_2$. For a dimer–monomer equilibrium (D ↔ M) these are

$$f(\alpha) \equiv \ln [(\alpha^2/(1-\alpha)] = \ln (C_{1/2}/2C) + p\Delta V/RT \quad (1)$$

$$K_2 = \exp(\Delta G/RT) = 2C_{1/2} \quad (2)$$

and for a tetramer–monomer equilibrium (T ↔ M) *that*

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‡ On leave from Shanghai Institute of Biochemistry, Academia Sinica, 320 Yue-yang Rd., 23000 Shanghai, China.

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involves no appreciable participation of an intermediate tetramer–dimer (T ↔ D) step

$$f(\alpha) \equiv \ln [\alpha^4/(1-\alpha)] = 3 \ln (C_{1/2}/2C) + p\Delta V/RT \quad (3)$$

$$K_4 = \exp(\Delta G/RT) = 32C_{1/2}^3 \quad (4)$$

K_2 and K_4 are, respectively, the dissociation constants of the dimer and tetramer into monomers. ΔV may be obtained from the slope of the plot of $f(\alpha)$ against pressure (eqs 1 and 3) or simply from the pressure span δp between the values of $\alpha = 0.1$ and $\alpha = 0.9$, employing the relation

$$\Delta V_P = nRT \ln (9)/\delta p \quad (5)$$

with $n = 3$ (dimer) or $n = 5$ (tetramer). ΔV may also be derived from the displacement of $f(\alpha)$ with concentration along the pressure axis. For two different concentrations C_1 and C_2, of which the pressures at $\alpha = {}^1/_2$ are p_1 and p_2, respectively, eqs 1 and 3 give

$$\Delta V_C = RT \ln (C_2/C_1)^m/(p_2 - p_1) \quad (6)$$

where $m = 1$ for the dimer and $m = 3$ for the tetramer. The subscripts P and C added to ΔV in eqs 5 and 6 serve to distinguish the two methods of determination of ΔV, by the dependence of the dissociation upon the pressure at constant concentration and by the dependence of the dissociating pressure upon the concentration at constant degree of dissociation, respectively.

MATERIALS AND METHODS

Glycogen phosphorylase A from rabbit muscle (GPA)[1] was from Boehringer Mannheim (Lot 108 651). The enzymes were dissolved in the standard buffer: 0.05 M Tris, pH 7.5, 0.001 M in both EDTA and DTT. Particulate matter of size greater than 2 μm was removed by a syringe filter. Protein concentration was determined spectrophotometrically using a specific absorptivity at 280 nm of 12.7 OD units for 1-cm depth of 1% solution (Graves & Wang, 1972). Enzyme activity was assayed by the method of Illingworth and Cori (1953). At 30 °C and pH 6.8 the specific activity was found to be 79 units/mg, one unit being defined as the amount of enzyme causing the release of 1 μmol of inorganic phosphate from glucose 1-phosphate per minute. AMP, glycogen, and glucose phosphate were from Sigma; 2-(dimethylamino)naphthalene-5-sulfonyl chloride (DNS-Cl), 1-anilinonaphthalene-8-sulfonate (ANS), succinimidopyrene butyrate (SPB), and fluorescein 5-isothiocyanate (FITC) were from Molecular Probes. All other reagents were of analytical grade. Distilled water was further purified by a Millipore system to a resistance of 18 MΩ.

Labeling of proteins with FITC was carried out by incubation of the protein for 1 h at room temperature with 10-fold molar proportion of the isothiocyanate in the standard buffer. Unreacted dye was removed by passage through Sephadex G-25 followed by dialysis against the standard buffer for 3 h. The concentration of dye bound to the enzyme was determined on the basis of a molar absorptivity of 63 000 cm²/mmol at 492 nm. Protein concentration of the FITC conjugates was determined by absorptivity at 278 nm, after subtracting the contribution by the fluorophore at this wavelength on the basis of a molar absorptivity of 21 000 cm²/mmol. A ratio of dye to protein ≃4, or an average of 1 per subunit, was aimed at obtaining the largest possible depolarization by energy transfer between fluorophores attached to different subunits. Pressurization was carried out as described by Paladini and Weber (1981a,b) and fluorescence polarization under pressure was measured employing the L format of the polarization photometer and the corrections for depolarization by birefringency there described. Absorption spectra were determined with a DU70 Beckman spectrophotometer; fluorescence spectra, with a Hitachi 3010 spectrometer. The kinetics of fluorescence changes was followed with a multichannel diode spectrometer (OSMA, Princeton Instruments). Electrophoresis at high pressure was carried out in the apparatus of Paladini et al. (1987) with the modifications described by Erijman et al. (1993).

Previous attempts to study the pressure dissociation of phosphorylase A (P. Tauc, unpublished results) were unsuccessful; the separated subunits aggregated readily and a precipitate was uniformly observed on decompression. In view of the general stabilizing action of glycerol upon proteins in solution (Gekko & Timasheff, 1981a,b), its effect was tested, and it was found that 8% glycerol (v/v) was sufficient to prevent precipitation under pressure and to ensure reversibility after decompression. All the results described below refer to solutions in the standard buffer with addition of that amount of glycerol.

RESULTS AND DISCUSSION

Dissociation of GPA Tetramer. SDS–PAGE was carried out by the procedure of Laemmli (1970), in the presence of 2% SDS and 2% mercaptoethanol. Heavily loaded samples of the muscle glycogen phosphorylase A employed showed a single compact band. Electrophoresis on Sepharose, performed at room temperature at 1.4 kbar (Paladini et al., 1987) without addition of glycerol, showed two well-defined bands that we assigned, from the experimental evidence described below, to the tetramer and monomers. The proportions of the two bands changed at 2 kbar as expected from the larger dissociation. A very small fraction of the protein that did not undergo migration was observed. This fraction was somewhat larger at 2 kbar than at 1.4 kbar.

Figure 1 shows the polarization of the intrinsic fluorescence of two GPA solutions of concentrations 2.6 and 0.26 μM subjected to increasing pressure as well as the decreasing pressure profile of the latter solution. Plots of $f(\alpha)$ according to eq 3 give $\Delta V_P = 199$ mL mol^{-1} for the higher concentration and 224 mL mol^{-1} for the lower one. Equation 5 gives for the two cases, respectively, $\Delta V_P = 217$ and 234 mL mol^{-1}. The displacement of the curves with concentration is 193 bar, while the theoretical displacement expected from eq 6 is 818 bar. The small influence of concentration results in $\Delta V_C = 791$ mL/mol, nearly 4 times larger than ΔV_P. The anomalous concentration dependence is obvious in Figure 2, which shows plots of the degree of dissociation and of ln $[f(\alpha)]$ against ascending pressure for the data of Figure 1. The full lines are theoretical for a tetramer with $\Delta V_P = 220$ mL/mol and the dashed lines are those expected when the concentration is decreased by a factor of 10. This discrepancy, observed also in solutions of LDH and GAPDH, was ascribed by Ruan and Weber (1989) to heterogeneity of the tetramer population and is dealt with in detail elsewhere (Weber, 1991; Erijman & Weber, 1991). The changes in molecular volume on

[1] Abbreviations: GPA, glycogen phosphorylase A; GAPDH, glyceraldehyde phosphate dehydrogenase; LDH, lactate dehydrogenase; DNS, 2-(dimethylamino)naphthalene-5-sulfonic acid; SPB, succinimdopyrene butyrate; FITC, fluorescein isothiocyanate; ANS, 1-anilinonaphthalene-8-sulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

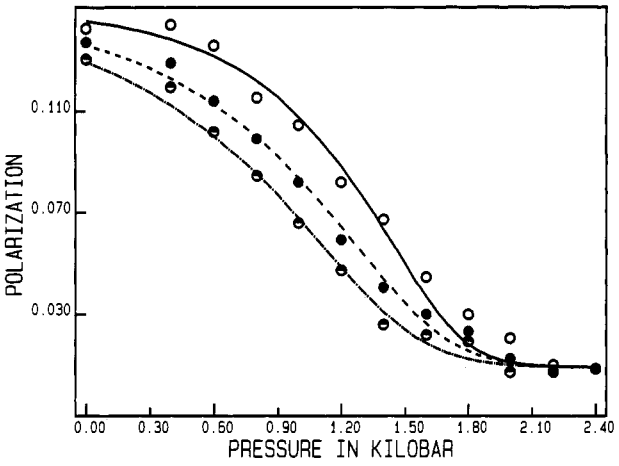

FIGURE 1: Polarization of the intrinsic fluorescence of GPA A at 2 °C. Excitation at 280 nm, emission detected through 0–54 Corning filter. Open circles: 2.6 μM solution at increasing pressures. Filled circles are for increasing pressure, and half-filled circles for decreasing pressures, for 0.26 μM solution. Curves are fittings by eq 3. (—) ΔV = 197 mL/mol, $C/C_{1/2(atm)}$ = 40; (- - -) ΔV = 150 mL/mol, $C/C_{1/2(atm)}$ = 26; (- · -) ΔV = 160 mL/mol, $C/C_{1/2(atm)}$ = 10.

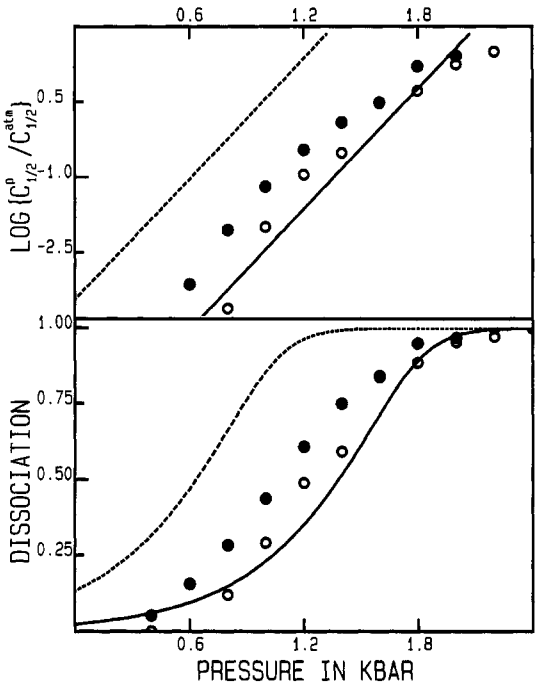

FIGURE 2: Data of Figure 1 for increasing pressures. Lower panel: Degree of dissociation vs pressure for a tetramer with ΔV = 220 mL mol^{-1}. The solid line is the theoretical, by eq 3 for $C_{1/2}/C_{1/2(atm)}$ = 70; the dashed line is for $C_{1/2}/C_{1/2(atm)}$ = 7. Upper panel: Linearized logarithmic plots, eq 3, of the same data.

association are, respectively, $\Delta V_{Pmol} = \Delta V_P/N$ = 380 Å^3 and $\Delta V_{Cmol} = \Delta V_C/N$ = 1320 Å^3. The surface of contact of the subunits in the tetramer, calculated from the $^2/_3$ power of the anhydrous volume of the subunits, is approximately 2900 Å^2 so that the linear contraction on dissociation equals 0.13 Å if calculated from the slope of the pressure profile and 0.45 Å if calculated from the shift in pressure profile with concentration. We recall that in the cases of hexokinase and β_2 tryptophan synthase, in which ΔV_P and ΔV_C are concordant values, the linear contraction of the subunits in contact with solvent is 0.4–0.5 Å. From this observation we conclude that ΔV_C = 791 mL mol^{-1} must be close to the actual volume change upon dissociation and that the anomalously small ΔV_P reflects the heterogeneity of the tetramer population.

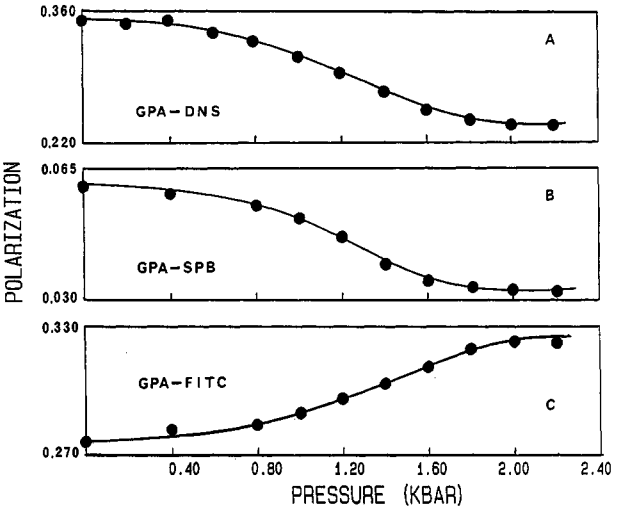

FIGURE 3: Demonstration of the concordance of the dissociation data of three methods that employ fluorescence polarization. (A) Dansylated GPA excited at 380 nm, emission detected through Corning filter 3–73. (B) Conjugate with succinimdopyrene butyrate excited at 340 nm, emission detected through Corning 0–52 and 3-mm layer of 2 M $NaNO_2$. (C) Fluorescein conjugate ($\simeq$4 labels/tetramer) excited at 480 nm, emission detected through 3–69 Corning filter and $NaNO_2$. In the two upper plots the decrease in polarization follows the change in macromolecular volume; in the lower plot the increase in polarization follows the diminishing energy transfer on dissociation.

Table I: Free Energy of Association and Standard Volume Change on Association from Dissociation of Glycogen Phosphorylase A by Hydrostatic Pressure

method[a]	C (μM)	ΔV_P (mL/mol)	$C_{1/2}$ (nM)	ρ_4[b] (ns)	ρ_1[c] (ns)	ρ_4/ρ_1
		(1) Tetramer–Monomer Equilibria				
A	0.26	199	10.8	414	132	3.2
A	2.61	234	32.0			
B	0.34	226	9.6			
B[d]	0.34	231	5.8			
B[e]	0.34	200	17.3			
C	0.34	240	9.1	455	98	4.6
D	0.35	195	10.5			
		(2) Dimer–Monomer Equilibria				
A	0.017	106	0.0273			
B	0.017	114	0.0404	240	94	2.5

[a] Method A = intrinsic fluorescence polarization; method B = polarization of dansyl conjugates; method C = polarization of pyrene butyrate conjugates; method D = polarization of fluorescein conjugates. All measurements at 2 °C. [b] ρ_4 = rotational relaxation time from polarization at atmospheric pressure and fluorescence lifetime. [c] ρ_1 = rotational relaxatin time from polarization at 2.4 kbar. [d] 0.05 M glucose added. [e] 0.001 M AMP added.

Like in previous cases, confirmation of the dissociation was sought by applying different methods: the pressure dependence of the rotational depolarization of the fluorescence of pyrene butyryl and dansyl conjugates and the increase in polarization upon dissociation of heavily labeled fluorescein conjugates of the protein. The general agreement of these three methods is illustrated in Figure 3. Rotational relaxation times were determined from Perrin's equation (Weber, 1952):

$$\rho = 3\tau(1/p_0 - {}^1/_3)/[(1/p) - (1/p_0)] \qquad (7)$$

where ρ is the Debye rotational relaxation time, p and p_0 are the observed and limiting polarizations, respectively, the τ is the fluorescence lifetime. Derived values of ΔV_P and ΔG are gathered in Table I.

No appreciable spectral shift of tryptophan was observed at the pressure of 2.4 kbar, at which all the other methods

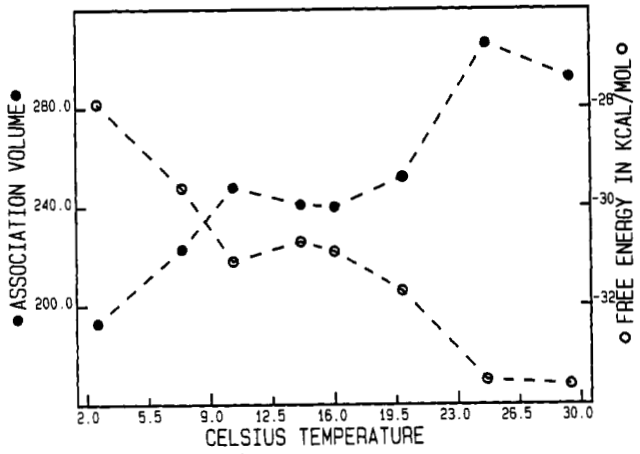

FIGURE 4: Association volumes ΔV and free energies ΔG in the temperature range of 2–30 °C. In the range of 10–20 °C smaller changes in ΔV and ΔG are observed. The discontinuous line joining neighbor points draws attention to the correlation between changes in free energy and volume.

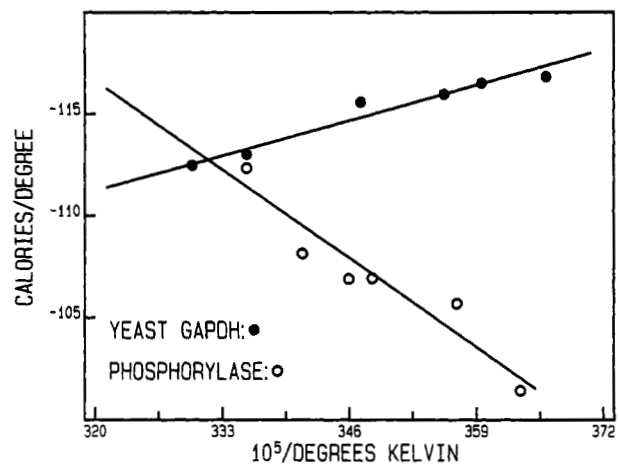

FIGURE 5: van't Hoff plots ($\Delta G/T$ vs $1/T$) for yeast GAPDH (Ruan & Weber, 1989) and GPA. Note that the plot minimizes the lack of uniformity of the temperature data of GPA shown in Figure 4.

demonstrated complete dissociation, and a similar lack of spectral change was apparent when the tetramer protein was sufficiently diluted to dissociate into dimers (see below). This spectral insensitivity indicates that the environment of the tryptophan residues responsible for the bulk of the fluorescence does not change in polarity upon dissociation and therefore that those tryptophan residues do not participate in the subunit boundary, although it is evident from the data of Figures 1 and 6 that the amplitude of the local rotations of these residues increases considerably in the monomer.

The pressure dissociation of solutions of GPA in the temperature range of 2–30 °C was investigated and the derived thermodynamic parameters ΔV_P and dG are plotted in Figure 4. In the range of 12–18 °C these parameters appear to vary slowly in comparison with the more rapid changes that take place between 2 and 12 °C and also between 18 and 30 °C. The protein stability measured by ΔG increases with temperature, but the simultaneous increase in volume of association results in a compensatory decrease of the stability toward dissociation by hydrostatic pressure. As a result, the dissociation of the protein at a fixed concentration remains approximately in the same range of pressures, regardless of temperature. While we expect the free energy and volume of association not to be drastically changed by the addition of 8% glycerol, it is not at all unlikely that the complex variation of ΔG and ΔV with temperature observed in this case may be due to some extent to the glycerol component. Figure 5 is a van't Hoff plot ($\Delta G/T$ against $1/T$). From this plot of the data of Figure 4, which minimizes the lack of uniformity of the temperature changes shown in that figure, we estimate the enthalpy change upon association $\Delta H = +33$ kcal/mol. This large positive value is indicative that the enthalpy of hydration of the subunit surfaces exposed on dissociation exceeds the sum of the energies of intersubunit bonds and solvent–solvent bonds formed on association. The aggregate is maintained by the large entropy increase on association, $T\Delta S$, which at 25 °C reaches +65 kcal/mol. These data must be contrasted with those obtained for GAPDH (Ruan & Weber, 1989) for which $\Delta H = -14$ kcal/mol and $T\Delta S = +19$ kcal/mol; data shown also in Figure 5. We note that in the neighborhood of 300 K the corresponding free energies of association are virtually equal for both proteins.

In all protein aggregates so far studied, the large and positive entropy of association is responsible for the large negative ΔG. We have previously addressed the question of the partition

Table II: Enthalpy Change on Association and Change in Association Volume with Temperature for Several Oligomers

protein	temp interval (°C)	enthalpy of association (kcal/mol)	volume change (mL/mol)
GAPDH	0–25	−14.0	−86
hexokinase	0–20	+9.0	+46
GPA tetramer	0–25	+33	+80
GPA dimer	0–20	+5.3	+18

of this large entropy increase between contributions owing to separate entropy changes in solvent and protein upon association (Ruan & Weber, 1988, 1989); the large difference observed between the entropies of association of GPA and GAPDH cannot be readily attributed to solvent contribution, and the largest part of the total must come from the larger entropy of the protein aggregate as compared to the separated subunits. This view is developed at length elsewhere (Weber, 1993).

Data for the enthalpy changes observed in β_2 tryptophan synthase, hexokinase, GAPDH, and GPA are gathered in Table II. Enthalpies of association can evidently be negative, as in GAPDH, or positive, as in the other cases. The thermal coefficient of ΔV, $d\Delta V/dT$, is positive if $\Delta H > 0$ and negative if $\Delta H < 0$, so that in the four cases shown in the table the product $\Delta H(d\Delta V/dT) > 0$. In the interval of 2 and 25 °C, $d\Delta V/d\Delta G = -26$ mL kcal^{-1} in GPA, while for GAPDH $d\Delta V/d\Delta G = +18$ mL kcal^{-1} (Ruan & Weber, 1989). Tentatively, we offer the following explanation of the influence of the sign of the enthalpy in the thermal coefficient of the association volume: If we assume that a negative enthalpy results from an important contribution to it from specific *intersubunit* bonds (hydrogen and electrostatic) that limit the approach of the subunits at other places, a decrease of these bonds by temperature would permit increasing approach of the subunit surfaces and a diminution of ΔV. If the enthalpy is positive and therefore the directed bonds are relatively unimportant, the opposite effect would happen because the unhindered dispersion forces will permit maximal subunit approach already at the lower temperatures.

Dissociation of Phosphorylase Dimer into Monomers. At a concentration of 20 nM, phosphorylase A is in the form of dimers (Wang & Graves, 1964). The dissociation of the tetramer into dimers was verified by the decrease in polarization of dansylated GPA by dilution from a value of 0.348 at $C = 340$ nM to a plateau of 0.315 at $C = 17$ nM, a change

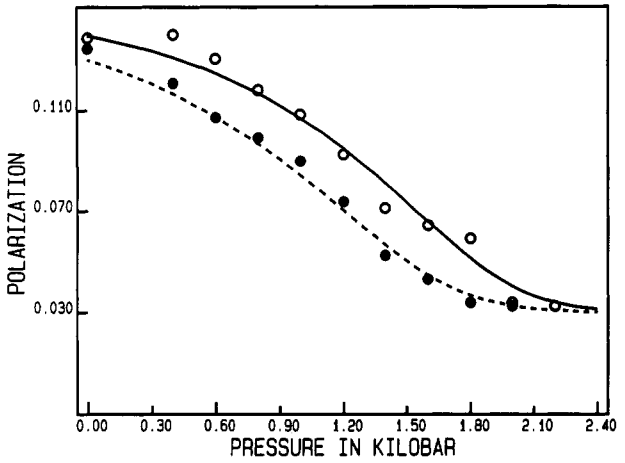

FIGURE 6: Intrinsic fluorescence polarization of 20 nM GPA, effectively in the dimer form, against pressure at 2 °C. Open circles, increasing pressures; filled circles, decreasing pressures. The curves are the theoretical for a dimer dissociation (eq 1). (—) $\Delta V = 130$ mL/mol, $C/C_{1/2(atm)} = 3000$; (- - -) $\Delta V = 120$ mL/mol, $C/C_{1/2(atm)} = 250$. Note the similarity of the range of polarizations with the tetramer, shown in Figure 1.

owing to a decrease in 1.8 times in rotational relaxation time. The dissociation constant of the tetramer into dimers reported by Huang and Graves (1970), 86 nM, agrees well with the dissociation range observed by fluorescence polarization. Figure 6 shows the pressure dependence of the intrinsic polarization of the fluorescence of a 17 nM solution of phosphorylase A. A plot according to eq 2 gives $\Delta V_P = 106$ mL mol^{-1} and $C_{1/2} = 27$ pM. We note that the changes in the polarization of the intrinsic fluorescence in the dimer occur in the same range of those of the pressure dissociation of the tetramer (Figure 1), indicating that the local rotations of the tryptophan are equivalent in dimers and tetramer. DNS conjugates of similar concentration give $\Delta V_P = 114$ mL mol^{-1} and $C_{1/2} = 40$ pM. The rotational relaxation times, 240 and 96 ns, are reasonable for a dimer and monomer of the known masses. The observed volume changes in tetramer and dimer are in the ratio of 2:1, in agreement with a similar ratio of the total contact areas of tetramer and dimer reported by Barford and Johnson (1992). From $C_{1/2}(T \leftrightarrow D) = 4.8 \times 10^{-8}$ and $C_{1/2}(D \leftrightarrow M) = 4 \times 10^{-11}$, we obtain $C_{1/2}(T \leftrightarrow D)/C_{1/2}(D \leftrightarrow M) \geq 1000$, implying that the pressure dissociation profile of the tetramer ought to consist of two well-separated steps, but the three different methods illustrated in Figure 3 do not show any indication of their existence. In fact, the data of Figure 3, *by themselves*, require $C_{1/2}(T \leftrightarrow D)/C_{1/2}(D \leftrightarrow M) \leq 0.1$. The simplest, and probably the only, explanation is to be found in the differences between the dimer species generated by dilution on one hand and hydrostatic pressure on the other. Specifically, the dimer generated by hydrostatic pressure dissociation must have a free energy of monomer association much smaller, in absolute value, than that generated on dilution. These differences in free energy of association must reflect specific boundary differences between the dimers obtained by hydrostatic pressure and by dilution. This view agrees with the observations of Barford and Johnson (1992) of the existence of large differences in the intersubunit contacts of dimer and tetramer in the crystals. We expect that on application of pressure the tetramer splits into monomers which are very similar in conformation to those within the tetramer. Failure to attain a conformation similar to that of the atmospheric pressure dimer could be due to either the stabilization of the water–subunit interface by the pressure or by a rate of rearrangement too slow to become significant within the time of the experiments. We note that a dimer intermediate was not detected in the pressure dissociation of any of the tetramers that have been studied. The dissociation of the dimer of GPA under pressure was studied at two temperatures: We obtained $\Delta V = 106$ mL mol^{-1} and $\Delta G = -13.0$ kcal mol^{-1} at 2 °C and $\Delta V = 124$ mL mol^{-1} and $\Delta G = -14.2$ kcal mol^{-1} at 20 °C. The enthalpy change was estimated from

$$\Delta H = \Delta G_1 - dG(T/\delta T) \quad (8)$$

where ΔG_1 is the free energy of association at the lower temperature T, $\Delta G_1 + dG$ is the free energy of association at the higher temperature, and δT is the temperature differential. We thus obtain $\Delta H = +5.3$ kcal/mol and $T\Delta S = +19.7$ kcal/mol.

Table III: Free Energies and Volumes Derived from Dissociation Recorded at Increasing and Decreasing Pressures

	increasing pressures		decreasing pressures	
temp (°C)	ΔG (kcal/mol)	ΔV (mL/mol)	ΔG^* (kcal/mol)	ΔV^* (mL/mol)
2	−28.1	185	−28.5	239
10	−31.5	227	−30.7	235
16	−31.2	222	−31.2	240
25	−33.9	271	−32.2	226

Hysteresis Effects of Pressurized Phosphorylase. Figure 1 shows that the polarizations recorded during increasing pressure at 2 °C were systematically larger than those recorded during decreasing pressure, so that some free energy of association appears to be lost after complete dissociation. At temperatures greater than 16 °C the decompression curve was shifted to *higher* pressures relative to the compression curve, and at 16 °C the two curves virtually coincided. The lack of complete reversibility of the pressure dissociation has been seen in some dimers and in all the tetramer proteins so far studied by this method. From this and other features it has been concluded that separation of the monomers leads to their "conformational drift". Reassociation on release of pressure results in an aggregate with new characteristic thermodynamic parameters, ΔV^* and ΔG^* [see Weber (1987, 1991) for reviews]. In phosphorylase A, unlike lactate dehydrogenase and glyceraldehyde phosphate dehydrogenase, the relation of the compression and decompression curves varies considerably with temperature. Table III shows free energies and volumes of association obtained from data of dissociation under increasing (ΔG and ΔV) and decreasing (ΔG^* and ΔV^*) pressure. These data show that ΔV has a large temperature coefficient while ΔV^* has a barely detectable one. Although the drifted form has a smaller free energy of association than the native form, it is stable at low temperature, just like LDH and GAPDH, in which conversion into this form is responsible for the cold inactivation (King & Weber, 1986; Ruan & Weber, 1989).

The differences between native GPA and the protein subjected to a cycle of compression and decompression can be demonstrated in several ways: The difference absorption spectrum (pressurized − native) shows an increase at 238 nm which can be attributed to a loss of hypochromicity of amide bonds, perhaps due to a decrease in effective helical content in the pressurized sample. Both native and pressurized protein bind 1-anilinonaphthalene-8-sulfonate, but fluorescence enhancement by the pressurized material was found to be 3-fold larger than for the native intact protein, when employing equal protein and dye concentration. Similar changes are observed in pressurized GAPDH (Ruan & Weber, 1989). The loss of

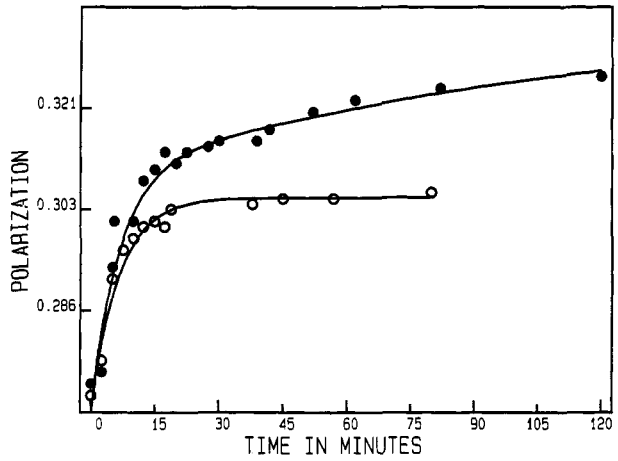

FIGURE 7: Time dependence of the polarization of 0.26 μM fluorescein–GPA conjugate of Figure 3, after a rapid rise in pressure to 1.4 kbar, at 2 °C. Filled circles, in the presence of a 10-fold excess of unlabeled GPA; open circles, by itself.

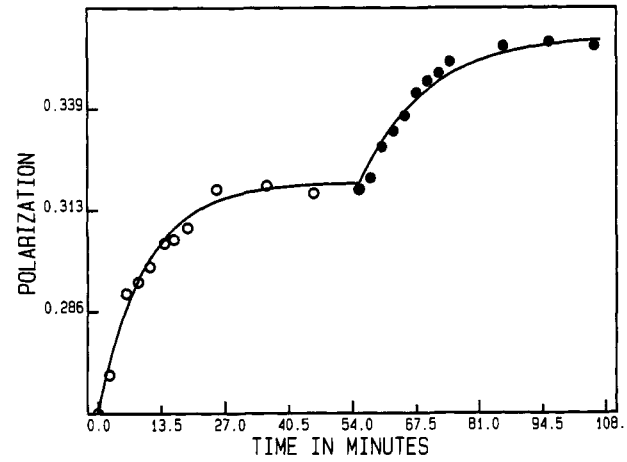

FIGURE 8: Time dependence of the polarization of 0.26 μM fluorescein–GPA conjugate subjected to two successive pressure increases: atmospheric to 1.4 kbar (open circles) and 1.4 to 2.4 kbar (filled circles). The corresponding relaxation times were T_1 = 9.0 min and T_2 = 14 min.

enzyme activity in GPA after pressurization is generally less than in GAPDH: After incubation at the dissociation pressure for 1 h at 2, 16, and 25 °C, the losses of enzyme activity were respectively 37%, 20%, and 17% of the original. Thus the pressure cycle results in tetramers with modified properties even at 16 °C. At this temperature an apparent absence of hysteresis is observed owing to the similarity of the thermodynamic parameters of the native and drifted species (Table III).

Character of the Equilibrium between GPA Tetramer and Subunits. Erijman and Weber (1991) have studied the subunit exchange in the tetramers of LDH and GAPDH by the use of energy transfer among subunits labeled with the same or different fluorophores. If the protein carries a fluorescein label in each subunit (homotransfer method) the polarization of the fluorescence is always lower than in tetramers labeled at only one subunit because a fraction of the excitations is emitted as fluorescence after transfer to a fluorophore that was not originally excited. Separation of the subunits by dissociation, or replacement of the labeled subunits for unlabeled ones, increases the fluorescence polarization toward that observed in the absence of energy transfer. The corrections owing to the different rotational rates of monomer and tetramer are very small in this case: The fluorescein lifetime is 4 ns, and with the ρ values of Table I, $3\tau/\rho = 0.03$ for the tetramer and 0.12 for the monomer. With such heavily labeled preparations of GAPDH, Erijman and Weber (1991) showed that when pressure is rapidly raised from atmospheric to that required for half-dissociation, the polarization increase shows two very different relaxations of about equal amplitude: the rapid one (minutes) corresponds to the dissociation of the tetramers and is followed by a second relaxation that requires several hours for completion. This latter relaxation must be assigned to the slow subunit exchange of the remaining tetramers. A similar result was observed with LDH employing energy transfer between different fluorophores. Use of the energy transfer method with GPA for the same purpose is demonstrated in Figure 7: When a solution of GPA labeled with ≈4 mol of fluorescein is subjected to a pressure that is known to result in half-dissociation (open circles), a rapid rise in polarization to a stable value is seen. It fits well a single exponential with a relaxation time of 5.5 min. When the same material is pressurized to the same extent in the presence of a large excess of unlabeled material, a secondary slow rise in polarization is observed (filled circles). The fast increase in polarization is completed in some 15 min and is followed by a much slower increase over the next 2 h. The points are fitted by a sum of two exponential terms with amplitudes of 0.59 and 0.41 and relaxation times of 5.5 and 118 min, respectively. The latter time is assigned to the exchange of labeled for unlabeled subunits in the undissociated tetramers, at equilibrium. From these observations we conclude that in GPA, just like in GAPDH and LDH, the rate of subunit exchange at equilibrium is at least an order of magnitude slower than the rate of attainment of the equilibrium. It follows that the population must be a very heterogeneous one with regard to the free energy of association and that the equilibrium between aggregate and subunits has deterministic rather than stochastic character. We note that the large difference between ΔV_C and ΔV_P provides independent evidence of the heterogeneity of the GPA tetramer population with regard to the pressure dissociation characteristics.

As shown by Erijman and Weber (1991), it is possible to estimate the relative effects of compression on the rates of association and dissociation by a measurement of the relaxation times, T_1 and T_2, after two fast, successive increases in pressure. The first increase, applied at atmospheric pressure, is calculated to raise the dissociation to nearly 50%. After this equilibrium is reached, pressure is raised once more to bring about almost complete dissociation. Log (T_1/T_2) is linearly related to the factor f, $(0 < f < 1)$, giving the relative weight of the increase in the rate of dissociation to the larger dissociation constant produced by a pressure increase. Figure 8 shows the time course of the fluorescence polarization of GPA multiple-labeled with fluorescein after two successive pressure increases. Log $(T_1/T_2) = -0.192$ from which f = 0.11, corresponding to a large predominance of the decrease in the rate of association as compared to the increased dissociation rate. In the case of a dimer, this experimental procedure is unequivocal in clearly assigning complementary fractions of the change of dissociation constant with pressure to the rates of association and dissociation involved. However, in a tetramer that dissociates into monomers, the influence of the pressure in the association, as determined by the described procedure, involves the entire set of processes by which the tetramer is reassembled, and this comprises not only the rates of association of monomers into dimers, trimers, and tetramer but also the rates of dissociation of the intermediate dimers and trimers. In agreement with the observations on dimers it seems much more likely that the association of particles of all orders is not appreciable influenced by pressure and that

the apparent observation to the contrary in tetramers is best explaned by the increased rate of dissociation under pressure of the intermediates in the association, dimers and trimers. The difference in stability of the dimers of GPA generated by dilution and by hydrostatic pressure examined above definitely points to the same effect.

CONCLUSIONS

The observations on the dissociation of the phosphorylase tetramer and dimer permit us to considerably reinforce the conclusions that we have previously reached by the study of these types of aggregates in previous work (King & Weber, 1986; Silva et al., 1986; Ruan & Weber, 1988, 1989; Erijman & Weber, 1991):

(1) Separation of the subunits leads to time-dependent changes in subunit affinity, spectroscopic properties, and enzyme activity that are attributed to a complex "conformational drift" that affects the parts of the molecule to different extents.

(2) The increase in entropy upon association is responsible for the stability of all the oligomers studied, be they dimers or tetramers. The enthalpy of association may be negative or positive, and the corresponding very high variation of the entropy from case to case is taken as an indication that the entropy contribution originates predominantly from changes in the protein and not in the solvent, as has been often proposed. [For a comparison of these contrasting opinions see Ben-Naim (1980) and Weber (1993).]

(3) The experiments that employ two successive relaxations show that in tetramers the promotion of dissociation by pressure is largely due to the apparent decrease in the rate of association, probably reflecting the increased rate of dissociation of the intermediate dimers or trimers under pressure rather than a hindered association. It may be expected that the importance of this effect increases with the number of subunits and the complexity of their arrangement in the oligomer. In agreement with this view, the dimer of GPA produced by dilution of the tetramer has a considerably larger free energy of association in comparison with the hypothesized dimer intermediate in the dissociation by hydrostatic pressure.

(4) The anomalous dependence of the pressure dissociation upon the tetramer concentration, and above all the experiments showing that the time for exchange of subunits in the half-dissociated population exceeds by more than an order of magnitude the time for the dissociation equilibration, show conclusively that the tetramer population is heterogeneous with regard to its free energy of association or association volume or both. This heterogeneity seems an inevitable consequence of the conformational drift of the separated subunits in equilibrium with the aggregate (Ruan & Weber, 1989).

Questions for the future include the character, compact or discontinuous, of the distribution of the tetramer population, the nature of the interactions that determine the remarkable persistence of the different tetramer conformations at low temperature, and most important, a clarification of the sources of the entropy that is responsible for the stability of the protein aggregates.

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REFERENCES

Barford, D., & Johnson, L. N. (1992) *Protein Sci. 1*, 472–493.
Ben-Naim, A. (1980) *Hydrophobic interactions*, pp 220–260, Academic Press, New York.
Erijman, L., & Weber, G. (1991) *Biochemistry 30*, 1595–1599.
Erijman, L., & Weber, G. (1993) *Photochem. Photobiol. 57*, 411–415.
Erijman, L., Paladini, A. A., Lorimer, G. H., & Weber, G. (1993) *Biochemistry* (submitted for publication).
Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667–4676.
Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677–4686.
Graves, D. J., & Wang, J. H. (1972) *The Enzymes VII* (Boyer, P. D., Ed.) pp 435–482, Academic Press, New York.
Huang, C. Y., & Graves, D. J. (1970) *Biochemistry 9*, 660–671.
Illingworth, B., & Cori, G. T. (1953) *Biochem. Prep. 3*, 1–9.
King, L., & Weber, G. (1986a) *Biochemistry 25*, 3632–3637.
King, L., & Weber, G. (1986b) *Biochemistry 25*, 3637–3640.
Laemmli, U. K. (1970), *Nature 227*, 680–685.
Liddington, R., Derewenda, Z., Dodson, G., & Harris, D. (1988) *Nature 331*, 725–728.
Paladini, A. A., & Weber, G. (1981a) *Biochemistry 20*, 2587–2593.
Paladini, A. A., & Weber, G. (1981b) *Rev. Sci. Instrum. 52*, 419–427.
Paladini, A. A., Silva, J. L., & Weber, G. (1987) *Anal. Biochem. 161*, 358–364.
Ruan, K., & Weber, G. (1988) *Biochemistry 27*, 3295–3301.
Ruan, K., & Weber, G. (1989) *Biochemistry 28*, 2144–2153.
Silva, J. L., Miles, E. W., & Weber, G. (1986) *Biochemistry 25*, 5781–5786.
Silva, M. M., Roger, P. H., & Arnone, A. (1992) *J. Biol. Chem. 267*, 17248–17256.
Wang, J. H., & Graves, D. J. (1964) *Biochemistry 3*, 1437–1445.
Weber, G. (1952) *Biochem. J. 51*, 155–167.
Weber, G. (1987) Dissociation of Oligomeric Proteins by Hydrostatic pressure, in *Chemistry and Biochemistry at High Pressures* (van Eldyk, R., & Jonas, J., Eds.) NATO ASI Series C197, pp 401–420, D. Reidel, Dordrecht, The Netherlands.
Weber, G. (1991) Pressure and Temperature Stability of Oligomeric Enzymes, in *New Trends in Biological Chemistry* (Ozawa, T., Ed.) pp 225–238, Japan Scientific Society Press-Springer, Tokyo-Berlin.
Weber, G. (1993) Relations of bond energies and entropy with volume, pressure and temperature in protein aggregates, in *High Pressure in Chemistry, Biochemistry and Material Science* (Winter, R., & Jonas, J., Eds.) NATO Advanced Science Institutes Series C401, Kluwer, Dordrecht, The Netherlands (in press).