Pressure-Induced Dissociation of Yeast Glyceraldehyde-3-phosphate Dehydrogenase: Heterogeneous Kinetics and Perturbations of Subunit Structure

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ABSTRACT: In studies of pressure-induced subunit dissociation of oligomeric proteins, the thermodynamic dissociation constant and the dissociation volume change are derived by assuming that high pressure itself does not significantly perturb the structure of both oligomer and isolated subunit. In this report, the intrinsic phosphorescence emission of Trp reveals that high-pressure dissociation of tetrameric yeast glyceraldehyde-3-phosphate dehydrogenase results in a dramatic shortening of the phosphorescence lifetime, from 300 to less than 2 ms, that is consistent with a profound loosening of the polypeptide structure about the phosphorescence probe. On pressure release, subunit reassociation occurs readily whereas recovery of the native phosphorescence properties is a very slow, thermally activated, process which goes hand in hand with the recovery of the catalytic activity. Further, the comparison between the kinetic traces that describe the degree of dissociation and the change in phosphorescence lifetime, at various applied pressures, has established the following: (1) that high pressure plays a direct role on the structural rearrangement, the extent of which increases with pressure; (2) that the conformational change in the monomer is concomitant with, or follows closely after, the break up of the tetramer, in any case long before an apparent tetramer−monomer equilibrium is established; (3) that native tetramers are highly heterogeneous with regard to their rate of dissociation. The influence of temperature, of protein concentration, of binding of NAD^+, and of the addition of 2 M urea on the dissociation/phosphorescence kinetic profiles was also examined. The complications arising from these conformational changes for the derivation of the dissociation free energy change as well as their relevance for understanding the lack of concentration dependence of the degree of dissociation are discussed.

The application of hydrostatic pressure, p, has become an increasingly common tool for promoting the dissociation of oligomeric proteins, or even larger protein assemblies such as viral capsids, into individual subunits (Weber, 1992, 1993a; Jonas & Jonas, 1994; Silva et al., 1996). In principle, the method affords the determination of very small dissociation constants, that are beyond the limits of the traditional dilution method, and, consequently, of the thermodynamic parameters ($\Delta H°$, $\Delta S°$ and ΔV) that characterize very strong subunit associations.

The pressure dependence of the dissociation free energy, $\Delta G(p)$, is described by

$$\Delta G(p) = \Delta G° + p\Delta V$$

where $\Delta G°$ is the dissociation free energy at atmospheric pressure, p is the applied pressure above 1 atm, and ΔV is the volume change of the reaction. As ΔV is invariably negative, half-dissociation is achieved at a pressure $p_{1/2}$ for which $p_{1/2}\Delta V = \Delta G°$. In practice, $\Delta G°$ and ΔV are estimated from the linear plot assuming that both parameters are pressure-independent (Silva & Weber, 1993). Of course, a necessary condition for this to be true is that the structure of reactants and products, namely, oligomer and isolated subunit, not be affected by hydrostatic pressure and also that their compressibility be negligibly small. Generally, in oligomer dissociation studies, neither of these assumptions have been rigorously tested. Further, the dissociation of oligomeric proteins by hydrostatic pressure exhibits complexities not encountered in ordinary chemical reactions. Peculiar features of these equilibria are an apparent decrease in subunit affinity at larger degrees of dissociation (α) and pronounced hysteresis in the α/p diagrams (Weber, 1992). In addition, as the number of subunits in the aggregate increases beyond 2, α becomes less sensitive to protein concentration (Silva & Weber, 1988; Bonafe et al., 1991; Da Poian et al., 1993; Erijman & Clegg, 1995).

Recent studies on some monomeric (Eftink et al., 1991; Samarasinghe et al., 1992; Royer et al., 1993; Cioni & Strambini, 1994) and dimeric proteins (Peng et al., 1993; Rietveld & Ferreira, 1996; Cioni & Strambini, 1996) raise the concern that at the hydrostatic pressures required to cause subunit dissociation pressure itself may directly influence the conformation of the oligomer and/or isolated subunit and that such perturbations might be responsible for some of the peculiarities of dissociation equilibria.

Isolated subunits obtained by pressure dissociation of oligomeric proteins are generally unstable, and conspicuous changes in their structure are often observed. However, the direct influence of pressure on the destabilization of dissociated forms has not been proven yet. Until now, the prevailing view is that pressure effects are negligible and that the change in the monomer structure is primarily driven by the hydration of newly exposed surface area (Ruan & Weber, 1989; Weber, 1992; Silva & Weber, 1993). This progressive deterioration of the monomer structure was termed conformational drift, and a detailed model of oligomer

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dissociation was developed (Weber, 1986; Ruan & Weber, 1989) according to which the drift would account for both hysteresis effects and the decrease in subunit affinity of pressurized samples. An assumption of this model, that is crucial for the derivation of $\Delta G°$ and ΔV, is that the drift is slow relative to the time needed to establish the dissociation equilibrium (10−40 min). As such, the two processes, dissociation and drift, are separated in time and effectively do not interfere with each other.

In previous reports from this laboratory, the structural sensitivity of the phosphorescence emission of internal Trp residues has been instrumental for revealing pressure-induced perturbations of the native fold of some monomeric and dimeric proteins (Cioni & Strambini, 1994, 1996). The aim of the present study is to characterize the alterations in the structure of isolated subunits obtained by pressure-induced oligomer dissociation and address the following issues: (1) the role of high pressure in destabilizing the native fold of newly formed monomers; (2) the time evolution of the monomer structure as compared to the kinetics of subunit dissociation. The latter will provide a test for the validity of the conformational drift hypothesis. A suitable protein system to address these questions is glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] from yeast. For this tetramer, high-pressure dissociation equilibria and kinetics, as well as subunit exchange rates, have been studied in great detail under a variety of conditions (Ruan & Weber, 1989; Erijman & Weber, 1991; 1993). Also, among the tetramers examined so far, GAPDH dissociation exhibits the largest hysteresis effects (Weber, 1992). Another interesting aspect of GAPDH dissociation is that at low temperature the degree of dissociation, α, is insensitive to protein concentration but the expected reaction order tends to be restored at higher temperature (Ruan & Weber, 1989). Such lack of concentration dependence of α, which can be total in much larger protein aggregates (Silva & Weber, 1988; Bonafe et al., 1991; Da Poian et al., 1993; Erijman & Clegg, 1995), has been rationalized in terms of a heterogeneous distribution of dissociation free energy/volume among the tetramer population (Silva et al., 1989; Ruan & Weber, 1989; Weber et al., 1996). As the heterogeneity is expected to persist during the time of the experiment, such a distribution could manifest itself in heterogeneous phosphorescence properties of the sample. Finally, yeast GAPDH exhibits intense and long-lived room-temperature phosphorescence whose interpretation in terms of changes in protein structure and conformational polymorphism is facilitated by the fact that a single Trp residue is emitting in each subunit (Gabellieri & Strambini, 1989) and its location is also relatively well-known. In fact, although there are three Trp residues present in each subunit (W84, W193, W310), single Trp mutants of the enzyme from *Bacillus stearothermophilus* have shown that room-temperature phosphorescence is due entirely to Trp-84 located in the catalytic domain (Gabellieri et al., 1996). Because of the very high sequence and structural homology between bacterial and yeast GAPDH (Harris & Waters, 1976), Trp-84 is likely to be the phosphorescing residue also in the latter protein. The strong similarity in spectral energy and phosphorescence lifetime between the two proteins (Gabellieri & Strambini, 1989; Gabellieri et al., 1996) confirms that both the chemical composition and the dynamic structure of the chromophore's environment are equivalent in them, a finding that further supports the assignment of room-temperature phosphorescence to Trp-84.

This paper reports drastic changes in the phosphorescence emission of GAPDH at dissociating pressures that attest to a remarkable loosening of the isolated subunit structure, a reorganization of the native fold that occurs concomitantly with dissociation and whose extent is clearly pressure-dependent. The effects of urea, of complex formation with ATP and with NAD^+, and of raising the temperature are also examined.

MATERIALS AND METHODS

All chemicals were of the highest purity grade available from commercial sources, and water doubly distilled over quartz was employed throughout. GAPDH from yeast was purchased from Sigma Chemical Co. (St. Louis, MO). To remove NAD^+ from GAPDH, the enzyme was treated with activated charcoal as reported before (Gabellieri & Strambini, 1989b). Our apo-GAPDH preparation had an absorbance ratio, A_{280}/A_{260}, of 1.75−1.85.

Protein stocks were prepared by dialysis in Tris-HCl (50 mM, pH 7.5), a buffer whose pH is one of the least sensitive to pressure. Prior to pressure experiments, freshly prepared dithioerythritol (DTE) and EDTA were added to each protein sample to a final concentration of 1 mM. The protein concentration in all luminescence experiments was typically 4.5 μM, unless otherwise specified.

Enzyme activity was assayed at 25 °C and pH 7.5 by measuring the increase in absorbance at 340 nm caused by the reduction of NAD (Allison & Kaplan, 1964).

Luminescence Measurements. Fluorescence and phosphorescence spectra, intensities, and phosphorescence decay kinetics were obtained with a home-made apparatus whose detailed description was given elsewhere (Cioni & Strambini, 1994). Briefly, continuous excitation for fluorescence and phosphorescence spectra is provided by a xenon lamp, and the excitation wavelength, which was 295 nm for Trp luminescence, is selected by a monochromator with a 10 nm band-pass. The emission collected at a right angle is analyzed by a monochromator with 3 nm band-pass and is detected by an EMI9635QB photomultiplier. The excitation is modulated by a light chopper, and the transition from fluorescence to phosphorescence detection is accomplished simply by shifting the position of the sliding chopper such that both excitation and emission beams are cut in an alternative fashion. Spectra were corrected for instrumental response.

Phosphorescence decays were obtained with pulsed excitation as provided by a frequency-doubled flash-pumped dye laser tuned at 292 nm. The pulse duration is 1 μs, and the light energy per pulse is typically 1−10 mJ. The phosphorescence signal collected at a right angle from the excitation beam is filtered (410−470 nm band-pass) and detected by a blue-sensitive photomultiplier. An electronic shutter arrangement protects the photomultiplier from the intense fluorescence pulse and permits the delayed emission to be detected 2 ms after the excitation pulse. The photocurrent is amplified by a current to voltage converter, and digitized by a computerscope system (ISC-16, RC Electronics) capable

[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase from yeast; DTE, dithioerythritol; τ, phosphorescence lifetime; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5'-triphosphate; NAD, oxidized nicotinamide adenine dinucleotide.

of averaging multiple sweeps. The intensity of each phosphorescence decay was normalized by a signal proportional to the excitation intensity. In general, the phosphorescence intensity of individual sweeps exhibited satisfactory signal to noise ratios, and therefore signal averaging was only necessary with dilute samples or under quenching conditions. All phosphorescence decays were analyzed in terms of a sum of exponential components by a nonlinear least-squares fitting algorithm (Global Unlimited, LFD, University of Illinois).

Luminescence measurements under pressure were carried out by placing the sample cuvette in a pressure cell (ISS-NOVA, ISS Inc.) provided with sapphire windows and employing highly pure ethanol as pressurizing fluid. For phosphorescence measurements, it is paramount to rid the solution of all traces of O_2 and isolate the sample from the pressurizing fluid. Deoxygenation was obtained by adding an enzymatic system composed of 80 nM glucose oxidase, 16 nM catalase, and 0.3% glucose. Satisfactory deoxygenation was achieved in 30 min. The thoroughness of O_2 removal was tested by the dependence of the triplet lifetime on the amount of excitation (Strambini, 1983).

The degree of dissociation was determined from the displacement of the center of mass of the Trp fluorescence spectrum as described elsewhere (Ruan & Weber, 1989). Briefly, the center of spectral mass (ν_g) is defined by the equation:

$$\nu_g = (\sum \nu_i F_i)/(\sum F_i)$$

where F_i is the fluorescence emitted at wavenumber ν_i.

Considering that the pressure-induced dissociation equilibrium for GAPDH does not involve a stepwise dissociation of tetramer into dimers and of these into monomers but simply tetramers and monomers (Ruan & Weber, 1989), the degree of dissociation (α) is related to ν_g by the expression:

$$\alpha = [1 + Q((\nu_g)_p - (\nu_g)_M)/((\nu_g)_T - (\nu_g)_p)]^{-1}$$

where Q is the ratio of the quantum yields of monomer and tetramer, $(\nu_g)_p$ is the center of spectral mass at pressure p, and $(\nu_g)_M$ and $(\nu_g)_T$ are the corresponding quantities for monomer and tetramer.

All luminescence measurements, unless otherwise stated, were carried out 5 min after any pressure change, the minimum time required for thermal equilibration.

RESULTS

The effects of high pressure on the Trp fluorescence spectrum and intensity have already been described in detail by Ruan and Weber (1989). At 2.4 kbar, when the protein is fully dissociated, the spectrum center of mass, ν_g, is red-shifted by 8−10 nm, and the intensity is about 50% of its initial value. These alterations were shown to accompany the dissociation of the tetramer into monomers, and throughout this study, ν_g was employed as an indirect measure of α. When the experimental conditions adopted here (pressure = 0.001−2.5 kbar, temperature = 0 or 30 °C, and protein concentration = 0.45 and 4.5 μM) were similar to those of Ruan and Weber (1989), our esteem of α was found to be in good agreement with that report.

Pressure Effects on the Phosphorescence Lifetime of GAPDH Tetramers. The decay kinetics of GAPDH phosphorescence in fluid solutions are nonexponential. The heterogeneity in the kinetics is even more evident under applied pressure (Figure 1). Since only one Trp contributes to the long-lived emission (Gabellieri & Strambini, 1989), the lifetime heterogeneity is attributed to heterogeneity in the subunit structure that persists for times longer than τ. In general, phosphorescence decays were fitted satisfactorily in terms of two discrete lifetime components, but, in a few circumstances, an additional component improved the fitting. The decay parameters, lifetime (τ_i) and amplitudes (a_i), obtained at 0 and 30 °C for the 0.001−2.4 kbar pressure range are collected in Table 1. Applied pressure decreases the average lifetime, $\tau_{av} = \sum a_i \tau_i$, of the tetramer particularly in the higher range and at the lower temperature. The maximum reduction is 45% at 0 °C and 26% at 30 °C. A reduction of τ means that the protein structure about Trp-84 is more flexible under applied pressure (Gonnelli & Strambini, 1995). The change in τ_{av} is the result of both a decrease in τ_1 and τ_2 and a shift in their relative amplitude. Pressure modulation of the amplitudes, which reveals a modulation

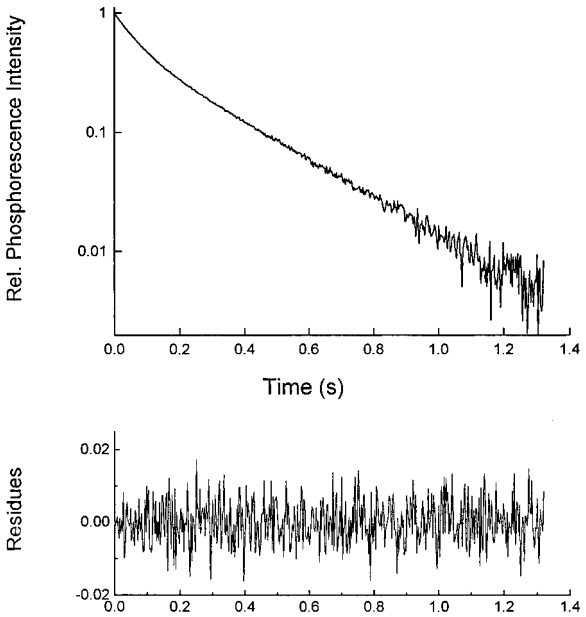

FIGURE 1: Example of primary phosphorescence decay data and fitting statistics. The sample is 4.5 μM GAPDH in Tris-HCl (50 mM, pH 7.5), 1 mM EDTA, and 1 mM DTE at 0 °C and at 2.4 kbar. The decay signal is from a single sweep. In the bottom panel are displayed the residues.

Table 1: GAPDH Phosphorescence Decay Kinetics at Different Solvent, Temperature, and Pressure Conditions

solvent composition	pressure (kbar)	T (°C)	τ_1 (ms)	τ_2 (ms)	a_1	τ_{av} (ms)	χ^2
buffer	0.001	0	300	612	0.94	319	0.9
buffer	1.0	0	279	528	0.87	311	0.8
buffer	1.5	0	240	420	0.53	325	0.9
buffer	2.0	0	167	358	0.40	282	1.0
buffer	2.4	0	72	281	0.50	177	0.9
buffer	0.001	30	70	−	1	70	0.8
buffer	1.0	30	43	77	0.18	71	0.9
buffer	1.5	30	34	66	0.18	60	0.9
buffer	2.0	30	27	67	0.28	56	1.1
buffer	2.4	30	48	107	0.93	52	1.0
+ATP, 1 mM	0.001	30	62	−	1	62	1.1
+ATP, 1 mM	2.4	30	50	−	1	50	1.0
+urea, 2 M	0.001	30	49	85	0.87	54	1.2
+urea, 2 M	2.4	30	50	−	1	50	1.1
+NAD, 19 μM	0.001	30	18	−	1	18	0.8
+NAD, 19 μM	2.4	30	17	−	1	17	0.8

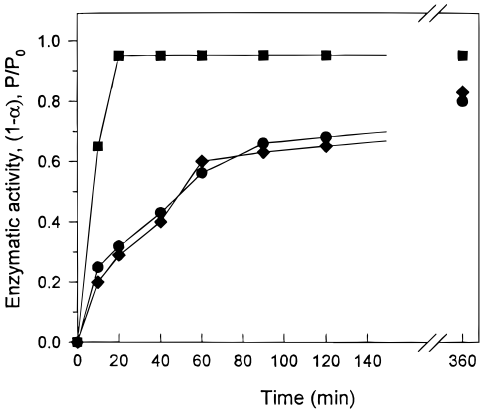

FIGURE 2: Comparison between the kinetics of recovery of the tetrameric form of GAPDH (1 − α) (■), of its specific enzyme activity (◆), and of its normalized phosphorescence intensity (P/P_0) (●) after the protein sample, at 0 °C, has been compressed for 20 min at 2.4 kbar. Recovery was carried out at 20 °C. The protein concentration was 4.5 μM.

in the GAPDH conformers distribution, is particularly evident at 0 °C. Time profiles show that the phosphorescence decay is stable for hours, varying by not more than 10% even at pressures that cause tetramer dissociation and pronounced phosphorescence quenching. On decompression, the native lifetime is promptly recovered, indicating that the increased structural flexibility simply represents an elastic response of the protein structure to the external perturbation.

Effects of High-Pressure Dissociation on GAPDH Phosphorescence. At 0 °C and 2.4 kbar, the GAPDH tetramer is completely dissociated in about 15 min. In the course of monomer formation, the phosphorescence intensity drops rapidly to zero, and after 10 min of pressurization, no residual emission is detected. This implies that, at 0 °C and 2.4 kbar, the monomer is not phosphorescent; i.e., its emission is too short-lived (τ < 2 ms, the time resolution of the apparatus) for detection. Such drastic reduction in τ, from about 320 ms in the tetramer to <2 ms in the monomer, reveals a dramatic loosening of polypeptide fold in the region of the chromophore, the local structure attaining a mobility comparable to that of unfolded chains (Gonnelli & Strambini, 1995). The concomitant red-shift of the fluorescence emission does indicate that the chromophore has become at least partly exposed to the aqueous solvent.

On release of applied pressure, GAPDH monomers reassociate and recover the original fluorescence spectrum and intensity in less than 20 min. In contrast, recovery of the phosphorescence properties is a very slow process, requiring days at 0 °C or several hours at 20 °C (Figure 2). This shows that the phosphorescence lifetime of tetramers formed from the reassociation of monomers remains very short (<2 ms) for a long time, and emphasizes the permanence of the structural alterations induced by high-pressure dissociation. For these tetramers, Ruan and Weber (1989) found a larger rotational mobility of the Trp residues and enhanced the binding of 1-anilino-8-naphthalenesulfonate, features that are fully consistent with the enhanced structural flexibility reported by τ. Finally, Figure 2 also points out that the recovery of the enzymatic activity follows closely that of the phosphorescence intensity, P/P_0 (it refers to the intensity in the phosphorescence decay at time zero following pulsed excitation), the close correlation between the two parameters confirming that the structural alterations responsible for the

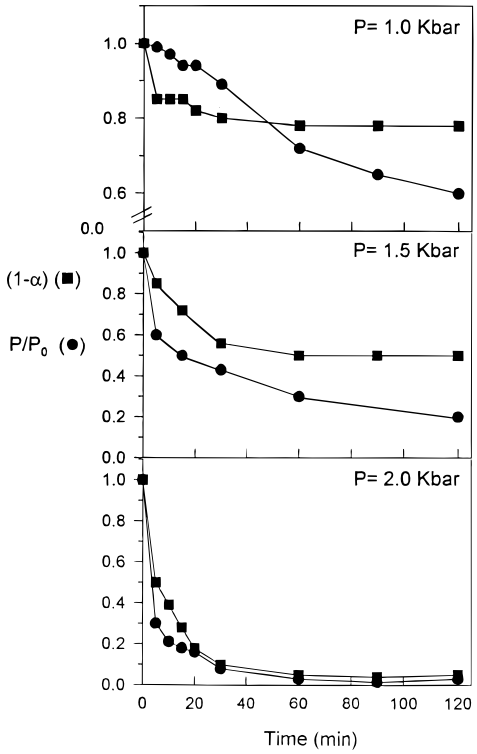

FIGURE 3: Time course of the decrease in phosphorescence intensity (P/P_0) (●) and in the tetramer fraction (1 − α) (■) of GAPDH (4.5 μm) at various dissociating pressures. The buffer is 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTE. The temperature is 0 °C.

quenching of the phosphorescence are highly inhibitory of the catalytic activity.

Time Profiles of GAPDH Phosphorescence at Partially Dissociating Pressures. Above, it was shown that at 2.4 kbar (0 °C), monomers are not phosphorescent, implying that their structure has been affected by either subunit dissociation or high-pressure conditions or both. In order to assess the relative importance of these two potential forces driving monomer degradation, the time profiles of α, P/P_0, and τ_{av}/τ_{av0} were obtained at more moderate pressures between 1 and 2 kbar, a range where tetramer dissociation is only partial. These kinetic runs show that, depending on the applied pressure, dissociation is accompanied by a more or less drastic decrease of P/P_0 whereas τ_{av} remains practically constant for as long as the phosphorescence intensity is sufficient to permit its precise determination. Thus, at any time of the reaction, a fraction of GAPDH subunits equal to (1 − P/P_0) is nonphosphorescent (τ < 2 ms) while the remainder preserves an unperturbed phosphorescence lifetime. The time course of P/P_0 at 1, 1.5, and 2 kbar is compared to that of the tetramer fraction (1 − α) in Figure 3. At each pressure, whereas the fraction of tetramers invariably stabilizes to a plateau value in about 30 min, P/P_0 decreases in time in a multiphasic way until, eventually, all the emission is quenched (final points not shown). Thus, the first conclusion to be drawn is that, as time goes on, both monomers and tetramers become nonphosphorescent and therefore structurally modified. A comparison between (1 − α) and P/P_0 profiles during the initial 30 min of the reaction also points out that at 1.0 kbar P/P_0 > (1 − α) whereas at 1.5 and 2 kbar P/P_0 < (1 − α). The first inequality implies that at the lowest pressure the fraction of GAPDH subunits still phosphorescent is greater than that in the tetrameric state and indicates that, at 1 kbar, the monomer

is initially phosphorescent and probably native-like. On the contrary, at 1.5 and 2.0 kbar, P/P_0 is always less than the tetramer fraction. Hence, at these higher pressures, quenching of monomer phosphorescence occurs more rapidly and long before the attainment of the dissociation equilibrium. These observations establish that: (1) pressure is an important driving force of the change in monomer conformation because the alteration occurs more rapidly at higher pressure; and (2) depending on the pressure, the monomer structure can be modified before the apparent dissociation equilibrium is reached.

Another important aspect of the P/P_0 profile is its nonuniform decay, a feature particularly evident at 1.0 and 1.5 kbar. Clearly, were GAPDH tetramers homogeneous with respect to their dissociation characteristics (rate and free energy change), the P/P_0 profile would decay to zero (as reassociated subunits remain nonphosphorescent) in a monophasic way with a time constant comparable to the dissociation time constant. An analysis of the P/P_0 profile at 1.5 kbar, for example, shows that to fit the decay it requires at least two components with lifetimes of 11 and 60 min. Whereas the rapid phase has an amplitude and a time constant (0.5, 11 min) similar to those of the dissociation profile (0.5, 16 min) and should therefore be largely attributed to the formation of dark monomers, the slow phase demonstrates unequivocally that a substantial fraction of tetramers is unable to dissociate during the tetramer−monomer equilibration time indicated by the $(1 - \alpha)$ profile. The P/P_0 profile provides direct evidence that within the GAPDH tetramer population the rate of dissociation is highly heterogeneous.

To further test the hypothesis that the monomer structure is pressure-dependent, we measured the extent of prompt (2 min after release of high pressure) recovery of P/P_0 of pressurized samples on the premise that the greater the alteration of subunit structure the longer it should take to restore the folded, phosphorescent, state. The percentage of prompt recovery of P/P_0 after 20 min of pressurization at 0 °C was practically 100% at 1 kbar ($\alpha = 0.2$), 65% at 1.5 kbar ($\alpha = 0.35$), 18% at 2.0 kbar ($\alpha = 0.82$), and no recovery at 2.4 kbar ($\alpha = 1.0$). Again, these results confirm that the isolated subunit structure is markedly pressure-dependent and that irreversible transformations occur long before the dissociation equilibrium is established (i.e., α reaches a plateau).

Influence of Temperature and Protein Concentration on the P/P_0 Profile. The time course of P/P_0 was found to be sensitive to both temperature and protein concentration. Raising the temperature from 0 to 30 °C increases the stability of the aggregate toward dissociation, and raises the midpoint dissociation pressure (Ruan & Weber, 1989). For example, to achieve $\alpha = 0.2$, it requires 1 kbar at 0 °C and 2.4 kbar at 30 °C. For this degree of dissociation ($\alpha = 0.2$), the P/P_0 time profiles obtained at 0 and 30 °C are compared in Figure 4. The results show that the decrease in P/P_0 is considerably more rapid and pronounced at the higher temperature.

The effect of decreasing 10-fold the protein concentration (from 4.5 to 0.45 μM) on the P/P_0 profile at 2 kbar and 0 °C is shown in Figure 5. It is interesting to note that the phosphorescence intensity is quenched more gradually in the dilute sample in spite of the fact that its final degree of dissociation is larger ($\alpha = 0.6$ compared to $\alpha = 0.4$).

Effects of NAD$^+$, ATP, and Urea on the Phosphorescence and Dissociation Profiles of GAPDH. Binding of NAD$^+$ to

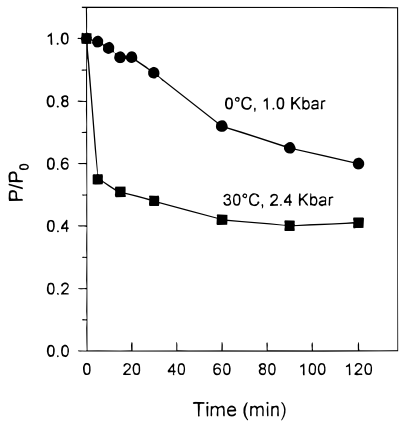

FIGURE 4: Effect of temperature on the time course of GAPDH (4.5 μM) phosphorescence intensity under dissociating conditions. Comparison between 0 °C (●) and 30 °C (■). The degree of dissociation is in either case 0.2.

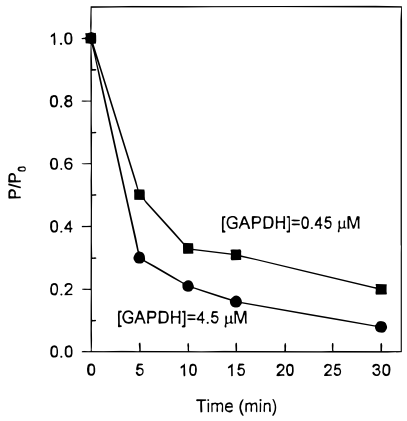

FIGURE 5: Effect of protein concentration on the time course of GAPDH phosphorescence intensity under dissociating conditions. The temperature is 0 °C, and the pressure is 2 kbar.

GAPDH shifts the pressure dissociation profile to higher pressure whereas complexation with ATP or addition of urea has the opposite effect (Ruan & Weber, 1989). The influence of the nucleotides and of urea on the phosphorescence and on the dissociation properties of GAPDH was monitored at 30 °C and at 2.4 kbar. At atmospheric pressure, the phosphorescence is little affected by 1 mM ATP or 2 M urea, the only differences being an 11 and 37% reduction in τ_{av} (Table 1), respectively. The lifetime reduction is considerably greater with the NAD$^+$ complex (19 μM, 50% saturation) where the decreased flexibility probably reflects the change in conformation associated with the apo to holo transition (Gabellieri et al., 1996).

Raising the pressure to 2.4 kbar decreases the lifetime of each sample (Table 1), as is the case with the protein alone. Under these conditions, all GAPDH samples partly dissociate, and α determinations confirm the stabilizing/destabilizing influence on the tetramer subunit affinity of NAD$^+$, ATP, and urea (Figure 6). In each case, monomer formation is accompanied by quenching of the phosphorescence intensity. The main effect of NAD$^+$ on the P/P_0 profile is to practically abolish the slow phase as, after the initial drop, the intensity remains constant for over 1 h (Figure 6). By contrast, the destabilizers ATP and urea both accelerate the decrease of P/P_0. However, it should be noted that in 2 M urea P/P_0 exceeds $(1 - \alpha)$. For instance, although dissociation is complete ($\alpha = 1$) in about 30 min, at this time there is still 20% residual phosphorescence intensity. This is possible

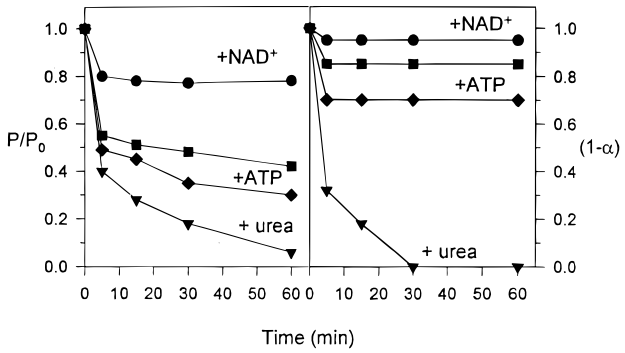

FIGURE 6: Influence of nucleotides and of urea on the phosphorescence intensity, P/P_0, and the dissociation profiles of GAPDH (4.5 μM) at 30 °C and 2.4 kbar. (■) GAPDH alone; (●) +19 μM NAD^+; (◆) +1 mM ATP; (▼) +2 M urea.

only if the monomer is temporally phosphorescent, that is, if in urea-aided pressure dissociation the integrity of the subunit structure is better preserved.

DISCUSSION

The phosphorescence lifetime of native GAPDH is relatively long, in the 100 ms range, and from the correlation between τ and the local viscosity (Strambini & Gonnelli, 1995), it may be deduced that the polypeptide about Trp-84 is quite rigid as is typical of well-packed chains rich in secondary and tertiary structure. The chromophore is located in the catalytic domain at a site quite removed from the subunit interface (Leslie & Wonacott, 1983). Consequently, its environment is not altered by subunit dissociation per se, and changes in τ will necessarily reflect variation in the conformation of the catalytic domain.

A main concern in the analysis of pressure-induced dissociation of oligomeric proteins is whether high pressure has an influence on the structure of the molecular species involved in the oligomer−monomer equilibrium, because then, depending on the kinetics of these structural alterations, the thermodynamic parameters $\Delta G°$ and ΔV derived from these equilibria may not correctly describe the interaction among the subunits at atmospheric pressure. The sensitivity of τ to changes in the flexibility of the chromophore's environment has revealed that high pressure does perturb the structure of both GAPDH tetramer and GAPDH monomer. According to the lifetime parameter, pressure effects on the native tetramer, i.e., prior to subunit dissociation or exchange, are distinct but modest. They involve a reduction in τ_{av} (about 40% at 2.4 kbar) and a shift in the relative amplitude of the lifetime components, changes that indicate a moderate loosening of the polypeptide and a shift in the equilibrium distribution of tetramer conformations. These effects appear to be simply elastic adaptations to pressurization as they are readily reversed on decompression. The decay characteristics of the tetramer are stable in time even at pressures that cause extensive dissociation and phosphorescence quenching. Unlike for the tetramer, the change in τ is dramatic for the isolated subunit. The more than 100-fold reduction of τ, and consequent quenching of the phosphorescence intensity, reveals a drastic increase in local mobility that is estimated as an increase in local viscosity of over 10^3-fold (Strambini & Gonnelli, 1995). As tightly packed cores of the globular structure, such as this one hosting Trp-84, are invariably responsible for the stability of entire domains, it is likely that the structural changes observed by the phosphorescence probe extend to beyond its immediate environment. This alteration of the environment is accompanied by a pronounced red-shift in the Trp fluorescence spectrum which confirms extensive solvation of the chromophore. Another indication that the drastic reduction of τ is probably associated to important deteriorations of the native fold is the close correspondence between the loss of phosphorescence intensity and that of catalytic activity. Indeed, even if at atmospheric pressure the monomers reassociate rapidly and the fluorescence characteristics are quickly restored, recovery of the enzymatic activity is a very slow and thermally activated process that occurs concomitantly with the recovery of the original phosphorescence intensity and lifetime (Figure 2).

Potentially, two factors may contribute to destabilize the monomer structure: (1) breaking of the subunit−subunit interactions with subsequent hydration of the interface; and (2) high pressure itself. The results obtained over a range of pressures suggest that the structural rearrangement of the monomer is not merely a consequence of dissociation. For instance, when dissociation occurs at low pressure, 1 kbar as opposed to 1.5 or 2 kbar, the inequality $P/P_0 > (1 - \alpha)$ (Figure 3) entails that newly formed monomers are temporally phosphorescent and presumably native-like. Another indicator that monomers are more destabilized at higher pressure is the smaller extent of prompt recovery of the phosphorescence intensity when pressure is released. For freshly formed monomers, i.e., after a relatively brief period (20 min) of pressurization such as to minimize any long-term reorganization of the subunit structure, recovery of their phosphorescence is practically complete in the 1−1.5 kbar range, but null at 2.4 kbar. This emphasizes that when monomers are formed in the lower pressure range they can rapidly recover the native fold in contrast to the higher pressure range where the transformations are presumably too drastic to be readily reversed.

An inescapable consequence of these pre- and postdissociational pressure effects on protein structure is that the free energy change describing oligomer dissociation at high pressure is a composite quantity with contributions from both the dissociation reaction and pressure-dependent conformational changes. The minimal reaction scheme to describe the tetramer−monomer (T↔M) equilibrium of GAPDH under pressure must be

$$T' \leftrightarrow M'' \rightarrow M^*$$

where T′ and M″ represent pressure-dependent conformations of the tetramer and of newly formed monomer, respectively, whereas M* is the product of successive alterations of the subunit structure taking place after the apparent dissociation equilibrium is established. The corresponding dissociation free energy change, $\Delta G_{exp}(p)$, may then be represented as the sum of two contributions:

$$\Delta G_{exp}(p) = \Delta G_{diss} + \Delta G_{conf}(p)$$

where $\Delta G_{diss} = \Delta G° + p\Delta V$ represents the perturbation free dissociation free energy change while $\Delta G_{conf}(p) = \Delta G(T' \rightarrow T) + \Delta G(M \rightarrow M'')$ takes into account the pressure-dependent conformational free energy changes involved in the transformation of T′ into T and of M into M″. Thus, unless the conformational term is negligibly small, the subunit affinity will appear to be pressure-dependent. Since in general pressure-induced structural rearrangements of the monomer

are expected to be larger than those of the more stable oligomer, as is clearly the case with GAPDH, ΔG_{conf} will generally be negative and the subunit affinity will then decrease with pressure (or larger α) as commonly observed in high-pressure oligomer dissociation (Weber, 1992). Note that a nonnegligible conformational term could also explain the sometimes large discrepancy in dissociation free energy when high pressure and dilution studies are compared, as was inferred for the tetramer−dimer equilibrium of glycogen phosphorylase A (Ruan & Weber, 1993). In the case of triosephosphate isomerase, the conformational term is so large as to lead to the unfolding of the subunit prior to monomerization (Rietveld & Ferreira, 1996). Finally, a more or less large contribution of the conformational term might also account for a large variability in dissociation, $\Delta S°$, among protein oligomers, that, as was pointed out by Weber (1993b), is otherwise hard to rationalize in terms of simple hydration of the subunit interface.

Heterogeneity of GAPDH toward Subunit Dissociation and Lack of Concentration Dependence of α. The monomers of GAPDH obtained at pressures above 1.5 kbar, and also the tetramers formed by their reassociation, are not phosphorescent. Thus, at any time during the kinetic profiles, the phosphorescence intensity is exclusively from never dissociated tetramers, and the intensity ratio P/P_0 provides a convenient monitor of their concentration. The nonuniform, evidently multiphasic, drop in P/P_0 demonstrates unequivocally that not all tetramers dissociate at the same rate and that, therefore, the tetramer population is heterogeneous with regard to its dissociation characteristics. It should be recalled that heterogeneity in the conformation of GAPDH is also inferred from the multiplicity of phosphorescence lifetimes. However, the two heterogeneities are evidently not correlated with each other because the lifetime distribution remains largely invariant as more and more tetramers dissociate. The existence of fast and slow phases in the P/P_0 profile shows that at 1.5 kbar (Figure 3) dissociation rates differ by at least a factor of 5. Of course, this range in dissociation rates only represents a lower bound because subunit exchange between tetramers and monomers limits the permanence of particle individuality. Indeed, the slow phase of the P/P_0 profile does seem to reflect the disappearance of fully native tetramers through subunit exchange rather than by straight dissociation. In fact, P/P_0 decreases more rapidly at higher temperature and higher protein concentration (Figure 5), conditions that enhance subunit exchange but not unimolecular oligomer dissociation. In accord with this conclusion, the rate of exchange determined under similar conditions by the method of fluorescence energy transfer (Erijman & Weber, 1993) is not dissimilar.

A plurality of stable conformations of GAPDH has been invoked to explain the anomalous protein concentration dependence of α that is sensibly less than that predicted by the law of mass action, particularly at low temperature (Ruan & Weber, 1989; Erijman & Weber, 1991). With even larger protein aggregates, pressure dissociation profiles can be totally independent of protein concentration (Silva & Weber, 1988; Bonafe et al., 1991; Da Poian et al., 1993; Erijman & Clegg, 1995). Until now, the lack of concentration dependence has been rationalized in terms of a deterministic equilibrium where particle individuality would arise from a wide distribution of dissociation free energies, $\Delta G°_i$ (and/or volumes, ΔV_i), that is frozen in the time scale of the experiment (Ruan & Weber, 1989; Erijman & Weber, 1991; Silva et al., 1989; Weber et al., 1996). According to this model, at any pressure p those particles for which $p_i = \Delta G_i/\Delta V_i < p$ will dissociate whereas the remainder will stay undissociated. The true reaction order will be progressively restored, and the equilibrium acquire stochastic character, when particle individuality is lost during the time of the experiment. The model is formulated in purely thermodynamic terms and therefore implicitly assumes that reactants and products are in dynamic equilibrium. It must pointed out, however, that oligomer subunit dissociation can be a considerably slow process and that, therefore, in many cases, the reaction could be under kinetic rather than thermodynamic control. In a model based on "kinetic control", particle individuality manifests itself through a wide distribution of activation barriers toward dissociation such that a true T↔M equilibrium is never reached and α merely represents the fraction of T whose dissociation rate constant is $k_i > 1/t_0$, where t_0 is the typical duration of the experiment (∼1 h). Of course, on raising the pressure, α increases because the subunit−subunit interaction becomes weaker, the barrier height is smaller, and a larger fraction of the sample is able to dissociate during t_0.

The results with GAPDH do confirm that at partially dissociating pressures (between 1 and 1.5 kbar) a good portion of tetramers do not undergo dissociation during t_0, implying that they are not in dynamic equilibrium with the monomers. In comparing the validity of these two models, we note that a wide distribution of activation free energies is more plausible than a wide distribution in dissociation free energies. For one thing, the transition state being a transient species is not subject to the Boltzmann distribution which, instead, limits the range of conformational free energies attainable by the oligomer. Further, slight differences in subunit structure or the arrangement of the subunit in the oligomer can determine dissociation pathways with markedly different steric activation barriers. With complex structure, the range of distinct dissociation pathways is expected to grow steeply with the number of subunits in the aggregate and accentuate the deterministic character of dissociation, as is actually observed. Most importantly, however, a dynamic equilibrium between oligomers and the products of their dissociation is not compatible with the concentration independence of α. For α to be invariant of protein concentration, reassociation of the subunits must be inhibited; i.e., oligomer dissociation under pressure must be an irreversible process. In the absence of subunit reassociation, α will reach a relatively stable plateau in time only if a fraction $(1 - \alpha)$ of the oligomers never dissociates in the time scale of the experiment. Consequently, the discrimination between dissociating and nondissociating oligomers can only be on kinetic grounds. Of course, there is one exception to this and is represented by oligomers with such a pronounced particle individuality that the isolated subunits from the same aggregate can only reassociate among themselves, a case totally unrealistic. Thus, terms such as "deterministic equilibrium" and "failure of the law of mass action" should be more appropriately replaced with "deterministic kinetics" and with the more familiar "kinetic control", respectively.

Among the factors that are expected to hinder subunit reassociation, prominence should be given to (1) alterations in the isolated subunit structure that drastically reduce the subunit affinity and (2) unfavorable statistics of random

reassembly. The latter becomes an important factor only with aggregates that are made up of many subunits organized in complex topologies. As a general criterion, then, those experimental conditions that oppose alterations in the structure of the isolated subunit are also expected to enhance the reversibility of oligomer dissociation, and, with it, to restore the concentration dependence. Pressure-induced hydration and unfolding of polypeptides are generally countered by moderately high temperature, presence of stabilizing agents like glycerol, and binding to structuring metal ions or to DNA/RNA templates. In accord with this premise, the concentration dependence of GAPDH dissociation is largely restored on increasing the temperature from 0 to 30 °C (Ruan & Weber, 1989), and the reversibility of dissociation of some oligomers increases with the addition of glycerol (Dreyfuss et al., 1989; Bonafe et al., 1991) or binding to Ca^{2+} ions (Bonafe et al., 1991, 1994) or to RNA (Da Poian et al., 1994). Interestingly, reversibility was also found to be promoted by subdenaturing concentrations of urea (<5 M). An example is the high-pressure dissociation of R17 bacteriophage where urea makes the dissociation process reversible and concentration-dependent (Da Poian et al., 1993). This effect of the denaturant is not simply rationalized. If, on the one hand, by reducing the subunit affinity urea lowers the dissociating pressure and therefore the perturbation deriving from it, on the other, being a denaturant, it is expected to favor the unfolding of the polypeptide. The answer may lie in a combination of more factors: (1) As suggested by Weber et al. (1996), the dynamic interaction between molecules of urea and the oligomer will reduce the permanence of particle individuality. This structure homogenizing effect of urea renders the sample more uniform in the time window characteristic of subunit dissociation and limits the range of dissociation rates. (2) Moderate quantities of urea may even protect the monomer against pressure-induced unfolding and, thereby, promote the reversibility of dissociation. Significant, in this respect, is the effect of urea on the phosphorescence of the GAPDH monomer (Figure 6) as it demonstrates that the subunit integrity is better preserved in 2 M urea. Should this finding be of general validity, then moderate amounts of urea are predicted to increase the reversibility of high-pressure oligomer dissociation and restore thermodynamic control of the reaction through the removal of kinetic determinism and a better preservation of the native fold of the dissociation product. Consistent with this analysis, preliminary pressure dissociation profiles indicate that urea restores the correct concentration dependence of the reaction (work in progress).

In conclusion, the intrinsic phosphorescence of Trp residues has demonstrated that the high-pressure dissociation of GAPDH yields prompt and drastic alteration of the polypeptide structure about the chromophore whose magnitude is, among other factors, dependent on the applied pressure. Because of these alterations, the free energy change of the process will contain a conformational term whose relative importance is not readily estimated. Further, it is proposed that these conformational changes play a direct role in inhibiting subunit reassociation, and that this irreversibility of dissociation together with a wide distribution in dissociation rates accounts for the lack of concentration dependence of α.

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