Deterministic Pressure Dissociation and Unfolding of Triose Phosphate Isomerase: Persistent Heterogeneity of a Protein Dimer[†]

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ABSTRACT: Subunit dissociation and unfolding of dimeric rabbit muscle triose phosphate isomerase (TIM) induced by hydrostatic pressure were investigated. Changes in fluorescence emission of TIM (both intrinsic and of covalently attached probes) indicated that pressure ranging from 1 bar to 3.5 kbar promoted subunit dissociation and unfolding. Intrinsic fluorescence changes upon unfolding by pressure included a 27 nm red-shift of the emission, a decrease in fluorescence anisotropy from 0.14 to about 0.01, and a 1.5-fold increase in fluorescence quantum yield, similar to that observed in the presence of guanidine hydrochloride. Kinetics of pressure-induced fluorescence changes were slow ($t_{1/2} \approx 15$ min) and little dependent on pressure. In order to selectively monitor subunit dissociation, fluorescence resonance energy transfer (FRET) measurements were carried out with TIM that was separately labeled with 5-((((2-iodoacetyl)-amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) or fluorescein-5-isothiocyanate (FITC). FRET measurements indicated that subunit dissociation and unfolding took place concomitantly, both under equilibrium conditions and in kinetic experiments in which dissociation/unfolding was triggered by a sudden increase in pressure. Release of pressure caused monomer refolding and dimerization. Contrary to what would be expected for a process involving subunit dissociation, pressure effects on TIM were not dependent on protein concentration. Experiments involving a series of pressure jumps demonstrated persistent heterogeneity in sensitivity toward pressure in the ensemble of TIM dimers. This kind of deterministic behavior is similar to that exhibited by higher order protein aggregates and indicates that not all individual dimers are energetically identical in solution. The heterogeneity of native TIM revealed by sensitivity to pressure could not be detected by traditional means of protein separation, such as polyacrylamide gel electrophoresis (under both native and denaturing conditions) and size exclusion gel chromatography. This suggests that energetic heterogeneity originates from conformational heterogeneity of the protein. The possible biological relevance of the deterministic character of stability of TIM is discussed.

In the past 15 years an increasing number of reports has appeared on the use of hydrostatic pressure as a reversible thermodynamic variable in the study of protein−protein and protein−ligand interactions [for reviews, see Heremans (1982), Weber and Drickamer (1983), Weber (1987, 1992), and Silva and Weber (1993)]. Many oligomeric proteins undergo subunit dissociation by application of pressure below 3 kbar (Silva & Weber, 1993). On the other hand, the structures of monomeric proteins were found to be little or not affected by pressure in this range (Weber & Drickamer, 1983). Therefore, in the interpretation of pressure effects on oligomeric proteins it is generally considered that no unimolecular reactions (such as unfolding) of dissociated subunits are *directly* induced by pressure in the range required to produce dissociation.

Effects of pressure on the dissociation equilibrium of protein subunits can be described by assuming a standard volume change (ΔV) upon dissociation, causing a linear dependence of the Gibbs free energy of dissociation (ΔG) on pressure (p) (Weber, 1992):

$$\Delta G(p) = \Delta G_{atm} + p\Delta V \quad (1)$$

As a consequence, the equilibrium of subunit association is displaced by pressure as described by Silva and Weber (1993):

$$\ln[\alpha^n/(1-\alpha)] = p(\Delta V/RT) + \ln(K_{atm}/n^n C^{n-1}) \quad (2)$$

where α is the extent of reaction (dissociation) at pressure p, n is the number of subunits in the aggregate, C is the molar concentration of protein expressed as aggregate, and R and T have their usual meanings.

The thermodynamic description outlined above was originally developed for stochastic equilibrium between oligomers and dissociated subunits, i.e., a dynamic equilibrium between discrete species possessing well-defined chemical potentials. However, this was found to be fully applicable only to the simplest cases of protein associations, such as

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the dimeric hormone neurophysin (Rholam & Nicolas, 1981; Scarlata & Royer, 1986). For several other dimers, as well as for higher order protein aggregates, significant departures from this simple thermodynamic description have been observed. In many cases it was found that the chemical potentials of monomer and aggregate were dependent on the extent of dissociation, due to slow conformational changes of the monomers after dissociation (i.e., conformational drift of the subunits; Xu & Weber, 1982; Weber, 1986, 1992; Silva et al., 1992; Erijman et al., 1993). Another interesting finding was that in pressure dissociation of trimers and tetramers the dependence on protein concentration was significantly smaller than predicted by eq 2 (King & Weber, 1986; Ruan & Weber, 1989, 1993; Pedrosa & Ferreira, 1994). For higher order protein aggregates dependence on protein concentration was completely absent (Silva & Weber, 1988; Silva et al., 1989; Bonafé et al., 1991). These observations were explained on the basis of a persistent (relative to the time scale of experiments) energetic heterogeneity of association of protein subunits. This leads to deterministic dissociation which is less or not at all dependent on protein concentration (Silva & Weber, 1993). These conclusions were confirmed by experiments that compared the time required for subunit dissociation induced by pressure and for subunit exchange, which revealed the existence of subpopulations of protein aggregates differing in sensitivity toward pressure (Erijman & Weber, 1991).

The structural basis of heterogeneity of protein aggregates is not clear. It is generally accepted that proteins fold in a highly reproducible manner into a unique structure which displays only short-lived structural fluctuations (Richards, 1992; Creighton, 1994). Currently available techniques for protein separation might not be suited for characterizing conformational subpopulations, since they are generally based on properties like molecular charge and/or size, which are expected to be similar in the subpopulations that give rise to energetic heterogeneity. Thus, it seems of considerable interest to develop experimental approaches which allow for characterization of persistent molecular heterogeneity in protein structures.

In this work, subunit dissociation and unfolding of rabbit muscle triose phosphate isomerase (EC 5.3.1.1, TIM)[1] induced by pressure were investigated. TIM is a homodimer of 26 kDa subunits, and its catalytic mechanism has been thoroughly studied (Albery & Knowles, 1976a,b; Knowles, 1991; Sun et al., 1992a; Lodi et al., 1994). The primary structures of TIM from different organisms are considerably conserved, and crystal structures show remarkable similarities among TIMs from various sources (Banner et al., 1975; Lolis et al., 1990; Wierenga et al., 1991, 1992; Kishan et al., 1994). Unfolding of TIM by GdnHCl, as well as refolding following dilution of the denaturant, has been investigated (Waley, 1973; Sawyer & Gracy, 1975; Zabori et al., 1980; Schnackerz & Gracy, 1991; Sun et al., 1992b; Garza-Ramos et al., 1992; Fernandez-Velasco et al., 1995). Here we show that dissociation of TIM subunits induced by pressure was accompanied by extensive disruption of structure of the monomers. Furthermore, our results indicate the existence of persistent heterogeneity in the ensemble of TIM dimers, leading to deterministic subunit dissociation. The possible functional relevance of this deterministic behavior is discussed.

[1] Abbreviations: AEDANS-TIM, triose phosphate isomerase labeled with 1,5-IAEDANS; DTT, dithiothreitol; FITC, fluorescein-5-isothiocyanate; fluorescein-TIM, triose phosphate isomerase labeled with FITC; FPLC, fast protein liquid chromatography; FRET, fluorescence resonance energy transfer; GdnHCl, guanidine hydrochloride; 1,5-IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TIM, triose phosphate isomerase (EC 5.3.1.1.); Tris, tris(hydroxymethyl)aminomethane.

MATERIALS AND METHODS

Materials. Rabbit muscle triose phosphate isomerase (type X) was from Sigma and was analyzed to confirm purity and homogeneity by native and denaturing PAGE and by size-exclusion FPLC (see Results). Guanidine hydrochloride and fluorescein-5′-isothiocyanate were from Sigma. 1,5-IAEDANS was from Molecular Probes (Eugene, OR). All other reagents were of the highest analytical grade available.

Fluorescent Labeling. AEDANS-TIM was prepared by reaction of 200 μM TIM (concentration in monomers) with 10 mM 1,5-IAEDANS. Labeling with fluorescein was carried out with 25 μM TIM (monomers) and 5 mM FITC. In either case (AEDANS-TIM or fluorescein-TIM) reaction was carried out in 100 mM Tris-HCl, pH 7.6, at room temperature in the dark for 12 h. Unreacted probe was removed after the reaction by gel filtration through NAP25 pre-packed Sephadex G-25 columns (Pharmacia). Labeling stoichiometries were determined using the following extinction coefficients: 76 mM^{-1} cm^{-1} at 495 nm for fluorescein, 5.7 mM^{-1} cm^{-1} at 336 nm for AEDANS, and 33 mM^{-1} cm^{-1} at 280 nm for TIM. For fluorescein-TIM, determination of protein concentration was corrected for fluorescein absorption by subtracting 40% of the absorbance at 495 nm from the measured absorbance at 280 nm. For both AEDANS-TIM and fluorescein-TIM, labeling ratios of about 1.7:1 (probe/monomer) were obtained.

Intrinsic Fluorescence Measurements. Fluorescence emission spectra were measured at 23 ± 0.5 °C on an ISS GREG 200 spectrofluorometer (ISS Inc., Champaign, IL). Excitation was at 280 nm with 16 nm band-pass for excitation and emission. Fluorescence anisotropy measurements were carried out on the same instrument with polarizers in excitation and emission ports (excitation and emission band-passes of 4 and 8 nm, respectively). Fluorescence measurements under pressure were performed using a pressure cell originally described by Paladini and Weber (1981), equipped with sapphire optical windows. Fluorescence anisotropy measurements in the pressure cell required quartz windows and were corrected for birefringence of the windows according to Paladini and Weber (1981). Temperature of the pressure cell was kept constant by means of a jacket connected to a circulating bath and was monitored by a telethermometer. Unless otherwise indicated, all experiments were carried out in buffer containing 100 mM Tris-HCl, pH 7.6, and 1 mM DTT.

Fluorescence spectral center of mass (average emission wavelength, λ_{av}) was calculated with software provided by ISS Inc. as

$$\lambda_{av} = \sum \lambda I(\lambda) / \sum I(\lambda) \qquad (3)$$

where λ is emission wavelength and $I(\lambda)$ is fluorescence intensity at wavelength λ. Shifts in spectral center of mass were converted into extent of dissociation/denaturation (α)

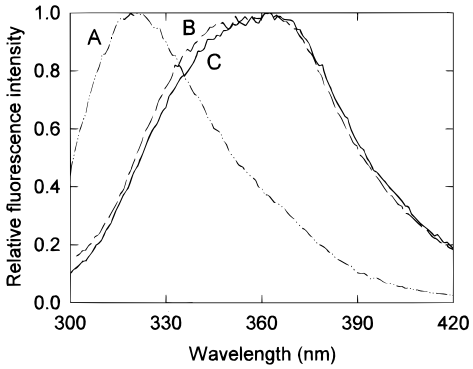

FIGURE 1: Intrinsic fluorescence emission spectra of native or unfolded TIM. (A) Emission spectrum of native TIM (3.2 μM); (B) emission spectrum of TIM (1.6 μM) incubated in 1.6 M GdnHCl for 2 h; (C) emission spectrum of TIM (3.2 μM) measured at 3.5 kbar after incubation for 1.5 h. Spectra are normalized for maximal emission intensity.

at each pressure according to (Weber, 1987)

$$\alpha_p = [1 + Q(\lambda_p - \lambda_U)/(\lambda_N - \lambda_p)]^{-1} \quad (4)$$

where λ_N and λ_U are spectral centers of mass of native or completely unfolded protein, respectively, λ_p is the spectral center of mass at pressure p, and Q is the ratio of fluorescence quantum yields of unfolded and native TIM.

Circular Dichroism. Ultraviolet circular dichroism was measured at 25 °C on a Jasco J 720 spectropolarimeter, using a 0.1 cm path-length quartz cell.

FRET Measurements under Pressure. The ratio of AEDANS fluorescence (emission at 476 nm) to fluorescein fluorescence (emission at 525 nm) when excited at 356 nm (F_{476}/F_{525}) was used as a measure of FRET efficiency between the two fluorophores. To verify whether this parameter was directly affected by pressure, regardless of changes in FRET, the following control experiment was performed. A sample containing a mixture of adducts of AEDANS-dithiothreitol and fluorescein-lysine was prepared at concentrations that were sufficiently low to exclude the possibility of significant energy transfer between fluorophores in solution. The ratio F_{476}/F_{525} was measured for this sample under pressure. The F_{476}/F_{525} ratio decreased more or less linearly as a function of pressure (approximately 2.6% per kbar; data not shown). All F_{476}/F_{525} values presented are corrected for this direct effect of pressure, which was small compared to the total change of F_{476}/F_{525} in the experiments.

Size-Exclusion FPLC. This was performed on a Pharmacia-LKB FPLC apparatus equipped with an LCC-500 controller, using a calibrated Superose 12 HR 10/30 column (10 × 300 mm; exclusion limits 1−300 kDa). The column was equilibrated with 50 mM Tris-HCl and 100 mM NaCl, pH 7.5. Elution was monitored by absorption at 280 nm. The flow rate was 0.5 mL/min. Column calibration was done with a set of eight proteins of known molecular weights.

RESULTS AND THEIR INTERPRETATION

Effects of GdnHCl and Pressure on the Intrinsic Fluorescence of TIM. Figure 1 shows intrinsic fluorescence spectra of native TIM (trace A), TIM in the presence of 1.6 M GdnHCl (trace B), and TIM under 3.5 kbar of hydrostatic pressure (trace C). Red-shifts of fluorescence emission are indicative of increased solvent exposure of tryptophan residues (Lakowicz, 1983). The spectra in the presence of GdnHCl and at high pressure indicate that under both conditions similar degrees of solvent exposure of tryptophan residues were obtained. In either case (GdnHCl or high pressure) the fluorescence quantum yield of TIM increased about 1.5-fold relative to native TIM (data not shown). Furthermore, spectral changes were accompanied by a decrease in fluorescence anisotropy from 0.14 to 0.04 or to 0.01 upon denaturation induced by GdnHCl or pressure, respectively (data not shown). The average fluorescence quantum yield of TIM was 92% that of L-tryptophan (not shown), indicating that the five tryptophan residues of TIM contribute appreciably to the emission and that the observed fluorescence changes reflect global conformational changes. At or above 1 M GdnHCl, dimeric TIM dissociates into unfolded monomers (Waley, 1973; Sawyer & Gracy, 1975; Zabori et al., 1980; Schnackerz & Gracy, 1991). Taken together the fluorescence changes indicate that application of pressure induced unfolding of TIM to a state qualitatively similar to (but not necessarily exactly the same as) that induced by GdnHCl.

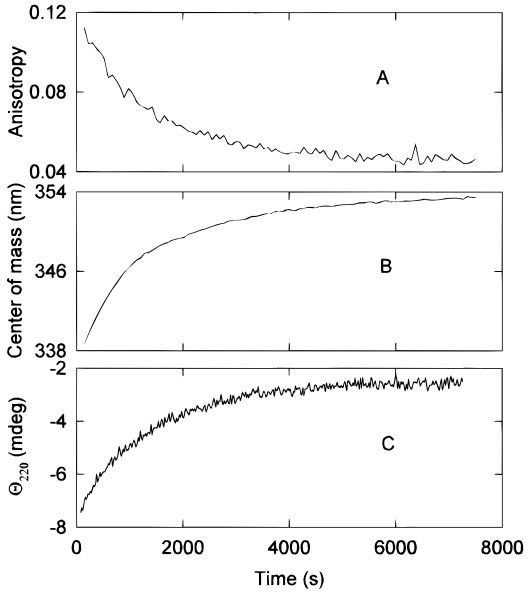

FIGURE 2: Kinetics of unfolding induced by GdnHCl. Unfolding was triggered as described in the text. The reaction mixture contained 50 mM $NaHCO_3$, pH 7.6, 1 mM DTT, 1 M GdnHCl, and 13.5 μM TIM, 25 °C. (Panel A) Intrinsic fluorescence anisotropy was measured at 75 s intervals. (Panel B) Spectral centers of mass of emission spectra (measured from 300−400 nm, 5 nm steps) were acquired at 75 s intervals. Excitation and emission were measured through vertically aligned polarizers. (Panel C) Circular dichroism (ellipticity at 220 nm) was measured at 75 s intervals.

Figure 2 shows the time course of unfolding of TIM by GdnHCl. In this experiment, after addition of GdnHCl the sample was quickly divided into two aliquots. One aliquot was placed in the fluorometer and both fluorescence anisotropy (Figure 2A) and emission spectra (Figure 2B) were simultaneously measured. The other aliquot was placed in a spectropolarimeter and ellipticity at 220 nm was recorded (Figure 2C). The dead-time for starting measurements after addition of GdnHCl was approximately 1 min. The time courses observed in panels A−C indicate that changes in fluorescence parameters take place in parallel with disruption of secondary structure of the protein.

In order to further compare denaturation of TIM by GdnHCl or pressure, we measured the kinetics of unfolding

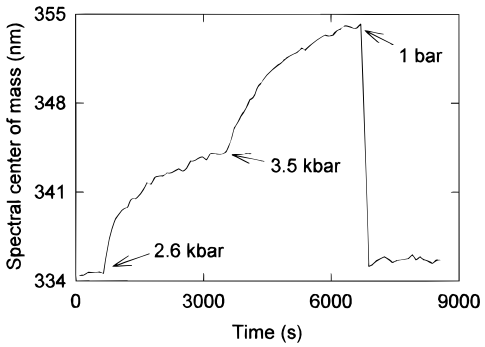

FIGURE 3: Kinetics of unfolding induced by hydrostatic pressure. Spectral centers of mass of emission spectra (measured from 300−400 nm, 5 nm steps) were acquired at 90 s intervals. Pressure was applied or released as indicated by arrows. [TIM] = 2.6 μM.

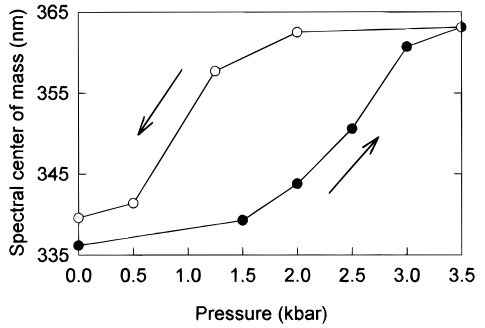

FIGURE 4: Spectral shift of intrinsic fluorescence of TIM versus pressure. Closed symbols: spectral centers of mass (emission spectra from 300 to 420 nm, 1 nm step) during compression. Open symbols: spectral centers of mass during decompression. [TIM] = 6.4 μM. Equilibration time at each pressure was at least 80 min.

following an increase in pressure from 1 atm to 2.6 kbar (which promoted approximately 50% red-shift of intrinsic fluorescence; see Figure 4), and after that to 3.5 kbar (Figure 3). Pressure-induced unfolding occurred in a time scale comparable to unfolding by GdnHCl (Figure 2). It is noteworthy that the rates of unfolding were similar upon raising pressure from 1 atm to 2.6 kbar or from 2.6 to 3.5 kbar, with half-times t_1 and t_2 of 15 and 20 min, respectively (Figure 3). This type of result has previously been observed in pressure-induced dissociation of higher order protein aggregates (tetramers or larger; Erijman & Weber, 1991) and has been related to deterministic subunit association. On the other hand, for stochastic equilibria the kinetics in experiments similar to that shown in Figure 3 display two clearly different half-times for two pressure jumps, the second being significantly faster (Silva et al., 1986; Erijman & Weber, 1991). So far, stochastic equilibria have been found for all dimers whose dissociation was investigated by application of pressure (Erijman & Weber, 1991; Silva & Weber, 1993).

Conformational Changes Induced by Pressure. Equilibrium data for unfolding of TIM as a function of pressure are shown in Figure 4. Intrinsic fluorescence spectra were measured after an equilibration of at least 80 min at each pressure. Fluorescence changes mainly took place between 1.5 and 3.0 kbar (Figure 4, closed symbols). Figure 4 (open symbols) also shows recovery of spectral changes when pressure was released stepwise toward 1 atm. A large hysteresis was observed in recovery, shifting $p_{1/2}$ (pressure at which $\alpha = 0.5$) approximately 1.5 kbar toward lower pressures. Hysteresis upon decompression has been reported in dissociation of other proteins (0−0.8 kbar for dimers, 0.3−1.0 kbar for tetramers; Silva & Weber, 1993) and has been attributed to loss of free energy of subunit association due to conformational changes of dissociated subunits (Weber, 1992). The large hysteresis observed for TIM (1.5 kbar) indicates large conformational changes in the separated subunits.

Control measurements of the ratio of Rayleigh scatter to fluorescence intensity during compression and decompression were performed. This ratio remained constant and low (<10%), ruling out formation of large, nonspecific protein aggregates during any part of the experiment. Size exclusion FPLC analysis confirmed the absence of protein aggregates, both before and after pressurization (not shown).

An interesting observation was that recovery of fluorescence properties of TIM after pressure release was dependent on the presence of dithiothreitol (DTT). Greater than 85% reversibility was observed in the presence of DTT (Figure 4). Conversely, in the absence of DTT reversibility was <45% (not shown). Reduced reversibility in the absence of DTT was confirmed by size exclusion FPLC analysis: nonpressurized TIM eluted as a single, sharp, symmetrical peak in the retention volume expected for a 52 kDa dimer, and a sample pressurized in the presence of DTT gave the same result. A sample pressurized in the absence of DTT exhibited an additional peak compatible with a monomeric species (not shown). Thus, incomplete recovery of fluorescence spectral shift upon decompression in the absence of DTT appears related to formation of monomers that are modified and have partially lost their capacity of forming dimers. Examination of crystal structures of TIM may provide an explanation for this observation. Crystal structures of yeast and chicken muscle TIM are known (Banner et al., 1975; Lolis et al., 1990). Though these enzymes share a primary sequence homology of only 53%, remarkable structural conservation is observed between them (RMS difference of 1.0 Å when C_α's are compared; Lolis et al., 1990). Since chicken muscle TIM has 89% sequence homology with rabbit muscle TIM, structural information from chicken TIM is likely to be applicable to rabbit TIM, of which the structure is not known. Rabbit TIM contains five free cysteine residues and no disulfide bridges. The crystal structure of chicken TIM reveals that cysteine residues are far apart and that their approximation for formation of disulfide bonds would require disruption of the inner α/β barrel. Thus, the reduced reversibility of fluorescence changes in the absence of DTT can be explained by formation of disulfide bonds under pressure, indicating disruption of the α/β barrel.

Lack of Concentration Dependence of Pressure Effects. For a stochastic equilibrium between dimer and monomer (whether folded or unfolded) a displacement of the pressure dissociation curve with changes in protein concentration is expected (Silva & Weber, 1993). In order to test whether this applied to TIM, we carried out pressurization at different protein concentrations. Figure 5 shows that pressure-induced fluorescence spectral changes were not dependent on the concentration of TIM. Data obtained at two concentrations of TIM differing by a factor of 20 were analyzed by fitting with a model reflecting possible stochastic equilibrium between monomer and dimer (eq 2). The same value of ΔV (59 mL) was recovered for both protein concentrations, while K_{atm} was found to be dependent on protein concentration (1.3

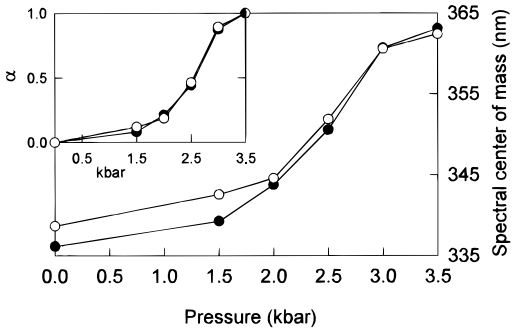

FIGURE 5: Lack of concentration dependence of spectral shift of intrinsic fluorescence of TIM. Pressure-induced red-shift of intrinsic fluorescence was measured at two different TIM concentrations: 6.4 μM (closed symbols) or 0.32 μM (open symbols). Conditions were as in Figure 4. (Inset) Extent of reaction α (calculated as described in Materials and Methods) as a function of pressure.

$\times 10^{-9}$ M^{-1} or 2.3×10^{-8} M^{-1} for 0.16 or 3.2 μM TIM, respectively). These results clearly reveal the incompatibility of the stochastic dissociation model with experimental data. Furthermore, ΔV was used to calculate the predicted displacement of the pressure curves upon dilution of the sample (Silva & Weber, 1993), as

$$\Delta p = (RT/\Delta V) \ln(C_2/C_1) \quad (5)$$

where C_2 and C_1 are the two concentrations of TIM used. Contrary to a calculated displacement of about 1.3 kbar, the experimental curves show no appreciable displacement. The lack of predicted concentration dependence again illustrates the inability of the stochastic dissociation model to account for the data.

Pressure-Induced Subunit Dissociation Revealed by FRET. The effects of pressure on TIM suggested that native dimers were dissociated into unfolded monomers. However, the lack of protein concentration dependence in the pressure curves might be explained if intrinsic fluorescence changes reflected a unimolecular unfolding reaction, rather than subunit dissociation. In order to directly determine if and when TIM dimers dissociated upon application of pressure, fluorescence resonance energy transfer (FRET) experiments were conducted. TIM solutions were separately labeled with 1,5-IAEDANS or FITC as a suitable donor/acceptor pair (Vanderkooi et al., 1977; Cheung, 1991). With this pair, the ratio of fluorescence intensities of donor to acceptor (F_{476}/F_{525}) was used to monitor subunit association/dissociation. When AEDANS-TIM and fluorescein-TIM were simply mixed together at a molar ratio of 1:1 at atmospheric pressure, no change in F_{476}/F_{525} could be detected for at least 6 h after mixing, indicating that spontaneous monomer exchange between the two populations of labeled dimers was negligible on this time scale (not shown).

When an equimolar mixture of AEDANS-TIM and fluorescein-TIM was submitted to a compression/decompression cycle, an irreversible decrease in F_{476}/F_{525} occurred. In the experiment shown in Figure 6 a sample was unfolded by 3.5 kbar of pressure and refolded by returning to 1 bar. The spectral center of mass of intrinsic fluorescence (upper panel) and the ratio F_{476}/F_{525} (lower panel) were simultaneously monitored as a function of time. The upper panel shows that kinetics of unfolding of labeled TIM after a pressure jump from 1 atm to 3.5 kbar were similar to unfolding of unlabeled TIM (Figure 3). In addition, im-

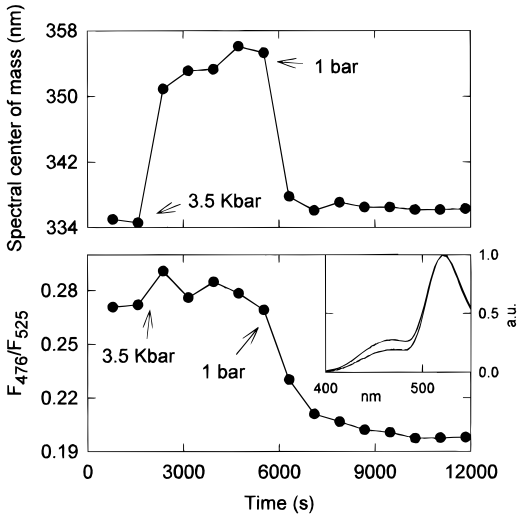

FIGURE 6: Kinetics of pressure-induced subunit exchange of AEDANS-TIM and fluorescein-TIM. Identical concentrations (5 μM) of AEDANS-TIM and fluorescein-TIM were mixed immediately before the experiment. The sample was then subjected to changes in pressure as indicated by arrows. (Upper panel) Spectral shift of intrinsic fluorescence (emission from 300 to 400 nm, 1 nm step). (Lower panel) Changes in FRET efficiency monitored by F_{476}/F_{525}. (Inset) Normalized emission spectra of the sample before compression (upper trace) and 2 h after decompression (lower trace).

mediate refolding of labeled TIM following pressure release from 3.5 kbar to 1 atm was observed. F_{476}/F_{525} (lower panel) showed no appreciable change during unfolding at 3.5 kbar, whereas during refolding it decreased, indicating subunit exchange and FRET between the two fluorescent probes.

An interesting point shown in Figure 6 is that different kinetics were observed for recovery of intrinsic fluorescence spectral shift and decrease of F_{476}/F_{525} ratio following pressure release from 3.5 kbar to 1 bar. While recovery of intrinsic fluorescence was complete within about 13 min (upper panel), subunit reassociation revealed by FRET occurred over a period of slightly over 1 h (lower panel). This result is in line with the model proposed for refolding of TIM after unfolding by GdnHCl (Waley, 1973; Zabori et al., 1980; Garza-Ramos et al., 1992). The unfolded state is monomeric and quickly refolds in a unimolecular reaction (revealed by the spectral center of mass of intrinsic fluorescence). Subsequent dimerization, during which FRET develops, is slower.

"Hybrid" TIM dimers in which one subunit was labeled with AEDANS and the other subunit with fluorescein were obtained through a compression/decompression cycle (Figure 6). Figure 7 shows kinetics of unfolding/subunit dissociation of such "hybrid" dimers. Panel A shows red-shift of intrinsic fluorescence, and the inset shows extent of reaction α calculated as described in Materials and Methods. The decrease in energy transfer (increase in F_{476}/F_{525}) shown in panel B reflects dissociation of TIM subunits. The inset shows normalized values of α. Comparison of the two insets (panels A and B) shows that pressure-induced dissociation and unfolding occurred in parallel.

In addition to the kinetics shown in Figure 7, equilibrium FRET data for subunit dissociation as a function of pressure were obtained (data not shown). These measurements showed that subunit dissociation (revealed by an increase in F_{476}/F_{525}) occurred between 1.5 and 3.5 kbar, in parallel with

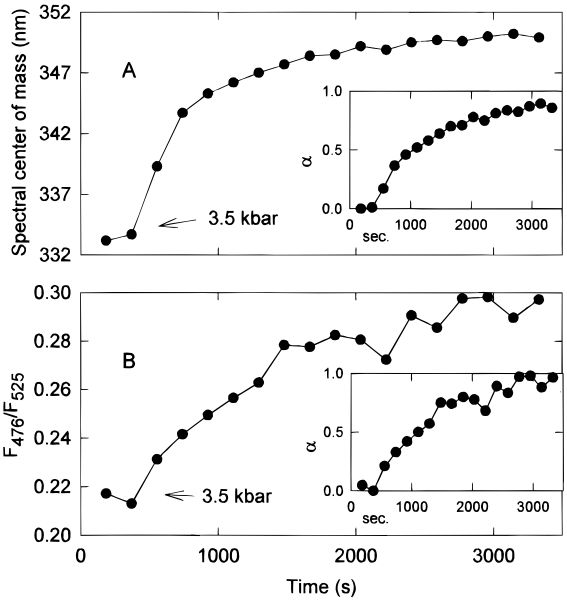

FIGURE 7: Kinetics of unfolding and blocking of FRET. A sample containing equimolar concentrations of AEDANS-TIM and fluorescein-TIM (5 μM) was previously subjected to a pressure cycle (as shown in Figure 6) and then diluted to 0.67 μM and pressurized at 3.5 kbar at the time indicated by an arrow. (Panel A) Changes in spectral center of mass of the intrinsic fluorescence (emission from 300−400 nm, 5 nm step). (Inset) Extent of reaction α. (Panel B) Changes in FRET (as in Figure 6). (Inset) Extent of reaction α, normalized from the FRET data.

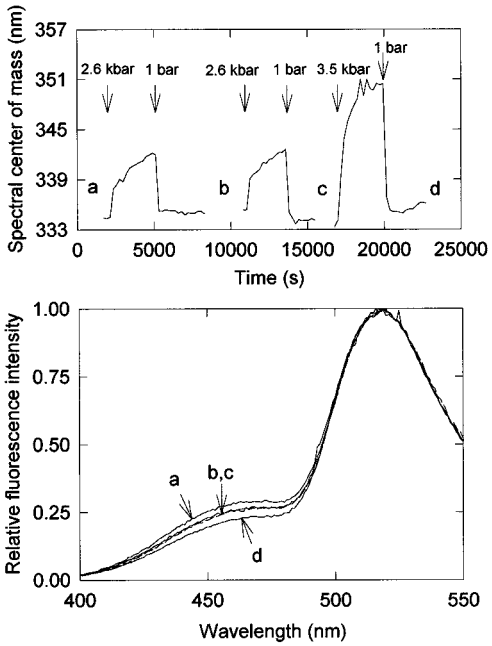

FIGURE 8: Consecutive compression−decompression cycles of a mixture of AEDANS-TIM and fluorescein-TIM. (Upper panel) A sample containing equimolar (2.2 μM) AEDANS-TIM and fluorescein-TIM was prepared immediately before the experiment and submitted to pressure changes as indicated by arrows. Unfolding was monitored by spectral shift of intrinsic fluorescence (emission from 300 to 400 nm, stepsize 5 nm). At the times indicated by letters a−d, FRET spectra were also measured as shown in the lower panel. (Lower panel) FRET spectra were measured before application of pressure (a), after the first pressure cycle (b), after the second pressure cycle (c, dashed line), and after the final pressure cycle (d).

unfolding (revealed by intrinsic fluorescence red-shift). Equilibrium measurements also confirmed the lack of dependence of subunit dissociation on the concentration of TIM (not shown).

The data presented so far suggested that pressure induced a nonstochastic transition from native TIM dimer to unfolded monomer. Similar anomalous behavior was previously reported for higher order protein aggregates and was attributed to deterministic heterogeneous behavior of the particles (Silva & Weber, 1988; Silva et al., 1989; Bonafé et al., 1991). The following experiment was conducted to determine whether pressure-induced dissociation of TIM was deterministic. A freshly made 1:1 mixture of AEDANS-TIM and fluorescein-TIM was submitted to two consecutive cycles of compression/decompression sufficient to promote approximately 50% unfolding (at 2.6 kbar) and to a final cycle that caused complete unfolding (at 3.5 kbar) (Figure 8, upper panel). Between pressure cycles sufficient time (one hour) was allowed for refolding and reassociation of the protein.

Before the experiment (Figure 8, lower panel, trace a) and after each consecutive pressure cycle (traces b, c, and d) fluorescence spectra were measured to monitor energy transfer. After the first compression/decompression cycle the ratio of donor to acceptor fluorescence had decreased (trace b), indicating that part of AEDANS-TIM and fluorescein-TIM dimers had dissociated and exchanged during reassociation. The second pressure cycle induced unfolding to a similar extent as the first cycle, as judged from intrinsic fluorescence spectral shift (Figure 8, upper panel). However, exchange of donor-labeled and acceptor-labeled subunits did not increase, as revealed by a constant ratio in donor to acceptor fluorescence (Figure 8, lower panel, traces b and c). The third pressure cycle finally led to full unfolding (Figure 8, upper panel) and complete subunit exchange (lower panel, trace d).

From these observations we conclude that those dimers that resisted dissociation and did not exchange subunits during the first pressure cycle again resisted dissociation during the second cycle. Only the fully unfolding third pressure cycle was able to promote complete subunit exchange. This means that a fraction of TIM dimers was persistently more resistant toward pressure than dimers that had already exchanged during the first cycle. Similar experiments to that shown in Figure 8 were repeated four times with different preparations of labeled TIM, giving the same results and indicating the reproducibility of these observations.

The results shown in Figure 8 possibly explain the origin of independence of pressure dissociation on protein concentration: dimer particles are divided in subpopulations that do not (or very slowly) exchange during the time and under the conditions of the experiments. These subpopulations differ in resistance toward pressure. Since the total percentage of unfolding is determined by properties of individual particles, protein concentration has no influence on this percentage.

In search for the origin of the observed energetic heterogeneity we analyzed the TIM preparations used by size exclusion FPLC and both nondenaturing and denaturing PAGE. In size exclusion chromatography, the sample eluted as a sharp, symmetric peak with the retention expected for dimeric TIM (52 kDa) (data not shown). SDS−PAGE showed a single, sharp band at 26 kDa (data not shown), indicating that the energetic heterogeneity could not be

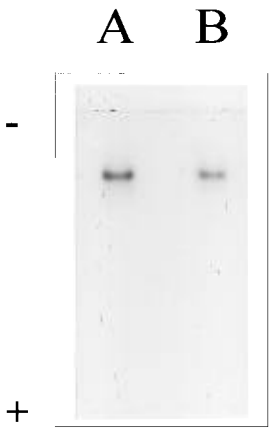

FIGURE 9: Nondenaturing PAGE analysis of TIM. Polyacrylamide (6%) gel electrophoresis was carried using the buffer system of Laemmli (1970) in the absence of SDS. Gels were stained with Coomassie Blue. Lanes A and B contained 3.5 or 2.5 μg of TIM, respectively.

explained by chemical heterogeneity. It is well established that mammalian TIM undergoes specific deamidation at asparagines 15 and 17 of each subunit (Yuan et al., 1981; Sun et al., 1992). These asparagine residues are located at the subunit interface, and deamidation leads to structural destabilization, which renders the enzyme susceptible to proteolytic degradation (Sun et al., 1992). The rate of deamidation of mammalian TIM is enhanced by incubation at 37 °C and alkaline pH (9.5) and appears to be markedly increased by active site occupancy and enzyme turnover (Sun et al., 1992). However, under the conditions employed in our study (pH 7.6, 23 °C, in the absence of substrate) no significant deamidation can be detected for several hours of incubation [see Sun et al. (1992)]. In any case, we have further examined whether partial deamidation could be the origin of energetic heterogeneity revealed in our experiments. Each deamidation introduces one negative charge into TIM, and multiple deamidation can be conveniently observed as multiple equally spaced bands on nondenaturing PAGE (Yüksel & Gracy, 1986; Sun et al., 1992). Nondenaturing PAGE analysis of our samples revealed a single band indicating that deamidation was not detected in our conditions (Figure 9). These results suggest that the energetic heterogeneity does not reflect heterogeneity in particle charge or size or chemical modification (deamidation) of the protein. We therefore ascribe the origin of energetic heterogeneity of TIM dimers to small, persistent differences in conformations of individual particles.

CONCLUSIONS

Pressure Destabilization of TIM. It is generally considered that dissociation of oligomeric proteins induced by pressure below 3−5 kbar can be described without taking into account the influence of pressure on unimolecular unfolding reactions. One important question was whether this assumption was valid for the anomalous pressure dissociation of TIM, namely, whether dissociation/unfolding was driven by pressure destabilization of intersubunit interactions or by destabilization of intramolecular tertiary and/or secondary interactions within the subunits. This question was motivated by our observations that (1) extensive conformational changes were produced by application of pressure on TIM (Figures 1, 3, and 4), suggesting full unfolding of the monomers and (2) there was no detectable dependence of the effect of pressure on protein concentration (Figure 5), in contrast to that observed with all other protein dimers dissociated by pressure so far (Silva & Weber, 1993).

Folded monomers (i.e., species having aromatic residues shielded from the solvent but no FRET between labeled subunits) were not observed either in kinetic (Figure 7) or in equilibrium pressure unfolding experiments (data not shown). This means that monomer structure was unstable under pressure, causing them to unfold concomitantly with dissociation. If the effect of pressure on TIM were due to destabilization of quaternary interactions only (followed by conformational changes of the monomers), this would mean that monomer instability could be extrapolated to atmospheric pressure. However, evidence indicates that under native conditions isolated monomers prefer a folded conformation over a random coil structure: studies of regain of enzymatic activity at different protein concentrations during refolding of TIM that had been previously unfolded by GdnHCl showed that the isolated monomer rapidly refolds in a unimolecular reaction and then dimerizes in a slower time scale (Waley, 1973; Zabori et al., 1981; Garza-Ramos et al., 1992; Sun et al., 1992b; Fernandez-Velasco et al., 1995). In line with this model is our observation that, during refolding of the pressure-induced monomer, protection of aromatic residues of TIM from solvent recovers quickly, while establishment of FRET is slower (Figure 6). Furthermore, recent studies have shown that stable, compactly folded monomeric TIM variants can be created by appropriate site-directed mutagenesis of residues at the subunit interface (Borchert et al., 1994, 1995). In one case (Borchert et al., 1994) 15 residues comprising the major interface loop were replaced by another eight-residue fragment, generating a stable, active monomer which was called monotim. A stable, monomeric variant of TIM has also been created by replacing histidine 47 by an asparagine residue (Borchert et al., 1995). Thus, it appears that the structure of the monomer is inherently stable at atmospheric pressure. Combined with the fact that during pressure dissociation no folded monomers could be detected, these observations indicate that pressures required to dissociate TIM destabilize the folded conformation of the isolated monomer.

Considering the above, it seems unlikely that extensive disruption of structure of the α/β barrel of TIM is solely caused by rearrangements of protein structure due to loss of quaternary interactions upon dissociation. Such rearrangements, indirectly induced by pressure, have been described as a conformational drift of the isolated subunits (Silva & Weber, 1993). We suggest that there is significant energetic contribution of direct pressure destabilization of tertiary and/or secondary structure to the overall process of pressure dissociation/unfolding of TIM. It is interesting to note that recent theoretical work indicates that pressure-induced protein transitions are driven by preferential destabilization of weak hydrophobic bonds (Weber, 1993). A large number of such bonds are present in the hydrophobic core of the TIM barrel (Lolis et al., 1990).

Pressure destabilization of TIM monomers contradicts the initial belief in high pressure biophysics that pressures below 5 kbar in general do not disrupt secondary or tertiary protein structure. A few cases have been described of unimolecular pressure transitions at low pH (Brandts et al., 1970; Hawley, 1971; Royer et al., 1993), at high temperatures (Zipp &

Kauzmann, 1973), or with mutant proteins (Royer et al., 1993; Eftink et al., 1991). In addition, the monomeric coat protein isolated from the capsid of bacteriophage P22 becomes pressure labile after having lost its stabilizing quaternary interactions (Prevelige et al., 1994).

Subunit Exchange under Pressure. Dealing with the kinetics of pressure-induced subunit exchange, Erijman and Weber (1991) pointed out three reasons why these kinetics might be slow: (1) reduction of the rate of association by pressure; (2) conformational drift after dissociation causing low reassociation affinity; and (3) high order of the aggregate, requiring the unlikely event of many particles meeting in one moment for reassociation. Especially the last reason was thought to be important, since a correlation was found between the order of the aggregate and the deterministic character of pressure-induced dissociation (Erijman & Weber, 1991).

For dimeric TIM the decreased affinity of association due to large changes in structure of the monomer (indicated by a large hysteresis in the compression−decompression cycle; Figure 4) possibly explains the slow kinetics of reassociation under pressure. Slow dynamics of monomer−multimer exchange under pressure are required for detection of persistent heterogeneity of the native state in the type of experiment depicted in Figure 8 (Erijman & Weber, 1991). In the anomalous TIM transition, slow monomer−dimer exchange allowed us to reveal heterogeneity of the native dimer. Thus, the apparent correlation between existence of heterogeneous subpopulations and order of the aggregate (Erijman & Weber, 1991) might be due to the generally slower monomer−multimer kinetics of higher aggregates. Persistent structural heterogeneity in proteins might be a more general phenomenon than has previously been thought.

Deterministic Character of TIM Dimers. TIM dimers have very slow kinetics of subunit exchange at atmospheric pressure, as demonstrated by the stability of the ratio F_{476}/F_{525} after mixing of AEDANS-TIM and fluorescein-TIM (no detectable subunit exchange after 6 h; data not shown). Furthermore, native TIM is extremely stable toward spontaneous dissociation under physiological conditions, as dilution down to 70 pM is unable to produce any monomers (Zabori et al., 1980). This means that the equilibrium association constant K_{ass} is larger than 10^{12} M^{-1}. Indeed, Lolis et al. (1990) estimated K_{ass} for yeast TIM to be of the order of 10^{16} M^{-1}, on the basis of buried surface areas between monomers. In terms of a stochastic equilibrium of association, K_{ass} equals the ratio between the rate constants for monomer association and dimer dissociation (k_{ass}/k_{diss}). By investigating refolding of rabbit muscle TIM from GdnHCl solutions Zabori et al. (1980) found $k_{ass} = 3 \times 10^5$ M^{-1} s^{-1} at 0 °C. Using the lower limit of the measured value of K_{ass} of 10^{12} M^{-1} we calculate that k_{diss}^{-1} is longer than 40 days, while the estimated value of K_{ass} of 10^{16} M^{-1} predicts k_{diss}^{-1} to be about a thousand years. This suggests that, once folded, the TIM dimer will not undergo dissociation during its lifetime in the cell.

Native TIM is quite resistant to prolonged incubation with various proteases, while unfolded TIM is vulnerable to proteolysis (Waley, 1973; Sun et al., 1992). Also, the five cysteine residues of native TIM are stable and do not form disulfide bridges, while the unfolded monomer in the absence of a disulfide reducing agent readily forms irreversibly modified species that are unable to refold properly (Figure 4 and text). This indicates that spontaneous, temporary unfolding of native TIM dimers does not occur, since this would lead to gradual digestion of native TIM in the presence of proteases or irreversible formation of disulfide bridges.

These considerations outline a "frozen", deterministic model of stability of TIM. In this view, folding and dimerization are unique events in the lifetime of an individual protein particle, and no dynamic nature needs to be attributed to these processes. Once an individual monomer is folded and has associated with a partner, it never again during its lifetime loses this individual partner, creating a stable, resilient dimer. This view opposes a dynamic stochastic model of constant renewal of particles from "old" ones that transiently dissociated and/or unfolded in equilibrium. The deterministic condensation model is compatible with persistent conformational heterogeneity of the native dimer (Figure 8): during folding of TIM subtle structural differences in individual particles might be generated, and, because of lack of exchange through dynamic pathways, these individual differences get "frozen" into the structures, giving rise to a persistently heterogeneous ensemble of dimers.

Regarding the possible biological significance of deterministic stability, we note that the role of TIM is a very simple one, namely, making sure that isomerization between dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate is always near its thermodynamic equilibrium. For this purpose, TIM should provide a sufficient and stable enzymatic capacity. TIM is not a key regulatory enzyme in the glycolytic pathway and therefore does not need effector controlled conformational flexibility, nor should any control on metabolic fluxes be mediated through the concentration of TIM in the cytoplasm. The characteristics of the deterministic condensation model that we propose are in line with these requirements: during folding/dimerization TIM dimers become "frozen" in a set of conformations that are resistant to proteolysis, thus supplying the cell with a stable enzymatic capacity. *In vivo*, chemical modification (deamidation of asparagine residues) of these remarkably stable species is possibly necessary in order to enable proteolytic turnover of TIM (Sun et al., 1992).

Recognition of deterministic behavior of protein structures may have consequences for practical problems in structural biology. For example, Lolis et al. (1990) proposed that drugs or peptides binding at the subunit interface of TIM might be designed as effective and specific inhibitors of TIM from pathogenic organisms, as enzyme activity is lost upon dissociation of the dimer. One would indeed expect a dimer in stochastic equilibrium with monomers to be inactivated by an inhibitor whose binding energy to the monomer exceeds the self-association energy. In contrast to this, the deterministic condensation model predicts that, regardless of the binding energy of such an inhibitor, it would never have a chance to perturb dimerization, since there will be no monomer (in dynamic equilibrium with native dimers) with which the inhibitor could form an inactive complex.

In 1992, Weber wrote: "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 are reasons to expect, or suspect, that at some level in the biological organization determinism at the molecular level replaces stochastic behavior. At present we have no clear idea as to how such deterministic behavior arises or the

degree of complexity of the system at which it becomes important." Our finding of deterministic behavior in dimeric TIM may help outlining the answer to the latter question, while future investigations of this relatively simple system might help solving the former.

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