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Thermodynamics and Kinetics of the Pressure Unfolding of Phosphoglycerate Kinase[†]

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ABSTRACT: Due to the relationship between compressibility and volume fluctuations, high-pressure studies provide vital insight into protein dynamics and function. Most high-pressure experiments were performed on small and fast folding proteins or model peptides. Here we show that a detailed kinetic study is necessary to extract reliable information from the high-pressure-induced structural conversion of large, slowly folding proteins. The pressure-jump unfolding kinetics of yeast phosphoglycerate kinase was recorded at pressures between 50 and 150 MPa. The time dependence of the conformational state of the protein was followed by tryptophan fluorescence measurements from 30 s to 2 h. The observed changes were described by a three-state model, and the volume change and the activation volume as well as the midpoint pressure of the transitions between the folded, intermediate, and unfolded states were determined. An interesting feature of the pressure unfolding of phosphoglycerate kinase was that the unfolding process speeds up with increasing pressure, which is the consequence of negative activation volumes for the folded → intermediate, intermediate → unfolded, and unfolded → intermediate transitions.

A unified view of protein folding and dynamics should be general enough to interpret the diverse experimental findings of the field. Thermodynamics offers such a universal approach. To gain experimental insight into the thermodynamics of proteins, stability and folding studies alter one or more of the intensive parameters (temperature, pressure, etc.) and detect the shift of the equilibrium or the kinetics of the repartitioning between structural states (*1*−*3*). By following structural changes induced by the variation of an intensive quantity, one can gain information about the conjugate extensive parameter (e.g., entropy change, volume change).

High pressure is often used as external perturbation in protein studies (*4*−*14*). Equilibrium pressure measurements allow to determine the volume and the compressibility difference between conformational states, while kinetic studies provide the activation volume of the transitions between them (*15*, *16*). Due to the close relationship between compressibility and volume fluctuations, such studies provide important information to understand dynamics and biological function (*17*, *18*).

In a search for simplicity of the experimental and theoretical work, most high-pressure experiments were performed on small single domain proteins and on model peptides. The experimental methodology was also adapted to the investigation of small protein molecules, in which equilibrium is reached relatively fast. Most proteins, however, are larger than 100 amino acids, have multidomain structure, and fold or unfold through kinetic intermediates. There is a need for a deeper understanding of the folding of large proteins, as well as the adaptation of the experimental procedures to their study.

Yeast phosphoglycerate kinase (PGK)[1] is a 415 residue long enzyme which is comprised of two domains. Several experiments addressed the sequential order of the folding of the domains and the kinetics of the folding and unfolding of this protein (*19*, *20*). Beechem and co-workers have shown that the unfolding of PGK happens through an intermediate which has a largely unfolded carboxy-terminal and an altered amino-terminal domain (*21*). PGK was one of the first proteins in which highly nonexponential folding kinetics was observed (*22*, *23*). Elaborate analysis of the refolding of PGK gave experimental evidence of the existence of hierarchic folding energy landscapes, without making any a priori assumptions about the thermodynamics of the protein (*24*).

Using PGK as model, here we show that a detailed kinetic study is necessary to gain insight into the high-pressure-induced structural events of large proteins, and we present a thermodynamic model of the pressure unfolding of PGK.

METHODS

A histidine-tagged version of yeast (*Saccharomyces cerevisiae*) phosphoglycerate kinase was expressed and purified as described earlier (*23*). Polyacrylamide gel (Nu-PAGE Bis-Tris gel; Invitrogen) electrophoresis showed that the purified protein was virtually contamination-free. The purified protein was concentrated to 40 μM, dialyzed in 50 mM Tris, 1 mM EDTA, and 1 mM DTT, pH 7.8, buffer, and stored at −80 °C until use.

PGK samples were placed in a temperature-controlled high-pressure optical cell (Unipress) equipped with sapphire windows and exposed to pressures between 50 and 150 MPa at 4 °C, as previously described (*25*).

Structural changes of PGK were followed by tryptophan fluorescence excited at 295 nm with 5 nm bandwidth. Emission spectra were recorded between 302 and 400 nm, with 1 nm step size and 1 nm bandwidth, on a FluoroLog3 luminometer

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[1]Abbreviations: PGK, yeast (*Saccharomyces cerevisiae*) phosphoglycerate kinase (UniProtKB/Swiss-Prot accession number P00560); EDTA, ethylenediaminetetraacetic acid disodium salt; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

(Jobin-Yvon). The center of weight of the spectra was calculated using the formula:

$$CG = \frac{\sum_{i=1}^{n} I_i \lambda_i}{\sum_{i=1}^{n} I_i}$$

where I_i is the measured fluorescence intensity at wavelength λ_i.

RESULTS

High-pressure-induced unfolding of PGK was monitored by recording the fluorescence of the two native tryptophan residues (W308 and W333) excited at 295 nm wavelength. Figure 1A shows the emission spectrum of PGK 30, 1470, and 7200 s after initiating unfolding by a jump to 85 MPa hydrostatic pressure. The W333 is surface exposed in the native structure and does not undergo fluorescence change upon unfolding of PGK (*22*, *23*). Because of this, the red shift of the fluorescence emission of PGK upon unfolding is rather small. The spectral shift was quantitated by the calculation of the center of gravity of the observed spectra. The unfolding kinetics of PGK was constructed by recording 37 emission spectra from 30 to 7200 s. Figure 1B presents the shift of the center of gravity of the tryptophan emission as a function of time. Similar unfolding kinetics were measured at 13 different pressures. None of these unfolding kinetics could be fit by a simple two-state unfolding mechanism, which is analogous with earlier findings by denaturant stopped flow measurements (*21*). Combining all the kinetic traces, we obtained a surface which shows the position of the center of gravity as the function of two parameters: hydrostatic pressure and time elapsed after the pressure jump (Figure 1C).

To gain a deeper insight into the thermodynamics of the unfolding of PGK, the experimentally obtained surface was fit by the three-state mechanism proposed by Beechem and co-workers (*21*):

$$F \underset{k_{IF}}{\overset{k_{FI}}{\rightleftharpoons}} I \underset{k_{UI}}{\overset{k_{IU}}{\rightleftharpoons}} U$$

where F, I, and U denote the folded, intermediate, and unfolded structures, respectively.

The differential equation describing the above model was analytically solved assuming that all of the protein was in the folded state at time $t = 0$. The position of the center of gravity of the emission spectrum was calculated from the time dependence of the states F, I, and U. Since conformational changes are accompanied by volume changes, the k_{FI}, k_{IF}, k_{IU}, and k_{UI} monomolecular rate constants depend on the pressure:

$$k = k^0 \exp(\Delta V p / RT)$$

where k denotes the monomolecular rate constant, k^0 the monomolecular rate constant at 0 MPa, ΔV the activation volume, p the pressure, R the universal gas constant, and T the absolute temperature.

The equality received for the experimentally expected surface for the time and pressure dependence of the center of gravity contained 13 parameters: the center of gravity of the spectra corresponding to the folded, intermediate, and unfolded states (CG_F, CG_I, CG_U), the relative fluorescence efficiency of the intermediate and unfolded states compared to the folded state (Φ_I, Φ_U), the rate constants of the forward and backward reactions connecting the three states of the protein at 0 MPa (k^0_{FI}, k^0_{IF}, k^0_{IU}, k^0_{UI}), and the activation volume of these transitions ($\Delta V^{\#}_{FI}$, $\Delta V^{\#}_{IF}$, $\Delta V^{\#}_{IU}$, $\Delta V^{\#}_{UI}$).

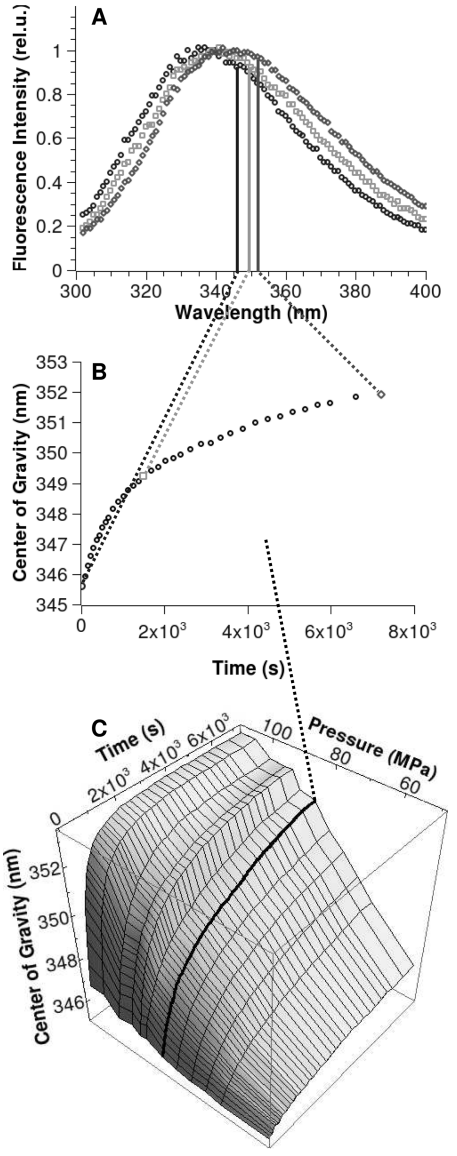

FIGURE 1: Pressure and time dependence of the fluorescence emission of PGK. Panel A shows the spectrum of the fluorescence emission excited at 295 nm in 20 μM PGK 30, 1470, and 7200 s after a pressure jump to 85 MPa from atmospheric pressure. Panel B indicates how the measured spectra contribute a point in the fluorescence-detected unfolding kinetics of the protein. Panel C combines all of the measured kinetic traces to construct a pressure and time dependence of the center of gravity of the emission spectrum of the PGK. The kinetics observed at 85 MPa is highlighted. All measurements were done at 4 °C in 50 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT, pH 7.8.

The center of gravity of the spectra containing folded, intermediate, and unfolded protein molecules was calculated as follows:

$$CG = \frac{[F]CG_F\Phi_F + [I]CG_I\Phi_I + [U]CG_U\Phi_U}{[F]\Phi_F + [I]\Phi_I + [U]\Phi_U}$$

where [F], [I], and [U] denote the concentration of the folded, intermediate, and unfolded species, respectively.

The theoretical model was fit to the experimentally obtained surface using Marquardt's algorithm, and the $\Delta V^{\#}$ activation volumes were determined. Figure 2A shows the best fitting theoretical surface of the experiments shown in Figure 1C, while Figure 2B represents the residuals obtained as the difference between the measured and fitted surfaces. Table 1 shows the p^m_{FI}, p^m_{IU} midpoint pressures at which the backward and forward

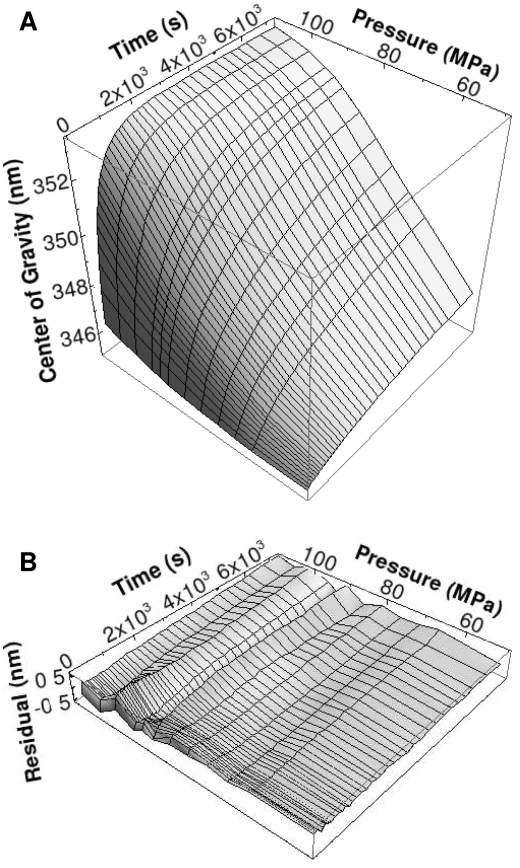

FIGURE 2: Model fit of the pressure and time dependence of the fluorescence emission of PGK. Panel A shows the best fit to the data shown in Figure 1C. Panel B represents the residuals received as the difference between the measured (Figure 1C) and calculated (panel A) center of gravity.

Table 1: Thermodynamic and Kinetic Parameters of the Pressure Unfolding of PGK Obtained from the Fit of the Measured Surface by a Three-State Model[a]

p^{m}_{FI} (MPa)	70.5 ± 4.7
p^{m}_{IU} (MPa)	63.4 ± 6.4
k^{m}_{FI} (s^{-1} M^{-1})	$6.6 \times 10^{-5} \pm 2.0 \times 10^{-5}$
k_{m}^{IU} (s^{-1} M^{-1})	5.2 ± 4.4
$\Delta V^{\#}_{FI}$ (cm^3/mol)	−185 ± 7.3
$\Delta V^{\#}_{IF}$ (cm^3/mol)	114 ± 67
$\Delta V^{\#}_{IU}$ (cm^3/mol)	−288 ± 44
$\Delta V^{\#}_{UI}$ (cm^3/mol)	−126 ± 70
ΔV_{FI} (cm^3/mol)	299 ± 6.3
ΔV_{IU} (cm^3/mol)	162 ± 41

[a]Here, p^{m}_{FI} and p^{m}_{IU} denote the midpoint pressures at which the forward and backward reactions between the folded and intermediate (k^{m}_{FI}) as well as between the intermediate and unfolded species (k_{m}^{IU}) become equal. $\Delta V^{\#}_{FI}$, $\Delta V^{\#}_{IF}$, $\Delta V^{\#}_{IU}$, and $\Delta V^{\#}_{UI}$ are the activation volumes obtained for the transitions between the folded and intermediate, intermediate and folded, intermediate and unfolded, and unfolded and intermediate states, respectively. ΔV_{FI} and ΔV_{IU} are the volume differences between the folded and intermediate and the intermediate and unfolded states, respectively.

reactions become equally fast, the k^{m}_{FI}, k^{m}_{IU} actual reaction rates at these pressures, the ΔV_{FI}, ΔV_{IU} volume differences between the different states, and the $\Delta V^{\#}_{FI}$, $\Delta V^{\#}_{IF}$, $\Delta V^{\#}_{IU}$, $\Delta V^{\#}_{UI}$ activation volumes determined from the fit.

DISCUSSION

In the present work, we uncover both the dynamic and the equilibrium behavior of the unfolding of yeast phosphoglycerate kinase (PGK), a large multidomain protein. To our knowledge, this is the first combined pressure study in which the pressure dependence of the folding/unfolding kinetics was systematically recorded in more than 1 dozen pressure steps.

The pressure-induced unfolding of PGK was investigated before (*26*), but the kinetic aspect of the unfolding was not included in that work. We believe that this is the reason for the higher stability of the protein observed in those experiments. We found that the characteristic time for the onset of equilibrium changed from several thousand seconds at 50 MPa to several hundred seconds at 120 MPa. At p^{m}_{FI} = 70.5 MPa pressure both the forward and backward reactions of the F ⇌ I transition have a rate constant of k^{m}_{FI} (s^{-1} M^{-1}) = 6.6×10^{-5} s^{-1} M^{-1}, which corresponds to roughly 2 h characteristic time for the onset of the equilibrium between the two states at this pressure. The onset of the equilibrium between the folded and unfolded states is pressure dependent, becoming faster as the pressure increases. The equilibration between the intermediate and unfolded states was orders of magnitude faster in the studied pressure range. An inadequately short waiting time between the measurements of an "equilibrium" study will result in an apparently higher unfolding pressure and sharper transition between the folded and unfolded states. The importance of the kinetic aspect in the pressure-induced protein folding/unfolding has been demonstrated by Lange's group as well (*27*, *28*). They found that the pressure-jump-induced conformational changes of photosystem II can be as slow as a few hundred or thousand seconds.

The pressure where the unfolding of PGK was observed is in the lower region of the typical unfolding pressures (*29*−*32*). Pressure denaturation of small proteins typically takes place in the 500−1000 MPa range, but pressure stabilities below 200 MPa have also been reported (*27*).

To gain a thermodynamic view of the unfolding mechanism of PGK, we wanted to fit the measured data with a simple model. A fit with a two-state mechanism (F ⇌ U), however, gave large differences between the measured and the predicted kinetics. We found that a simple three-state model (F ⇌ I ⇌ U) can describe the measurements within the error of the experiment. This is in accordance with the findings of Beechem and co-workers, who used a similar model to describe the chemical unfolding of PGK (*21*). A global fit of the whole data set resulted in the activation volumes and equilibrium volume changes of the conformational transitions (Table 1). Figure 3 shows the pressure dependence of the equilibrium distribution of PGK between the native, intermediate, and unfolded structures, as calculated from the model with the obtained thermodynamic parameters. The thin black lines visualize the effect of the error of the thermodynamic parameters on the calculated equilibrium. In this representation it is possible to discern a weakly populated intermediate at 70 MPa. This also underlines the importance of kinetic studies in revealing the transient accumulation of important intermediate states, which may appear only in small amount in the equilibrium studies.

It is obvious that the equilibrium volume change of both steps of the unfolding (F → I and I → U) should be negative, since pressure drives the system toward the unfolded state. The volume change accompanying the unfolding (F → U) reaction of several proteins has already been determined (*33*). Typical values found for globular proteins range between −50 and −100 cm^3/mol. The unfolding of myoglobin was found to cause a −70 cm^3/mol volume change (Smeller and Heremans unpublished results). A larger unfolding volume change of −150 to −188 cm^3/mol was

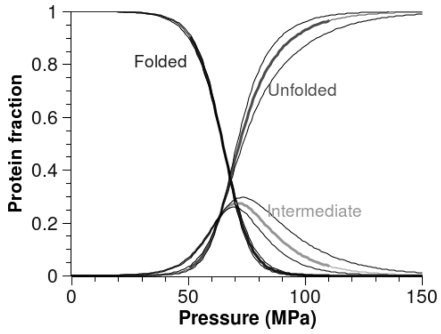

FIGURE 3: Calculated equilibrium fractions of different states as a function of pressure. The fraction of the folded, intermediate, and unfolded protein structures was calculated in the 0−150 MPa range using the parameters shown in the Table 1. These parameters were obtained from a global model fit to pressure-jump measurements in the 50−110 MPa range (thick line). Thin black lines indicate the error in the fractions of the different species that comes from the error of the thermodynamic parameters of the model indicated in Table 1.

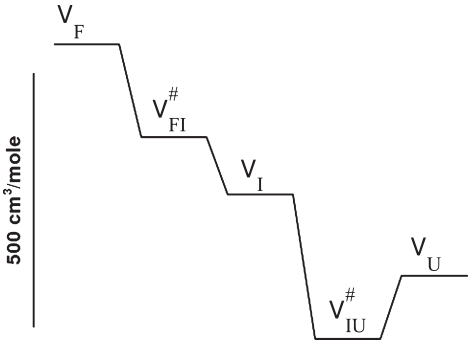

FIGURE 4: Volume changes during the two-state unfolding of PGK. V_F, V_I, and V_U denote the volume of the folded, intermediate, and unfolded states, respectively. $V^{\#}_{FI}$ and $V^{\#}_{IU}$ represent the volume of the activation state for the folded → intermediate and intermediate → unfolded transitions, respectively.

obtained for the tryptophan repressor at different temperatures (*34*). In both cases, the volume change increased if the temperature was lowered, easily explained based on the elliptic $p-T$ phase diagram (Hawley theory) of proteins (*30*, *35*). It can be shown that if the $\Delta V_{FU}(p,T) = 0$ line of the $p-T$ phase diagram has higher slope (dp/dT) than the $\Delta G(p,T) = 0$ phase boundary curve (*30*), cooling leads to a larger volume change. It has been suggested that the underlying mechanism behind this is the increased thermal volume of the unfolded state, which also has a larger thermal expansion coefficient (*36*). The volume changes found for the F → I and the I → U transitions of PGK (Table 1) are somewhat larger than the typical values obtained earlier for globular proteins, probably for two reasons. First, PGK is bigger than the globular proteins previously studied (*33*). With its 415 residue length and 45 kDa molecular mass, PGK is more than twice as big as myoglobin (17 kDa) or the tryptophan repressor (22 kDa). The large size of the protein implies also larger protein−solvent interface. It is known that the protein surface is covered by a dense layer of water, which decreases the volume of the whole system. Transient increase of the protein surface in the transition states could also increase the amount of the bound water, which can contribute to the activation volume. Second, the relatively low temperature of 4 °C where our experiments were carried out probably also increased the volume change. The low temperature was needed to avoid the irreversible aggregation after the pressure treatment, which was observed at room temperature (data not shown). It is difficult to estimate the temperature dependence of the activation volumes, since thermal activation data are only available for the folded state and not for the unfolded and transition states. The temperature dependence of the unfolding volume change was measured for a few proteins. In the case of the tryptophan repressor the unfolding volume changes by 20% when the temperature is raised from 10 to 40 °C (*33*).

In most cases, large conformational changes of proteins are cross-barrier reactions, involving an activated state. If the volume of the activated state is larger than that of the native structure (positive activation volume), increasing pressure will slow the reaction. A smaller than native volume of the activated state (negative activation volume), on the other hand, will speed up the unfolding of the protein, as pressure is increased. Three of our activation volumes are negative (Table 1), which means that the corresponding processes speed up as pressure is increased. It is obvious at first glance in Figure 1C that the overall unfolding rate dramatically increased as we increased the pressure. It is rather difficult to compare our results with previous studies, because of the lack of unfolding activation volume measurements in the literature. Activation volumes were mostly determined for catalytic reactions of enzymes (*37*, *38*). The activation volume measured in these systems is typically in the range of 0 −20 cm^3/mol. Kunugi and co-workers measured notably high negative activation volumes (−75 cm^3/mol) for the activity of thermolysin (*39*). Recently, Solymosi and co-workers found that the reorganization of the macrodomains of NADPH:protochlorophyllide oxidoreductase after phototransformation is associated with an activation volume of 35 and 39 cm^3/mol at 40 and 30 °C, respectively. The activation volumes obtained for PGK unfolding (Table 1) are large compared to the results listed above, which can be rationalized if we compare the size of the structural rearrangements. The conformational change, and consequently the activation volume of protein unfolding, is obviously much larger than that linked to catalytic activity or reorganization of domain structure.

CONCLUSIONS

In the study of the pressure-induced structural changes of large proteins it may be necessary to record a systematic kinetic map of the reaction, because slow conformational transitions can prevent equilibrium measurements.

A three-state model could describe well the kinetic map of the PGK unfolding and yielded the volume change and activation volume (Figure 4) as well as the midpoint pressure of the transitions between the folded, intermediate, and unfolded states (Table 1).

An interesting feature of the pressure unfolding of PGK is that the activation volumes of the unfolding reaction are negative for the folded → intermediate, intermediate → unfolded, and unfolded → intermediate transitions.

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