Temperature- and Pressure-Induced Unfolding and Refolding of Ubiquitin: A Static and Kinetic Fourier Transform Infrared Spectroscopy Study†

Heinz Herberhold and Roland Winter*

Physical Chemistry I, University of Dortmund, Otto Hahn Strasse 6, D-44227 Dortmund, Germany

Received November 5, 2001; Revised Manuscript Received December 17, 2001

ABSTRACT: Temperature- and pressure-induced denaturation of the protein ubiquitin was investigated using FT-IR spectroscopy. On the basis of IR spectral parameters, different states are distinguished and a pressure/temperature−stability diagram of the protein has been determined. The evolution of the secondary structures with temperature illustrates that the band intensities of disordered structures decrease at the expense of the formation of intermolecular β-sheets at 83 °C, pD 7, and ambient pressure, with the population of intramolecular β-sheets and α-helices remaining essentially unchanged. At ambient temperature ($T = 21$ °C) and pD 7, ubiquitin denatures at 5.4 kbar. Contrary to other proteins studied so far, features of secondary structure of ubiquitin remain distinct at high pressure, suggesting that part of this small protein rearranges and does not unfold to disordered structures. The secondary structural changes during compression and decompression are fully reversible, and no aggregation occurs. With corresponding measurements of the pressure-induced denaturation of ubiquitin at different temperatures, a p/T−stability diagram of ubiquitin could be obtained. Furthermore, kinetic FT-IR measurements were carried out using the pressure-jump relaxation technique. The denaturation process is shown to occur on a time scale which is about twice as long as that of the renaturation process, and both processes are much slower than the unfolding−refolding kinetics observed at ambient pressure.

Most studies dealing with protein unfolding and denaturation have been carried out at atmospheric pressure using various physicochemical perturbations such as temperature, pH, or chemical denaturants as experimental variables (*1*). Besides temperature and chemical potential, pressure represents a further important thermodynamic variable, and the volume changes that have been found, which are associated with the observed transition, correspond to a fundamental physical parameter of the protein−solvent system, the volume of unfolding, $\Delta V_u°$.[1] Compared to varying temperature, which produces simultaneous changes in both density (or volume) and thermal energy, the use of pressure to study protein solutions perturbs the environment of the protein in a continuous, controlled way by changing intermolecular distances. As such, pressure represents a potentially very informative approach to the study of protein structure and stability. A more complete description of the pressure-denatured states of proteins, therefore, should be quite useful for understanding protein folding phenomena. The structural properties of the denatured state that is achieved may depend on the method employed to perturb the native structure.

It has long been known that the application of hydrostatic pressure results in a change in the native protein structure (*2*, *3*) due to a decrease in the total volume of the protein−solvent system upon denaturation. A number of reviews on the effects of pressure on proteins discuss these volume changes in greater detail (*4*−*8*). Also, in a recent theoretical study, the pressure dependence of hydrophobic interactions has been shown to be consistent with the observed pressure denaturation of proteins (*9*).

In addition to being an important thermodynamic variable, pressure can be used as a valuable means of triggering and investigating the kinetics of the unfolding−refolding transition of proteins. The use of pressure as a kinetic variable for studying the kinetics of biomolecular phase transformations is a field which is developing rapidly (*8*, *10*). Generally, the pressure-jump technique has several advantages over other trigger mechanisms. First, it does not significantly change the solvent properties; second, pressure propagates rapidly so that sample homogeneity is no problem, and third, pressure jumps can be performed bidirectionally, i.e., in the pressurization and depressurization direction. There is yet another reason for systematic studies on the pressure unfolding of proteins related to the new theoretical approach by Bryngelson and Wolynes (*11*), which uses a statistical characterization of the energy landscape in analyzing protein folding. Different modes of denaturation, including pressure, may correlate differently with the roughness of the energy scale and slope of the folding funnel, and small changes in

† This work was supported by the Deutsche Forschungsgemeinschaft (DFG) and the Fonds der Chemischen Industrie.

* To whom correspondence should be addressed: Physical Chemistry I, University of Dortmund, Otto Hahn Str. 6, D-44227 Dortmund, Germany. Phone: +49 231 755 3900. Fax: +49 231 755 3901. E-mail: winter@steak.chemie.uni-dortmund.de.

[1] Abbreviations: FT-IR, infrared spectroscopy; IR, infrared; NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering; DAC, diamond anvil cell; Snase, staphylococcal nuclease; Rnase, ribonuclease; BPTI, bovine pancreatic trypsin inhibitor; τ, relaxation time; T, temperature; p, pressure; $\Delta G_u°$, standard Gibbs energy change of unfolding (denaturation); $\Delta V_u°$, volume change of unfolding (denaturation); k_u, reaction constant of unfolding (denaturation); $\Delta V_u^{\ddagger}$, activation volume of unfolding (denaturation); $\Delta V_f^{\ddagger}$, activation volume of folding (renaturation).

10.1021/bi012023b CCC: $22.00
Published on Web 01/18/2002

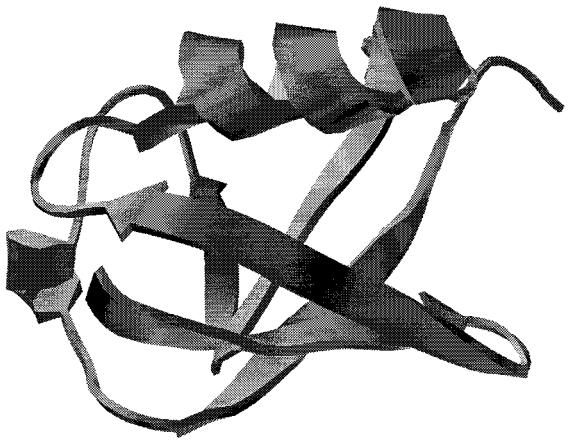

FIGURE 1: Tertiary structure of ubiquitin.

solvent temperature or pressure might lead to continuous changes in the populations of molecular conformations during folding. Such subtlety is not recognized in the classical modeling where specific intermediate structures and pathways are invoked.

In this paper, we used high-pressure FT-IR spectroscopy to study the reversible pressure-induced unfolding and denaturation of ubiquitin and we compare the results with those obtained for the temperature-induced transition. This work is a continuation of our studies on pressure-induced folding−unfolding reactions of proteins (*12*−*15*). For example, our recent pressure-jump relaxation FT-IR and SAXS studies on Snase indicated that a partial unfolding to disordered structures occurs at pressures above ∼2 kbar, and these studies provided a picture of the transition state in which a significant fraction of the protein has been removed from contact with the solvent. To characterize the nature of the transition state in the pressure-induced folding−unfolding reaction of ubiquitin, we also performed pressure-jump kinetic FT-IR studies on this protein.

Ubiquitin is a single-domain protein without disulfide bonds or bound cofactors that is bound covalently by a conjugating enzyme to proteins that are targeted for degradation by the 26S proteosome (*16*). This 76-amino acid protein with a molecular mass of 8433 Da exists in virtually all eukaryotes, including plants. The sequence is highly conserved, being identical in animal sources from insects to humans (*17*). The X-ray structure shows the extremely compact nature of the protein with approximately 87% of the polypeptide chain being involved in hydrogen-bonded secondary structure, involving a 5-strand mixed β-sheet (residues 1−7, 11−17, 40−45, 48−50, and 64−72), 3.5 turns of an α-helix (residues 23−34), a short piece of a 3_{10}-helix (residues 56−59), and 7 reverse turns (residues 7−11, 18−21, 37−40, 45−48, 51−54, 57−60, and 62−65) connecting the secondary elements (*18*, *19*). The β-sheet has a characteristic left-handed twist, and the α-helix fits into the cavity formed by the sheet. The protein crystalline state contains about 16% α-helices, 37% β-sheets, and 37% other structures (Figure 1) (*18*, *19*). On the other hand, circular dichroism (CD) experiments reveal 16% α-helices, 55% β-sheets, and 29% other structure elements (*20*). Four of the five β-strands form a β-sheet plane; above this plane is arranged the α-helix. Ubiquitin folds very rapidly under ambient-pressure conditions. Kinetic analysis of the renaturation of ubiquitin by nuclear magnetic resonance (NMR) spectroscopy using rapid mixing techniques indicates a three-state transition. The first 80% of ubiquitin folds with a time constant of ∼8 ms; 12% folds with a time constant of ∼100 ms, and 8% folds within ∼10 s (*21*).

MATERIALS AND METHODS

Ubiquitin was purchased from Sigma Chemical Co. and used without further purification. The protein was dissolved at a concentration of 5% (w/w) in 10 mM TRIS buffer (Sigma) with 99.9% D_2O (Sigma) at pD 7 for the high-pressure experiments. For the temperature-dependent experiments, we used a 10 mM phosphate buffer. The pD of the solution was adjusted to 7.0 using DCl. Before the measurements were performed, the solution was heated to 55 °C for 1 h to allow deuterium exchange of the labile amide protons of ubiquitin.

FT-IR spectroscopy has proven to be a powerful technique for determining the secondary structure elements under high-pressure conditions. The amide I band (between 1600 and 1700 cm^{-1}) was detected, which is mainly associated with the carbonyl stretching vibration (85%) of the amide groups of the amino acids, and which is directly related to the backbone conformation and hydrogen bonding pattern of the protein. The FT-IR spectra were recorded with a Nicolet MAGNA 550 spectrometer equipped with a liquid nitrogen-cooled MCT (HgCdTe) detector. The infrared light was focused by a spectra bench onto the pinhole of a diamond anvil cell (DAC) with type IIa diamonds (*22*). Each spectrum was obtained by co-adding 256 scans at a spectral resolution of 2 cm^{-1} and was apodized with a Happ−Genzel function. The sample chamber was purged with dry and carbon dioxide free air. Powdered α-quartz was placed in the pinhole of the steel gasket, and changes in pressure were quantified by the shift of the quartz photon band at 695 cm^{-1} (*23*). An external glycol thermostat was used for the pressure- and temperature-dependent measurements to control the temperature to within 0.1 °C. The equilibration time before recording spectra at each temperature and pressure was 20 min. Pressure jumps were obtained by turning the spring-loaded screw of the DAC. Within the accuracy of the experiment, an adiabatic temperature change in the course of the pressure jumps could not be observed within the dead time of the measurement (∼10 s), as the sample volume was very small (∼10 nL) and the DAC was effectively thermostated.

Fourier self-deconvolution of the IR spectra was performed with a resolution enhancement factor of 2.4 and a bandwidth of 12 cm^{-1}. The fractional intensities of the secondary structure elements were calculated from a band-fitting procedure assuming a Gaussian−Lorentzian line shape function (*13*, *14*). For the time-dependent measurements, 32 scans were co-added to one spectrum. We analyzed the I band by calculating the difference between the absorbance measured at time *t* and the absorbance measured before the pressure jump (*24*). It should be mentioned that the results of this method need to be treated with caution for the determination of the absolute values of the secondary structure elements, because their transition dipole moments may be different. Fewer problems arise, however, from the application of the fitting method to the study of changes in

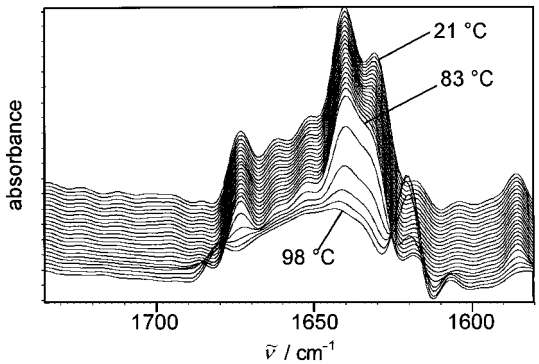

FIGURE 2: Deconvoluted FT-IR spectra of ubiquitin as a function of temperature at ambient pressure (pD 7).

Table 1: Wavenumbers of Secondary Structure Elements in the Amide I′ Band Region (*25*, *26*)

secondary structure element	wavenumber (cm^{-1})
intermolecular β-sheets	~1620
	~1685
intramolecular β-sheets	~1630
	~1674
disordered structures	~1641
α-helices	~1652
turns	~1663

conformations of the protein backbone, which was the primary goal of this study.

To determine the standard Gibbs energy change $\Delta G_u°$ and the volume change $\Delta V_u°$ $[=(\partial \Delta G_u°/\partial p)_T]$ of a two-state pressure-induced unfolding process, the equilibrium profiles obtained from the FT-IR spectra can be fitted to the relation $\Delta G_u° = -RT \ln K_{eq,u} = -RT \ln[(I_f - I_p)/(I_p - I_u)]$ using the data points at each pressure, I_p, and the asymptotic values of the fractional band intensities due to particular secondary structural elements for the folded and unfolded states, I_f and I_u, respectively.

RESULTS

Temperature and Pressure Dependence of the Amide I′ FT-IR Spectrum of Ubiquitin. Figure 2 shows the deconvoluted IR spectra of ubiquitin between 20 and 100 °C at pD 7.0 and ambient pressure after H−D exchange at 55 °C. As can be clearly seen, significant changes in the spectral shape of the amide I′ band occur above ~83 °C. At 20 °C, the maximum of the amide I′ band (the prime indicates that the solvent is D_2O) in the FT-IR spectrum appears at 1639 cm^{-1}. Up to ~83 °C, the band maximum increases slightly and then it shifts drastically to higher wavenumbers (Figure 2). Above 83 °C, two new bands appear, around 1620 and 1684 cm^{-1}. These bands are characteristic of the formation of intermolecular β-sheet structures and are thus indicative of the aggregation of the protein at these temperatures. The sharp rise in the amide I′ band maximum at 83 °C indicates the location of the temperature-induced unfolding of the protein.

The fractional intensities of the secondary structure elements were calculated as described above using the assignment to IR bands as summarized in Table 1 (*25*, *26*). The evolution of secondary structures of ubiquitin with temperature is illustrated in Figure 4. Within experimental error, the fractional intensities of the intramolecular β-sheets

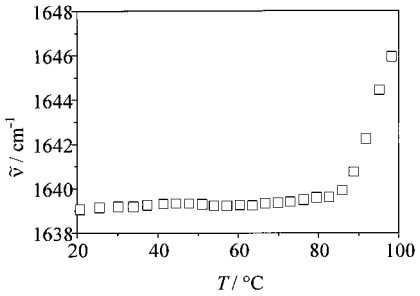

FIGURE 3: Temperature dependence of the maximum of the amide I′ band of ubiquitin at ambient pressure (pD 7).

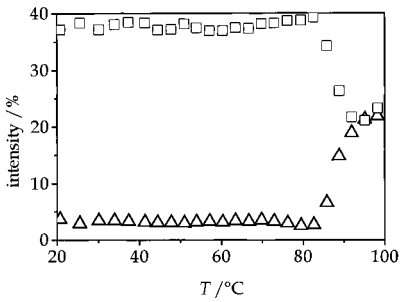

FIGURE 4: Relative intensities of secondary structure elements [(□) disordered and (△) intermolecular β-sheets] of ubiquitin as a function of temperature at ambient pressure (pD 7).

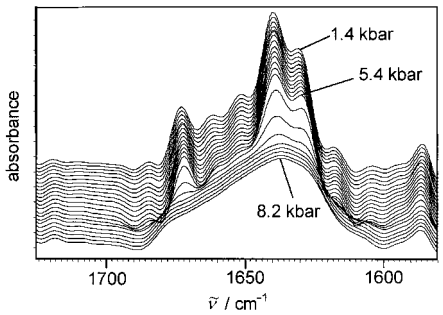

FIGURE 5: Deconvoluted FT-IR spectra of ubiquitin at different pressures at 21 °C and pD 7.

(~36%), α-helices (~12%), and turns (~15%) are constant, and are therefore not included in the plot. The temperature-induced increase in the band intensities of intermolecular β-sheets is clearly visible above 83 °C, which is accompanied by a corresponding decrease in band intensities of disordered (nonperiodic) structures. Without being significantly unfolded, ubiquitin aggregates by forming new intermolecular β-sheets when heated above 83 °C. An analysis of the IR band at 1516 cm^{-1}, which is due to the vibrational band of tyrosine 59, which is located at the end of the α-helix, indicates a small change in the tertiary structure of ubiquitin at ~83 °C only (data not shown).

To investigate also the effect of pressure on the secondary structures of ubiquitin, we measured the pressure-induced changes in the amide I′ region of the IR spectrum in the pressure range of 1 bar to 9 kbar. The pressure dependence of the deconvoluted FT-IR spectra of deuterium-exchanged ubiquitin at pD 7.0 and 21 °C is shown in Figure 5. Above ~5.4 kbar, drastic changes in the spectral shape of the amide I′ band are clearly visible. With increasing pressure, the amide I′ band maximum decreases from 1638.7 cm^{-1} at 1.4 kbar to 1637.6 cm^{-1} at 5 kbar, i.e., at a rate of −0.5 cm^{-1}/kbar (Figure 6), the negative slope being expected to occur

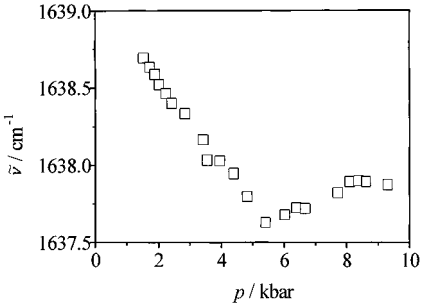

FIGURE 6: Pressure dependence of the amide I′ band of ubiquitin at 21 °C and pD 7.

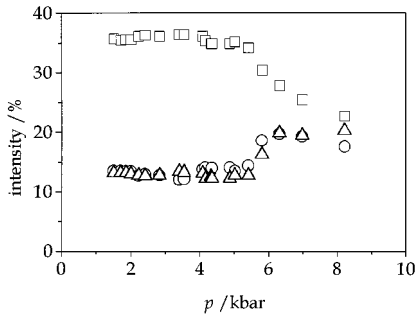

FIGURE 7: Relative intensities of secondary structure elements [(□) disordered, (○) α-helical, and (△) turns] of ubiquitin at different pressures at 21 °C and pD 7.

for hydrogen-bonded carbonyl groups (*27*). It should be clear that these continuous changes in pressure-induced wavenumber shifts only apply to the elastic regime, i.e., in the absence of conformational changes to the protein. Above 5.4 kbar, the band maximum shifts to larger wavenumbers. At this pressure, the pressure-induced denaturation of the protein takes place at 21 °C.

The pressure-induced changes in the fractional intensities of the different amide I′ subbands are presented in Figure 7. Within experimental error, the fractional intensity of intramolecular β-sheets is constant, and is therefore not included in the plot. The pressure-induced denaturation as judged by changes in the fractional band intensities also begins at 5.4 kbar, and the transition is complete at 6.4 kbar. At the transition, the fractional intensities of α-helices and turns slightly increase, whereas the population of disordered (nonperiodic) structures decreases. Assuming that the changes taking place in the transition region can be assigned to a simple two-state unfolding process, we can calculate the volume change during denaturation ($\Delta V_u°$) to be -50 ± 20 mL/mol.

Corresponding measurements of the pressure-induced denaturation of ubiquitin at different temperatures result in a $p/T-$stability diagram, which is shown in Figure 8 (the onset temperatures and pressures of the denaturing transition are given). At 31 °C, the point of maximum pressure stability is reached. Above 40 °C, the pressure-induced denaturation and partial unfolding are accompanied with some degree of aggregation (data not shown) during compression, as can be inferred from the occurrence of aggregation bands around 1620 and 1684 cm^{-1}.

Time Dependence of the Amide I′ FT-IR Spectrum of Ubiquitin. To study the unfolding and refolding kinetics of ubiquitin, pressure jumps from 3 to 8 kbar and in the reverse direction were performed. The dead time of these high-

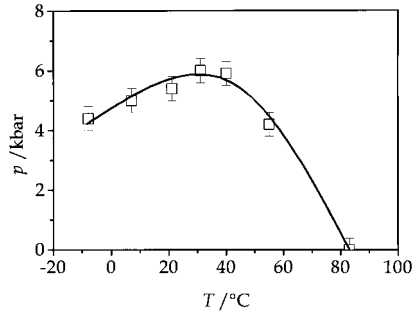

FIGURE 8: $p/T-$stability diagram of ubiquitin at pD 7.

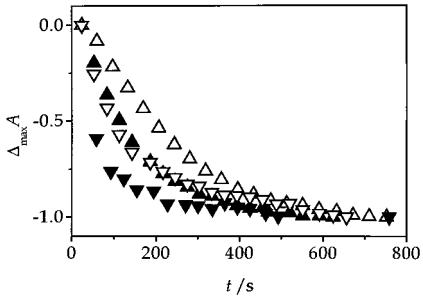

FIGURE 9: Intensity changes of the amide I′ band maximum of ubiquitin after pressure jumps at 21 °C [(△) from 3 to 8 kbar and (▽) from 8 to 3 kbar] and 31 °C [(▲) from 3 to 8 kbar and (▼) from 8 to 3 kbar] (pD 7).

pressure kinetic FT-IR experiments was 10 s. Figure 9 shows the intensity of the amide I′ band maximum of the difference spectra at 21 and 31 °C after pressure jumps from 3 to 8 kbar and from 8 to 3 kbar, respectively. The first data point is set to zero on the difference absorbance scale, and the end point for $t \rightarrow \infty$ is set to -1. The renaturation curves (pressure jumps from 8 to 3 kbar) are mirrored on the *x*-axis. The data reveal a significant change in the IR spectra on the time scale of 800 s after the pressure jump. A fast burst phase may not be resolved here. The relaxation times τ can be fit to single exponentials. The calculated relaxation time for denaturation of ubiquitin (τ) at 21 °C is 242 s; at 31 °C, it has decreased to 132 s. For the renaturation, the relaxation time (τ) is 115 s at 21 °C and 44 s at 31 °C. As expected, the denaturation and renaturation process at 31 °C is faster than that at 21 °C, and the renaturation process is a factor of ~2 faster than the denaturation process at the same temperature. An additional kinetic experiment with a pressure jump from 4.5 to 8 kbar, i.e., in a pressure region which is probably mainly determined by the reaction constant of unfolding, $k_u(p)$, revealed a much shorter relaxation time of ~23 s only at 21 °C, indicating that the activation volume of unfolding $\Delta V_u^‡$ is negative. Assuming for simplicity that τ is essentially determined by k_u, and assuming a simple two-state process, we can calculate an apparent activation volume of unfolding of approximately -38 mL/mol. This would indicate that the transition state has a structure which is close to the conformation of the pressure-denatured state. The corresponding activation volume for refolding, $\Delta V_f^‡$, has to be positive then, which would be consistent with a rate-limiting step for refolding involving minor secondary structural rearrangements and some degree of dehydration.

DISCUSSION AND CONCLUSIONS

The FT-IR measurements indicate that ubiquitin partially unfolds and aggregates above 83 °C. During aggregation,

the ubiquitin molecules form intermolecular β-sheet structures; α-helical structures are not involved in this process. The temperature-induced aggregation process turns out to be irreversible. This finding is in agreement with the results of Wintrode et al. (*28*−*30*), who pointed out that above pH 4.0 ubiquitin tends to aggregate and that ubiquitin has a transition temperature of ∼90 °C at pH 4.0.

Reversible pressure denaturation experiments were carried out at different temperatures in the pressure range from 1 bar to 9 kbar. The results indicate that the protein unfolds at pressures above ∼5 kbar in the temperature range from 7 to 55 °C. Interestingly, the pressure-denatured state contains more α-helical and turn structures than the native state, and the increase in the levels of these secondary structure elements is accompanied by a decrease in the levels of disordered (nonperiodic) structures. Application of high pressure thus results in no complete unfolding forming essentially disordered structures, but instead leads to a partial rearrangement of the secondary structure elements of this particular protein. This effect must arise from an overall volume decrease of the system. This is a surprising finding, because normally protein−water systems decrease their volume by a decrease in the levels of ordered structures to form, at least partially, unfolded states. In general, no random coil structures are observed for pressure-induced denatured states of proteins, however. To date, only a few other proteins which form ordered structures under high pressure are known. One example is the model protein L-polylysine, where pressure induces a transformation from an unordered to an α-helical form (*31*, *32*). In this case, the finding has been explained by the effect of pressure on the ionization of the side chains. A change in periodic conformation is also seen in BPTI (bovine pancreatic trypsin inhibitor), a 58-amino acid protein. Under pressure, it seems to transform α-helical and disordered structures into a β-sheet structure (*27*).

Interestingly, also in kinetic studies, a stable, partially structured state, the A state, has been observed for ubiquitin at pH 2 under particular solvent conditions. Two-dimensional NMR data revealed that ubiquitin in the A state contains a subset of the interactions present in the native state, in which a partially structured α-helix covers the hydrophobic face of the β-sheets, and this α-helix seems to be considerably more flexible than that in the native state. It has been suggested that the A state could plausibly lie on the folding pathway, with the A to native transition being a late step in the folding process (*33*). Pressure has indeed been shown in a series of studies now to be able to stabilize conformational substates which are observed in kinetic folding studies under ambient-pressure conditions (*8*, *34*). This could as well be the case in this particular situation.

The p/T−stability diagram of ubiquitin exhibits an elliptic-like curvature which is typically found for monomeric proteins, such as Snase and Rnase A (*8*, *12*−*14*).

To study the pressure-induced folding−unfolding kinetics of ubiquitin, additional pressure-jump relaxation experiments were performed across the phase transition region of the protein. The IR data reveal significant changes in the shape of the IR spectra after the pressure jump. Within the accuracy of the experiment, these data fit well to a model assuming one exponential relaxation process. The calculated relaxation time for denaturation of ubiquitin (τ) at 21 °C is 242 s; at 31 °C, it has decreased to 132 s. The renaturation process is considerably faster. The corresponding relaxation times are 115 s at 21 °C and 44 s at 31 °C. As the first data point in our current high-pressure experiments indicates that at 21 °C 45% of the ubiquitin molecules are denatured, we cannot rule out a faster burst phase during denaturation. For comparison, the process of folding induced by chemical denaturants is much faster. Kinetic analysis of the renaturation of ubiquitin by nuclear magnetic resonance (NMR) spectroscopy using rapid mixing techniques indicates a three-state transition; 80% of ubiquitin folds with a time constant of 8 ms, 12% with a time constant of 100 ms, and the final 8% with a time constant of 10 s (*21*). Our data clearly indicate that the pressure-induced relaxation times for denaturation and refolding are much slower. In our case, the kinetics is determined by the activation volume of the denaturation and refolding reaction, and not by activation energies as in the ambient-pressure studies using rapid mixing techniques.

REFERENCES

1. Fersht, A. (1999) *Structure and Mechanism in Protein Science*, W. H. Freeman and Co., New York.
2. Bridgman, P. W. (1914) *J. Biol. Chem. 19*, 511−512.
3. Suzuki, K., Miyosawa, Y., and Suzuki, C. (1963) *Arch. Biochem. Biophys. 101*, 225−228.
4. Weber, G., and Drickamer, H. (1983) *Q. Rev. Biophys. 16*, 89−112.
5. Heremans, K., and Smeller, L. (1998) *Biochim. Biophys. Acta 1386*, 353−370.
6. Silva, J. L., and Weber, G. (1993) *Annu. Rev. Phys. Chem. 44*, 89−113.
7. Gross, M., and Jaenicke, R. (1994) *Eur. J. Biochem. 221*, 617−630.
8. Winter, R., and Jonas, J., Eds. (1999) *High Pressure Molecular Science*, NATO ASI Series E 358, Kluwer Academic Publishers, Dordrecht, The Netherlands.
9. Hummer, G., Grade, S., García, A. E., Paulaitis, M. E., and Pratt, L. R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 1552−1555.
10. Winter, R., Erbes, J., Czeslik, C., and Gabke, A. (1998) *J. Phys.: Condens. Matter 10*, 11499−11518.
11. Bryngelson, J. D., Onuchic, J. N., Socci, N. D., and Wolynes, P. G. (1995) *Proteins: Struct., Funct., Genet. 21*, 167−195.
12. Panick, G., and Winter, R. (2000) *Biochemistry 39*, 1862−1869.
13. Panick, G., Vidugiris, G. J. A., Malessa, R., Rapp, G., Winter, R., and Royer, C. A. (1999) *Biochemistry 38*, 4157−4164.
14. Panick, G., Malessa, R., Winter, R., Rapp, G., Frye, K. J., and Royer, C. (1998) *J. Mol. Biol. 275*, 389−402.
15. Desai, G., Panick, G., Zein, M., Winter, R., and Royer, C. A. (1999) *J. Mol. Biol. 288*, 461−475.
16. Varshavsky, A. (1997) *Trends Biochem. Sci. Rev.*, 383−387.
17. Rechsteiner, M. (1984) *Ubiquitin*, Plenum Press, New York.
18. Vijay-Kumar, S., Bugg, C. E., Wilkinson, K. D., and Cook, W. J. (1985) *Biochem. J. 82*, 3582−3585.
19. Vijay-Kumar, S., Bugg, C. E., and Cook, W. J. (1987) *J. Mol. Biol. 194*, 531−544.
20. Love, S. G., Muir, T. W., Ramage, R., Shaw, K. T., Alexeev, D., Sawyer, L., Kelly, S. M., Price, N. C., Arnold, J. E., Mee, M. P., and Mayer, R. J. (1997) *Biochem. J. 323*, 727−734.
21. Briggs, M. S., and Roder, H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2017−2021.
22. Reis, O., Winter, R., and Zerda, T. W. (1996) *Biochim. Biophys. Acta 1279*, 5−16.
23. Siminovith, D. J., Wong, P. T. T., and Mantsch, H. H. (1987) *Biochemistry 26*, 3277−3287.
24. Arrondo, J. L. R., Muga, A., Castresana, J., and Goñi, F. M. (1993) *Prog. Biophys. Mol. Biol. 59*, 23−56.
25. Byler, D. M., and Susi, H. (1986) *Biopolymers 25*, 469−487.

26. Prestrelski, S. J., Byler, D. M., and Liebman, M. N. (1991) *Biochemistry 30*, 133−143.
27. Goossens, K., Smeller, L., Frank, J., and Heremans, K. (1996) *Eur. J. Biochem. 236*, 254−262.
28. Wintrode, P. L., Makhatadze, G. I., and Privalov, P. L. (1994) *Proteins: Struct., Funct., Genet. 18*, 246−253.
29. Ibarra-Molero, B., Makhatadze, G. I., and Sanchez-Ruiz, J. M. (1999) *Biochim. Biophys. Acta 1429*, 384−390.
30. Ibarra-Molero, B., Loladze, V. V., Makhatadze, G. I., and Sanchez-Ruiz, J. M. (1999) *Biochemistry 38*, 8138−8149.
31. Wong, P. T. T., Mantsch, H. H., and Carrier, D. C. (1990) *Biochemistry 29*, 254−258.
32. Carrier, D., Mantsch, H. H., and Wong, P. T. T. (1990) *Biopolymers 29*, 837−844.
33. Harding, M. M., Williams, D. H., and Woolfson, D. N. (1991) *Biochemistry 30*, 3120−3128.
34. Jonas, J., Ballard, L., and Nash, D. (1998) *Biophys. J. 75*, 445−452.

BI012023B