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High-Pressure Solution X-ray Scattering of Protein Using a Hydrostatic Cell with Diamond Windows

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A hydrostatic high-pressure cell (maximum pressure 700 MPa) with synthetic diamond windows is applied to measure small-angle X-ray scattering of a protein at high pressure. Use of the present cell allows an accurate solvent background correction, providing quantitative analyses. The performance of the present cell for X-ray scattering is shown by using lysozyme as a sample solution. From the Guinier plot, values of the radius of gyration of lysozyme are evaluated to be 15.31 ± 0.09 Å at 1 atm (0.10 MPa) and 14.80 ± 0.15 Å at 400 MPa.

Keywords: high pressure; quantitative solution X-ray scattering; hydrostatic cells; lysozyme; radius of gyration.

1. Introduction

The history of the effects of pressure on proteins began with the observation of the pressure-induced irreversible denaturation of egg albumin by Bridgman (1914). It took, however, half a century before systematic studies of the pressure effects began. The reversible denaturation of a protein was first observed by Suzuki *et al.* (1963). Since the 1970s, the thermodynamic features of the pressure-induced denaturation of some proteins have been investigated by stoichiometric methods using UV or fluorescence spectroscopies (Bransts *et al.*, 1970; Hawley, 1971; Zipp & Kauzmann, 1973; Taniguchi & Suzuki, 1983). On the other hand, there have only been a small number of microscopic studies of the pressure effects because of experimental difficulties. Recently, high-pressure IR spectroscopic (Wong & Heremans, 1988) and NMR (Samarasinghe *et al.*, 1992) studies on reversible structural changes have been undertaken, which follow changes in the secondary and local tertiary structures of proteins, respectively. Only recently, reversible changes of ribonuclease A have been shown by FT–IR combined with resolution enhancement techniques (Takeda *et al.*, 1995) and NMR (Zhang *et al.*, 1995). These spectroscopic techniques are, however, insufficient for a comprehensive description of pressure-induced structural changes on the molecular level because these experiments cannot provide information about the global tertiary structure. Although the method of X-ray crystal diffraction is the most powerful technique for investigating the tertiary structure of proteins, it is very difficult to use this technique under high pressure because of the low resistance of protein crystals to pressure. Indeed, there has only been one X-ray crystal diffraction study of lysozyme at 100 MPa, by Kundrot & Richards (1987).

Small-angle X-ray scattering is another powerful technique for probing tertiary structural features of proteins. It provides information on the global structure, such as radius of gyration, volume, distance distribution function *etc*. An important advantage of this technique is that it is applicable for solution systems. Thus, solution X-ray scattering

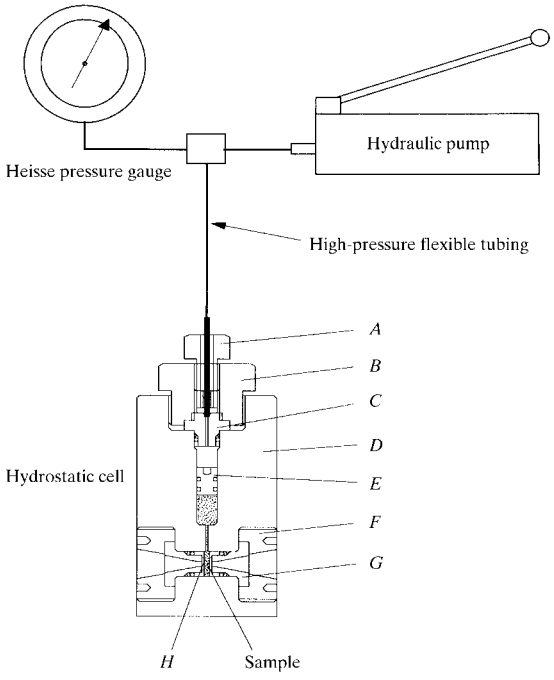

Figure 1
Schematic diagram of the high-pressure apparatus. The components of the hydrostatic cell are (*A*) high-pressure tubing guide, (*B*) screw plug, (*C*) tubing support plug, (*D*) cell body, (*E*) free piston (separator), (*F*) screw plug, (*G*) window support plug, (*H*) synthetic diamond window.

(SOXS) can be applied to measurements even at extreme sample conditions such as high pressure where the structure of proteins may change drastically.

Only recently a high-pressure SOXS study using a diamond anvil cell (DAC) technique was reported for biological systems including a protein solution (Czeslik *et al.*, 1996). Although a DAC is very convenient for generating high pressure, it has fundamental disadvantages for quantitative measurements. The most crucial problem is that a change in optical path length of the DAC with increasing pressure is not a reproducible function. This makes it very difficult to subtract accurately solvent scattering from sample scattering (Kato & Taniguchi, 1995). Thus, the use of a DAC is not suitable for systems in which solvent scattering is significant.

Some hydrostatic types of cells are better for high-pressure optical measurements requiring the solvent background correction. Preliminary measurements on lysozyme (Kato *et al.*, 1994) and on ATCase (Lorenzen *et al.*, 1994) have been performed using two different types of hydrostatic cells. The former group first reported the radius of gyration of a protein (Kato *et al.*, 1994, 1996, 1997). The latter group has reported X-ray scattering curves of ATCase at 1.0 kbar (100 MPa) and 2.9 kbar (290 MPa). Recently, Kleppinger *et al.* (1997) measured SOXS of ribonuclease A up to 9 kbar and estimated the radius of gyration with pressure.

The purpose of this work is to develop the quantitative high-pressure measurement of SOXS from protein solutions. In the present work, we apply a high-pressure hydrostatic cell with synthetic diamond windows to the high-pressure SOXS measurement of protein solutions. The present high-pressure apparatus allows us to obtain quantitative data even for a relatively low concentration ($\sim$10 mg ml^{-1}) of protein solution. We show the high performance of the high-pressure cell for SOXS, together with the results of high-pressure SOXS of lysozyme at 400 MPa.

2. Experimental

2.1. *High-pressure cell*

The correct evaluation of particle scattering requires the sample to be an infinitely dilute solution. This is because interparticle interference induces a decrease in scattering intensity at small angles. In general, a series of concentrations of the sample are measured in order to extrapolate to zero concentration. In the case of protein solutions, the concentration range of several tens to several mg ml^{-1} is typically accepted. At the low concentration of several mg ml^{-1}, background scattering (cell and solvent) is predominant over the total scattering. The accuracy in the estimation of the net scattering depends sensitively on the reliability of subtracting the background scattering from the sample solution scattering. A highly reliable subtraction is therefore necessary for quantitative analyses of protein solutions. In the case of high-pressure experiments, accurate subtraction will not be easy because increasing pressure may significantly alter both the path length of the cell and the density of the solvent. To perform an accurate background correction, we have to measure both SOXS from sample and reference solutions under exactly the same cell condition at each pressure. This condition requires high reproducibility of the path-length change by pressure. For this requirement, a hydrostatic high-pressure cell is considered to be most suitable, the path length of which would be marginally and reproducibly altered by pressure. In this study, therefore, we use a hydrostatic cell similar to that developed for optical measurements of fluids (Kato & Taniguchi, 1995) for high-pressure SOXS. Mechanical properties and a picture of the cell are given in detail elsewhere (Kato & Taniguchi, 1995; see also Kato, 1993, for a clearer cell picture).

Fig. 1 shows a schematic diagram of the present high-pressure apparatus. The cell, of which most components are made of SUS-630 stainless steel heat-treated to a hardness of 48 R_c, is designed to resist up to 700 MPa. The pressure of the cell is directly controlled by the transmitting fluid of a hand hydraulic pump (Hikari Co.; maximum pressure 1 GPa). A free piston (E) with a double O-ring seal separates the sample liquid from the pressure-transmitting media. Thus, we need no inner-cell assembly for sample measurements. This is a great advantage in terms of the background correction because we need not take account of both scattering from the pressure-transmitting fluid and the inner cell. The pressures were measured using a Heisse Bourdon tube gauge (full scale 700 MPa) with an accuracy of 1 MPa, while the typical accuracy is 100 MPa in the case of a DAC using ruby fluorescence (Piermarini & Block, 1975). Thus, we can measure both X-ray scattering for a sample and its solvent under high pressure under the same instrumental conditions, which is necessary for an accurate background subtraction. In addition, the cell is compactly designed (42 mm depth $\times$ 56 mm length $\times$ 86 mm height) because a pressure intensifier is not required. It can thus be used in established instruments without having to modify these instruments. The temperature of such a compact cell can also be easily controlled with a temperature-controlled cell jacket.

Here, we discuss another type of hydrostatic cell (Lorenzen *et al.*, 1993). It is a piston-cylinder-type cell combined with a pressure intensifier, which was first developed by Fishman & Drikamer (1956). An advantage of this type of cell is its ability to reach easily a relatively high pressure without using a high-performance hydraulic pump. Indeed, this cell is optimized for macromolecule measurements that require larger pressure and temperature ranges (1 GPa and 573 K) (Lorenzen *et al.*, 1996). Thereby it is not optimized for measurements in biological systems. This cell essentially requires a larger cell body size and more complicated equipment than the present cell. Its large body may need special instrument modifications for its X-ray camera, and controlling the sample temperature is also more difficult. A precise pressure determination

requires an additional sealing plug with manganin electrical resistance pressure gauges inside the cell, due to the significant friction between the piston and cylinder. Maintaining the reproducibility with the manganin gauge requires careful maintenance. Furthermore, an inner cell is used for biological solutions. Natural diamonds with a total thickness of 3.5 mm are employed as windows, whereas we use two synthetic (type Ib) diamonds (Sumicrystal, Sumitomo Denco Co.), each of 1.0 mm thickness and 5.0 mm diameter. The present thinner windows increase the transmission by a factor of 4.5 at 1.3 Å and by 9.4 at 1.5 Å. The use of thinner windows is advantageous, particularly for measurements using X-rays of large wavelength.

2.2. *SOXS measurement*

The SOXS experiments were performed at the solution scattering station installed at beamline 10C of the Photon Factory, Tsukuba, Japan (Ueki *et al.*, 1985). With the installed Si(111) double-crystal monochromator, the X-ray wavelength was tuned to 1.3 Å. The beam size at the sample position was reduced to 1.2 × 1.2 mm by guard slits. The X-ray scattering was recorded using a linear position-sensitive proportional detector with a 10 mm slit. The distance between the sample position and the detector was 55 cm, calibrated using meridional diffraction data of cholesterol. We also measured a direct beam attenuated by 1.3 mm Al foil at each pressure to estimate the pressure dependence of the X-ray absorption of water. The temperature of the cell was maintained at 298 K by using temperature-controlled water circulating into a cell jacket. Lysozyme (chicken egg white, 3 × crystallized) was purchased from Sigma. The protein was dissolved in 10 m*M* MES buffer (pH 5.7). The protein concentration of 13.4 mg ml^{-1} was determined spectrophotometrically using E_{282} (1 mg ml^{-1}) = 2.64. It took 300 and 600 s to obtain the SOXS profiles of proteins when using a standard quartz window cell and the present high-pressure cell, respectively. No radiation damage to samples was detected during each measurement.

3. Results and discussion

3.1. *Background correction from protein solution*

The reproducibility of observed scattering is a critical condition for quantitative SOXS measurements as described above. First, we show X-ray scattering of water at 1 atm to 500 MPa as an example to demonstrate the success of reproducibility using the present cell. An increase in pressure increases the path length and the density of the liquid. This causes a decrease in the X-ray transmission, which decreases the X-ray scattering intensity at all angles. Indeed, the decrease in the transmission was 37% at 400 MPa. We corrected each scattering profile using the transmission change factor estimated from each direct-beam measurement. Fig. 2 shows SOXS profiles of water up to 500 MPa after the intensity correction. These scattering profiles correspond to one another within the experimental error of 2.8% and show no line crossing. The change in the path length is fundamentally reversible in the pressure range within the elastic limit of the cell material. Hence we can quantitatively correct the protein solution scattering for the background scattering *via* subtraction of the scattering profiles of the buffer solutions measured at each pressure.

If high-pressure measurements proceed well without accident, the changes in transmission of the solution and the solvent should be identical. In high-pressure experiments of protein solutions, we do not accept a series of measurements where these transmission changes are not identical. Furthermore, we check whether there is no line crossing when overlaying the sample solution and the solvent profiles. We reject the scattering data when line crossing occurs. Line crossing means that instrumental situations such as beam position, cell window alignment and so on are different between the sample solution and the solvent measurements.

Here, we discuss the use of a DAC for the quantitative SOXS measurement. The background correction seems to fail when using a DAC. It uses the deformation of a metal gasket for generating pressure. Squeezing the gasket between diamonds leads to its plastic deformation, and thus increasing pressure irreversibly changes the path length and the radius of the aperture (Dunstan, 1989). The changes in path length are irreversible and do not show any correlation with pressure. The background correction cannot be performed without the X-ray transmission correction. Furthermore, it seems to be too difficult to maintain the parallelism between the culet faces through all the high-pressure measurements (Dunstan & Spain, 1989). A deviation from parallelism would cause critical problems, *e.g.* sample line crossing and buffer scattering profiles (Kato & Fujisawa, unpublished data). Even if all the difficulties are surmounted, the low accuracy in the pressure measurement causes problems. The error in pressure

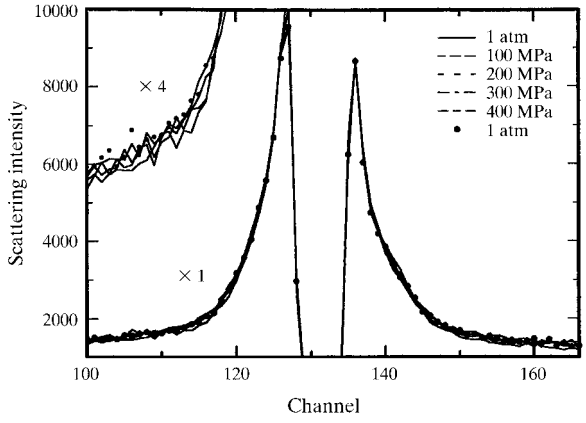

Figure 2
X-ray scattering of water at various high pressures. The pressure was increased in steps of 100 MPa. The dotted line represents the scattering at 1 atm after reducing the pressure. The specimen-to-detector distance is 55 cm and the conversion gain of the detector is 6.5%.

(typically 100 MPa) of the ruby fluorescence method is too large for aqueous solution systems, which are generally solidified at about 1 GPa. Furthermore, it is fundamentally impossible to control the pressure to correspond to the same values for the sample solution and the solvent measurements. It is an essential condition for background subtraction.

3.2. *High-pressure SOXS of lysozyme*

Here we show high-pressure SOXS measurements of a lysozyme solution as a sample, and discuss the performance of the present high-pressure system. Fig. 3 shows the SOXS profiles of lysozyme at 1 atm and 400 MPa, at which lysozyme is not denatured (Li *et al.*, 1976). We measured two SOXS profiles at 1 atm using a standard quartz window cell and the present high-pressure cell. The curves shown are corrected against the background. Furthermore, the profile at 1 atm using the standard quartz cell was scaled to the profile using the high-pressure cell by the transmission factor and the incident X-ray intensity. Both net intensities overlie each other within the experimental error. This suggests that the present high-pressure equipment causes no systematic error.

A number of structural parameters can be obtained directly from these X-ray scattering profiles. The radius of gyration, R_g, is one of the most basic parameters among them. Formally, it corresponds to the radius of inertia in mechanics. Thus, R_g is an intuitive probe for the spatial extension of the macromolecules. X-ray scattering intensities at the small-angle region are given by

$$I(S) = I(0)\exp[-(2\pi S)^2 R_g^2/3],$$

where $S = 2\sin\theta/\lambda$; 2θ is the scattering angle, λ is the X-ray wavelength. We obtain R_g from the slope of the ln $[I(S)]$ *versus* S^2 plot (Guinier plot).

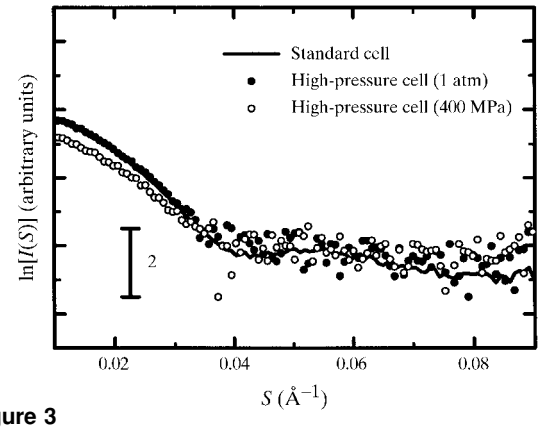

Figure 3
Background-subtracted intensities of the X-ray solution scattering of lysozyme at 1 atm and 400 MPa. The atmospheric pressure profiles were measured using a standard quartz window cell (line) and the present high-pressure cell (circles). The former profile was scaled to the latter profile by the transmission factor and the incident X-ray intensity. The inserted number and bar indicate the scale of the vertical axis. The solution conditions are described in the experimental section.

Fig. 4 shows the Guinier plot of the scattering curves of lysozyme at 1 atm and 400 MPa presented in Fig. 3. We obtained a linear behaviour in a relatively wide region of the scattering angle for both measurements. The Guinier plot is also suitable as an estimate of the performance of the present high-pressure SOXS measurements. It can be used as another check on high-pressure measurements. A failed background correction would cause a kink in the plot in the Guinier region. Thus, the results indicate the high accuracy of the present high-pressure measurement. From these slopes we obtained R_g = 15.31 $\pm$ 0.09 Å at 1 atm and R_g = 14.80 $\pm$ 0.15 Å at 400 MPa, where each range of R_g means a standard deviation of each least-square fitting in Fig. 4. An increase in pressure induces a significant decrease in R_g. The increase by 400 MPa decreases R_g by 0.51 Å. Furthermore, we also obtained R_g = 15.54 $\pm$ 0.02 Å at 1 atm from data (Fig. 3) obtained using the standard quartz window cell. At 1 atm, a SOXS study by Luzzati *et al.* (1961) reported R_g = 15.2 Å. The present values at 1 atm are comparable with this reported value. Light-scattering and sedimentation studies (Bruzzesi *et al.*, 1965) showed that lysozyme tends to associate at neutral pH and relatively high concentration but does not significantly associate at a pH of 6.8 and a concentration of 14 mg ml^{-1}. It therefore seems that the solution condition in this study does not significantly cause lysozyme to associate.

A high-pressure X-ray crystal diffraction study of lysozyme by Kundrot & Richards (1987), which is the only high-pressure study for proteins using this technique, showed that R_g is 13.72 Å at 1 atm and 13.67 Å at 1000 atm (~100 MPa). The crystal diffraction value at 1 atm is smaller by around 1.5 Å than the SOXS values. It is well known that R_g values obtained by SOXS give a somewhat larger value than those obtained by X-ray crystal diffraction. This is because R_g from SOXS also includes the

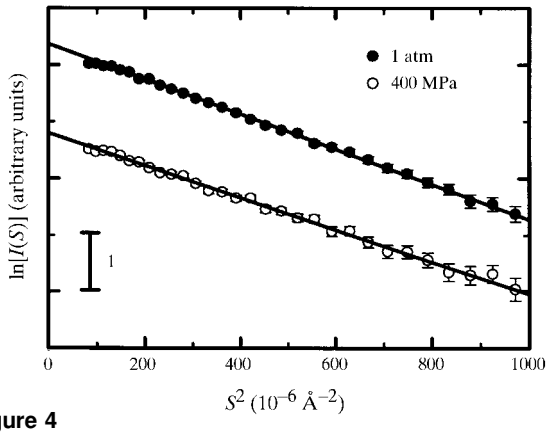

Figure 4
Guinier plots for lysozyme solution at 1 atm and 400 MPa. The intensity data (filled circles, empty circles) of Fig. 3 were used. The inserted number and bar indicate the scale of the vertical axis. For clarity, each plot is shifted along the vertical axis. Indeed, $I(0)$ at 400 MPa indicates 57.6% of $I(0)$ at 1 atm. This decrease is attributed mainly to a change in the transmission, which decreases by 37% when the pressure is increased to 400 MPa. Another factor may be a change in the density contrast between the solute and the solvent by pressure.

hydration shell around the particle whereas R_g from X-ray crystal diffraction includes only intrinsic atoms of the particle (Pavlov & Fedorov, 1983; Fujisawa *et al.*, 1994). The present difference in R_g values is close to the van der Waals radius of a water molecule. An interesting point is the remarkable difference in the pressure dependence of R_g between SOXS and X-ray crystal diffraction. The decrease in R_g per 100 MPa is 1.0% from SOXS but 0.33% from X-ray crystal diffraction.

The present result may be still preliminary because our data are obtained only at one concentration of the protein. In the present work, we consider no interparticle interference effects. For a better data collection, we have to make measurements at various sample concentrations in order to extrapolate to zero concentration. Such measurements are now in progress. More detailed results and discussions will be reported in the future.

In this paper, we applied a hydrostatic cell with diamond windows to quantitative X-ray solution scattering measurements of a protein under high pressure. The accurate correction of background scattering, which is a necessary condition for quantitative analyses, was successful using the present cell. Indeed, we showed that the radius of gyration of lysozyme decreases by 0.51 Å when increasing the pressure to 400 MPa. Our technique allows the investigation of various high-pressure phenomena such as protein folding/unfolding, compressibility of proteins, pressure-dependent dissociation/association of origomeric proteins, and protein crystallization. Some of these projects are now in progress.

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