High-pressure small-angle neutron scattering studies of glucose isomerase conformation in solution

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Small-angle neutron scattering (SANS) of solutions of glucose/xylose isomerase from *Streptomyces rubiginosus* was measured as a function of pressure. It is shown that the structure of the enzyme in solution as seen by SANS is practically the same as that in the crystal and does not change with pressure up to 150 MPa. This reflects the unusually high structural stability of this material, which makes it extremely interesting to use as a secondary standard for pressure-dependent SANS experiments. This lack of pressure dependence of the SANS data also indicates that any possible change in hydration of the protein induced by pressure is not visible in the SANS curves. An appropriate correction procedure must be used for the SANS data in order to account for the distortion of the intensity curve due to hard-sphere and electrostatic interactions. After this correction, the isomerase can be readily used as a secondary standard for SANS measurements.

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1. Introduction

Glucose/xylose isomerase from *Streptomyces rubiginosus* (D-xylose ketol-isomerase, EC 5.3.1.5) is an intracellular enzyme of exceptional stability catalysing the isomerization reaction of glucose and xylose. Four identical subunits, of 43.3 kDa each, form a homotetramer. Each monomer is composed of two domains, of which the larger one contains the (α/β)₈ motif of the triose phosphate isomerase barrel type.

The crystal structure of glucose/xylose isomerase from *Streptomyces rubiginosus* has already been deposited in the Protein Data Bank (PDB; Berman *et al.*, 2000), having been solved by X-ray diffraction up to atomic resolution, namely 0.094 nm (PDB code 2glk; Katz *et al.*, 2006) and 0.099 nm (PDB code 1mnz; Fig. 1), and by time-of-flight neutron diffraction with 0.18 nm resolution (PDB code 2gve; Katz *et al.*, 2006). The metal-free enzyme and a number of complexes of this protein with substrate analogues have been studied to an accuracy of 0.14–0.19 nm (Carrell *et al.*, 1989, 1994; Katz *et al.*, 2006).

This enzyme is one of the most widely used industrial enzymes and its activity remains at the same level even at temperatures of about 333 K (half-life of hundreds of days) (Visuri *et al.*, 1999).

Because of its high conformational stability on storage, thermal stability, radiostability during synchrotron radiation measurements and appropriate molecular weight (173 kDa), as well as the high reproducibility of its small-angle X-ray

scattering measurements, this enzyme has been proposed as a possible secondary standard of molecular weight in small-angle X-ray scattering measurements (Kozak, 2005).

The interfacial water molecules on the protein surface enter into a specific type of interaction, with the hydrophobic and hydrophilic amino acid residues forming a hydration shell whose physical properties differ significantly from those of bulk water. Correct recognition of the properties of this shell in different conditions determined by temperature, pressure and salt concentration are of key importance for explaining the mechanisms of protein folding, structure stability and protein–protein interaction. According to small-angle neutron and X-ray scattering (SANS and SAXS) experimental results, water surrounding the protein surface can have a density 15–20% greater than the bulk water (Svergun *et al.*, 1998). This phenomenon is, among other things, a consequence of the electrostriction effect accompanying the strong electrostatic field around the charged residues (Merzel & Smith, 2002, 2005; Danielewicz-Ferchmin *et al.*, 2003). Changes in the concentration of salt ions (Leberman & Soper, 1995), pressure or temperature affect the properties of the hydration shell, disturb the water–protein interactions and destabilize the protein structure. The external hydrostatic pressure up to 300 MPa has no significant effect on the tertiary structure of the majority of proteins; however, as predicted by Danielewicz-Ferchmin *et al.* (2007), it should influence the density of water molecules in the range of the strong electrostatic fields on the protein surface. Assuming structural stability in the

pressure range 10–300 MPa, changes in the hydration shell properties might be visible in the shapes of the SANS and SAXS curves.

Although hydrostatic pressure not exceeding 300 MPa has no significant effect on the tertiary structure of the majority of proteins, it can be sufficient to disturb their quaternary structure (if they have it).

The effect of pressure on dissociation of oligomers has been widely documented in the literature and concerns a wide range of particles: from dimers to multi-subunit structures such as ribosomes or viruses (Silva *et al.*, 1996; Boonyaratankornkit *et al.*, 2002). Dissociation of oligomers seems to be a universal phenomenon, although there are proteins that enhance their stability under elevated pressure. For instance, the tetramer of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from thermophiles or the hexamer of glutamate dehydrogenase (GDH) from the hyperthermophile *Pyrococcus furiosus* enhance their resistance to thermal inactivation under pressures up to 100 MPa (Hei & Clark, 1994; Sun *et al.*, 1999). Under pressures of similar, relatively low values, dissociation of dimers of lysozyme from hen egg white has been observed (Banachowicz, 2006). The tetramer of oxidase in the crystal state has been reported to begin to deteriorate close to 140 MPa (Colloc'h *et al.*, 2006). Tetramers of GAPDH from yeast, bovine and porcine lactate dehydrogenase, and malate dehydrogenase have been found to dissociate upon application of hydrostatic pressure up to 200 MPa (King & Weber, 1986; Ruan & Weber, 1989).

The quaternary structure of the isomerase seems stable even under pressures reaching 250 MPa (unpublished data).

Therefore, as our study is performed for pressures up to 150 MPa, it is reasonable to expect the structure in solution as seen by SANS to be practically the same as that in the crystal. Furthermore, the structure of this protein has been shown to be unaffected by temperature (see above) and by variations in the ionic strength induced by increasing the NaCl concentration. These observations reflect the unusually high degree of structural stability of this material, which makes it extremely interesting to use it as a secondary standard for SANS

experiments, especially in studies of protein conformation under elevated pressure. In order to correct for the relative changes in the protein scattering cross section with pressure, the pressure dependence of the scattering length density of D₂O must be taken into account. The use of the so-called secondary scattering standards has been so far restricted to synthetic polymers. Unfortunately, these materials are characterized by size polydispersity. Thus any data evaluation is complicated. The proposed use of the isomerase as a secondary standard allows problems with the size polydispersity to be avoided.

1.1. Sample preparation

Glucose/xylose isomerase from *Streptomyces rubiginosus* was purchased from Hampton Research (USA). The buffer was exchanged and the enzyme was additionally purified by gel filtration. The protein solution (25 mM Tris D₂O, 75 mM NaCl, pH 7.6) was concentrated by ultrafiltration using Amicon Ultra-4 30k filters (Millipore). The protein concentration was determined by UV absorption at 280 nm ($A_{0.1\%}^{280} = 1.059$; Gill & Von Hippel, 1989).

1.2. SAXS measurements

Synchrotron radiation scattering data were collected for the same sample at the EMBL SAS beamline X33 at the DORIS storage ring of the Deutsches Elektronen Synchrotron (Hamburg, Germany) as previously described (Kozak, 2005). Samples of glucose isomerase with protein concentrations of 3 and 6 mg ml⁻¹ were used to eliminate concentration artefacts. The background data for the buffer were collected before and after data collection for the protein samples. The scattering of the buffer was subtracted and the final scattering curve was obtained by merging the scattering data collected at lower concentration with the data recorded at a higher concentration using the program *PRIMUS* (Konarev *et al.*, 2003).

1.3. SANS measurements

The SANS experiments were performed at the SANS I instrument at the SINQ spallation source at the Paul Scherrer Institute (PSI) in Villigen, Switzerland (Kohlbrecher & Wagner, 2000). Thermal neutrons of wavelength $\lambda = 0.5$ nm with a wavelength spread $\Delta\lambda/\lambda$ of about 0.1 were used. Data analysis was performed using the *BerSANS* software package (Keiderling, 2002), which accounts for all necessary corrections for background, transmission, sample thickness, count rate/dead time ratio, masking and radial averaging of the raw data. A standard water sample (for discussion of the details of absolute calibration see §2.1) was used for calibration to absolute scattering intensities and also to account for non-uniform detector efficiency (Strunz *et al.*, 2000). The applied calibration procedure also included the correction for inelastic and multiple scattering contributions necessary if water is used as a secondary standard by correcting the water cross section with a wavelength-dependent term $g(\lambda)$, which at the SANS instrument used is practically identical to that of the SANS instrument D22 at ILL published by Lindner (2000). The $g(\lambda)$

Figure 1

Structure of glucose/xylose isomerase from *Streptomyces rubiginosus* (D-xylose ketol-isomerase, EC 5.3.1.5; PDB code 1mnz). The model was rotated clockwise by 90° around the y axis. The figure was prepared using the program *RASTOP* (Sayle & Milnerwhite, 1995).

dependence was obtained during commissioning of SANS I at PSI by comparing the incoherent scattering of H₂O with the forward scattering of a polystyrene sample of very narrow molecular weight distribution. After this correction and the calibration procedures, the scattering cross sections ($d\Sigma_s/d\Omega(q)$) in cm^{-1} were obtained, and these are used throughout the paper. As an input to the *CRYSON* program (Svergun *et al.*, 1998), the primary beam profile was measured and was used to account for smearing effects. The measurements were performed at two different detector positions at 2 and 6 m from the sample, which gave access to a q range of $0.02 < q < 3.5 \text{ nm}^{-1}$, where q is the scattering vector, defined as $q = |\mathbf{q}| = (4\pi/\lambda) \sin(\theta/2)$, λ being the neutron wavelength and θ being the scattering angle.

1.4. Pressure cell

A custom-made high-pressure cell was used, which allows measurements of liquid samples at pressures up to 500 MPa and temperatures from 278 to 323 K (Kohlbrecher *et al.*, 2007). Care was taken to separate the sample of approximately 1 mm thickness from the oil transmitting hydrostatic pressure, generated by a Nova-Swiss moving piston hand pump. The pressure cell was equipped with large sapphire windows to withstand the pressure. The transmission of the cell is about 0.6 for $\lambda = 0.5 \text{ nm}$ and causes no considerable excess scattering in the q range used.

2. Analysis of the SANS data

2.1. Absolute calibration: the problem

Various scattering standards have been used for SANS measurements. Among them, the use of light water samples is most frequently chosen. A rather extensive discussion on that issue has been presented by Wignall & Bates (1987). In general the calibration standards can be divided in two groups: the so-called incoherent scatterers, such as water, Lupolen (which is partly crystalline polyethylene), poly(methyl methacrylate) or vanadium, and the coherently scattering standard samples, such as polymers in solution. If the molecular weight of the polymers has been established by other methods, then from the measurement of the forward scattering a calibration can be obtained. Porous samples with a calibrated internal surface can be also used. One approach is the so-called direct beam method. All these methods have specific advantages and drawbacks. For incoherent scatterers, the error in values of the absolute intensity is about 2% according to Jacrot & Zaccai (1981). Primary standards suffer from the fact that they scatter little; moreover, inelastic effects in water as discussed by Gosh & Rennie (1990) and deviations from isotropic scattering in the case of Lupolen, due to crystallinity, have to be taken into account.

Another approach is to use the so-called secondary standards. Lindner (2000) has discussed the use of secondary standards such as narrowly distributed poly(styrene) samples (characterized by stronger scattering) for verification of the water calibration. The scattering from a polymeric standard

with a molecular weight of about 40 kDa at low q is easily one order of magnitude higher than scattering from primary standards. Scattering can be increased, of course, if higher molecular weights are used. Among the coherently scattering secondary standards the use of polymeric materials suffers especially from their polydispersity and hence from the preparation conditions, which in turn renders the reproducibility complicated. Moreover, as well as the molecular weight distribution, the molecular volume of the polymer in solution rather than the molecular volume of the bulk polymer has to be known. In principle this can be obtained by a precise pycnometric (or density) measurement. Here the use of biological molecules is favoured, since they are monodisperse and their structure and volume do not change upon solvation in water. This is a reasonable assumption as the protein is a globular and compact object in contrast to polymers in solution. In this paper we propose the use of glucose isomerase as a secondary scattering standard, which is by its nature monodisperse, can be easily obtained by standard preparation procedures, and in solution is stable across large ranges of temperature, pressure and ionic strength. Its molecular weight of about 173 kDa and thus its size ensure that the plateau of the scattering curve is reached at q values smaller than 0.2 nm^{-1} , which allows a reliable determination of the forward scattering. The q value of 0.2 nm^{-1} is readily accessible by all SANS machines.

2.2. Data evaluation of isomerase

The raw SANS data were analysed in the usual way using the *BerSANS* software package, as already outlined in §1.3.

The scattering cross section ($d\Sigma_s/d\Omega(q)$) is related to the physical properties of the protein in solution *via*

$$\begin{aligned} I_s(q)K_N &= \frac{d\Sigma_s}{d\Omega}(q) = \frac{N}{V_{\text{tot}}}(b - V\rho_0)^2 P(q)S(q) \\ &= c \frac{N_A}{M_w}(b - V\rho_0)^2 P(q)S(q), \end{aligned} \quad (1)$$

where b is the scattering amplitude (scattering length) of the isomerase, V is its volume, ρ_0 is the scattering length density of the solvent, N is the number of particles in the illuminated volume and $c = (NM_w)/(V_{\text{tot}}N_A)$ is the particle concentration in g cm^{-3} , V_{tot} being the illuminated sample volume, N_A Avogadro's number and M_w the molecular weight. $P(q)$ is the form factor, which is normalized to 1 for $q = 0$, and $S(q)$ is the structure factor, which takes into account the inter-particle correlations. The constant K_N depends on experimental details. $I_s(q)$ is the experimentally measured scattering curve in arbitrary units and has to be multiplied by the constant K_N , which includes all instrumental parameters (Wignall & Bates, 1987), to convert it to absolute units of cm^{-1} . As we are interested in the determination of ($d\Sigma_s/d\Omega(q)$) for $q = 0$, we have to take into account that $S(q = 0)$ can take values considerably lower than 1, depending on the ionic strength, concentration and charge. Consequently, we have to take these effects into account in the determination of ($d\Sigma_s/d\Omega(q)$). In order to proceed we have divided the data

treatment procedure according to equation (1) into three steps:

- (i) determination of the form factor $P(q)$ for the isomerase from the crystallographic data,
- (ii) evaluation of the structure factor $S(q)$ of the isomerase solution, and finally
- (iii) calculation of the scattering cross section ($d\Sigma_{\text{S}}^{\text{corr}}/d\Omega(q)$) corrected for the structure factor as well as the estimation of the protein volume V .

3. Results and discussion

3.1. The form factor $P(q)$

A comparison of the experimental scattering curves $I_S(q)$ measured by means of SANS (concentration of 22.7 mg cm^{-3}) and SAXS (concentrations of 3 and 6 mg cm^{-3}) is shown in Fig. 2.

The fit of the SAXS data and the crystal structure (PDB code 1mnz) was obtained for $\delta\rho = 10 \text{ e nm}^{-3}$, while the fit of the SANS data and the crystal structure was obtained for a contrast of the solvation shell of 0.4314 and a D_2O fraction in the solvent of 0.7. As one can see in Fig. 2, all of the important features of the shapes of these curves are practically identical, although the neutron beam is by a few orders of magnitude less monochromatic ($\Delta\lambda/\lambda = 0.1$) than the X-ray beam from the synchrotron. Both curves are shifted vertically in order to compare the two methods, and they are therefore not presented in absolute scales. The lack of sharp minima in the calculated curves results from the non-spherical shape of the protein; however, the ellipticity is only of a few percent. Small deviations from a spherical shape result in strongly smeared out minima; such an effect can be observed for spheres with a

Figure 2
SANS: isomerase concentration 22.7 mg cm^{-3} (25 mM Tris in D_2O , 75 mM NaCl, pH 7.6), solid circles indicate a sample-detector distance of 6 m and open circles 2 m. SAXS: open squares represent the same salt as before (Kozak, 2005). Solid lines are the results of CRYSON and CRYSQL simulation for SANS and SAXS, respectively, based on the crystal structure. The SANS data lie below the curve predicted by CRYSON for low q owing to the influence of $S(q)$. Curves are shifted vertically for clarity.

shape variance of just 2%. The positions of the major minima and maxima in the SAXS and SANS experimental curves are in agreement with the curves calculated for the same isomerase crystal structure using CRYSQL (Svergun *et al.*, 1995) and CRYSON, respectively. In this way a reliable determination of the form factor of the protein is made and its value will be used in further calculations. The SANS data (open circles) deviate from the calculated form factor (solid line) in the low- q range because at higher concentrations the structure factor is substantially different from 1. As the concentration of the protein in the SAXS experiment was lower by almost one order of magnitude than that for the SANS experiment, the experimental and calculated curves are identical, indicating $S(q) = 1$. This effect is discussed in detail in the next section.

Both the SANS and the SAXS curves deviate slightly at high q from the lines simulated for the isomerase crystal structure using CRYSON and CRYSQL, respectively. This deviation might be due to the fact that the hydration water shell has not been taken into account properly. In the CRYSON program a hydration shell of a uniform thickness and density over the entire protein surface is assumed. Theoretical studies (Merzel & Smith, 2005; Danielewicz-Ferchmin *et al.*, 2006), however, indicate that the hydration of proteins is not uniform: charged hydrophilic regions of the protein surface are strongly hydrated, while the hydration layer on the hydrophobic regions might be much thinner or nonexistent.

3.2. The determination of $S(q)$

The scattering curves obtained for isomerase solutions at concentrations of 4.54 and 22.7 mg cm^{-3} at ambient pressure and a temperature of 293 K are shown in Fig. 3 (symbols). In this figure the $P(q)$ curve calculated for the 1mnz crystal structure by means of the CRYSON program is shown as a

Figure 3
Experimental intensities $I(q)$ normalized with concentration for isomerase concentrations of 4.54 (open symbols) and 22.7 mg cm^{-3} (solid symbols) (25 mM Tris in D_2O , 75 mM NaCl, pH 7.6). The solid line is generated by CRYSON for the crystal structure of the isomerase. Experimental intensities are smaller than the CRYSON result at low q owing to the influence of $S(q)$.

solid line. A reasonable agreement between the calculated and measured curves can be seen for the low-concentration solution, indicating that the structure of the isomerase in solution is very close to that in the crystal. At high concentrations a strong systematic deviation of the calculated curve from the experimental one can be seen in the low- q range. A similar but much smaller deviation is also seen for the low-concentration curve.

The deviation at low q values (Fig. 3) of the experimental intensity curve measured at high concentration from that measured at low concentration and calculated using *CRYSON* can be explained as follows.

The scattered intensity for a protein solution [equation (1)] depends on the form factor $P(q)$, which depends on the size and shape of the protein, and the structure factor $S(q)$, which describes the radial distribution (radial ordering) of the proteins in solution, in the following way: $I(q) \propto P(q) S(q)$. In the case of non-interacting dilute protein solutions, the structure factor $S(q) = 1$ for all q values, and hence $I(q) \propto P(q)$. If, however, the proteins are interacting through long-range electrostatic interactions resulting from incomplete screening of the protein charges, $S(q) \neq 1$ and $I(q)$ is not proportional to $P(q)$ only, but $S(q)$ must also be included. The effect of electrostatic interactions on $S(q)$ can be estimated using the rescaled mean-spherical approximation (RMSA; Nägele, 1996). The $S(q)$, $P(q)$ and $I(q)$ curves calculated using the RMSA model for a protein of radius $R = 3.8$ nm and a charge of about 5.6076×283^{-18} C (35 elementary charges) at a salt concentration of 75 mM are shown in Fig. 4.

It can be seen that the theoretical $I(q)$ curve coincides well with the experimental data, and the deviation of the experimental $I(q)$ from the theoretical $P(q)$ curve calculated from the protein structure can be quantitatively explained and is due to the long-range interactions and the resulting $S(q)$. This

Figure 4

The $P(q)$, $S(q)$ and $I(q)$ curves calculated for a sphere using the RMSA model with the parameters $R = 3.8$ nm, $c = 22.7$ mg cm $^{-3}$, charge/protein = 5.6076×283^{-18} C (35 elementary charges), NaCl concentration 75 mM. Uncorrected (filled circles) and corrected (open circles) experimental data measured at a concentration of 22.7 mg cm $^{-3}$ are also shown. The corrected data are well fitted by the form factor $P(q)$.

means that, at the concentrations of isomerase used in this experiment, a salt concentration of 75 mM is insufficient to screen the long-range electrostatic interactions. It also means that the part of the $I(q)$ curve that deviates from $P(q)$ in the low- q range should be used neither for the fit using the *CRYSON* program nor for the Guinier plot.

The $S(q)$ correction depends on the protein charge and concentration as well as the salt concentration and can be calculated using the RMSA model. In order to illustrate this effect we have plotted $S(q = 0)$ versus isomerase concentration for different salt concentrations in the range from 1 to 500 mM and compared it with the curve calculated for the hard-sphere model (Fig. 5). The same data are plotted versus the salt concentration for protein concentrations in the range 1–100 mg cm $^{-3}$ (Fig. 6). In these calculations, the protein charge was fixed to 5.6076×10^{-18} C (35 elementary charges).

Figure 5

The dependence of the value of $S(q = 0)$, calculated using the RMSA model, on the isomerase concentration for salt concentrations in the range 1–500 mM, compared with data for a hard sphere. Solid lines from top to bottom: salt concentration 500, 200, 100, 75, 50, 25, 10, 5, 1 mM; dashed line: hard sphere.

Figure 6

The dependence of the value of $S(q = 0)$, calculated using the RMSA model, on salt concentration for isomerase concentrations in the range 1–100 mg cm $^{-3}$ and protein charge 5.6076×283^{-18} C (35 elementary charges). Solid lines from top to bottom: 1, 5, 10, 25, 50, 100 mg cm $^{-3}$, as indicated in the figure.

Table 1

The values of the experimentally measured scattering cross sections $(d\Sigma_S^{\text{exp}}/d\Omega)(q=0)$ and the corrected scattering cross sections $(d\Sigma_S^{\text{corr}}/d\Omega)(q=0)$ obtained using RMSA as well as the corresponding values of the molecular volume V and V_{corr} calculated using equation (5) and the values of $S(q=0)$ obtained from RMSA.

c (mg ml ⁻¹)	Not corrected		RMSA correction		
	$(d\Sigma_S^{\text{exp}}/d\Omega)(q=0)$ (cm ⁻¹)	V (nm ³)	$S(q=0)$	$(d\Sigma_S^{\text{corr}}/d\Omega)(q=0)$ (cm ⁻¹)	V_{corr} (nm ³)
22.7	2.311	190	0.704	3.2827	206
4.54	0.628	204	0.928	0.6767	207
2.27	0.333	207	0.963	0.3457	209

Another approach taking into account the concentration effects is based on the analysis of the forward scattering. The value for $S(q=0)$ is related to the osmotic pressure Π of the particle solution,

$$S(q=0) = \frac{RT}{M_w} \left(\frac{\partial \Pi}{\partial c} \right)^{-1}, \quad (2)$$

where R is the gas constant and T the absolute temperature. We can expand the derivative to obtain

$$\Pi/(cRT) = 1/M_w + A_2 c + \dots, \quad (3)$$

where A_2 is the second virial coefficient, which depends on the interaction pair potential between particles in solution. Usually, A_2 is obtained from the slope of the linear fit:

$$1/S(c, q=0) = 1 + 2M_w A_2 c + \dots \quad (4)$$

In Fig. 7 we plot the reduced quantity $(1/c)[1/S(c, q=0) - 1] = 2M_w A_2$ versus the salt concentration.

Clearly for protein concentrations lower than 5 mg cm⁻³ all structure factors reduced in this manner coincide, thus indicating that for these low concentrations the structure factor is well described by equation (4), and A_2 is only dependent on the salt concentration and becomes independent of the isomerase concentration.

Figure 7

The dependence of the reduced value of $(1/c)[1/S(c, q=0) - 1]$ on salt concentration for isomerase concentrations in the range 1–100 mg cm⁻³. Dashed line: 1 mg cm⁻³; solid lines: 5, 10, 25 and 50 mg cm⁻³; dotted line: 100 mg cm⁻³ as indicated in the figure.

3.3. Determination of the correct $I_S(q=0)$ and the protein volume V

From the experimentally measured $I_S(q)$ curves one can obtain the value of $(d\Sigma_S^{\text{exp}}/d\Omega)(q=0)$, rewriting equation (1) for $q=0$ as

$$\begin{aligned} \frac{d\Sigma_S}{d\Omega}(q=0) &= c \frac{N_A}{M_w} \left(\frac{b}{V} - \rho_0 \right)^2 V^2 S(q=0) \\ &= c \frac{N_A}{M_w} (b - \rho_0 V)^2 S(q=0) = I_S(q=0) K_N, \end{aligned} \quad (5)$$

where $b/V - \rho_0 = \Delta\rho$ is the scattering contrast of the protein, b is the scattering length, V the volume, b/V the scattering length density of the protein and ρ_0 the scattering length density of the solvent. Note that the formulation of equation (5) with $\Delta\rho$ implies that we have two volume-dependent terms on the right-hand side of the equation, whereas the use of $(b - \rho_0 V)^2$ avoids that problem and makes the determination of V unambiguous. The length b can be obtained from the atomic composition of the protein using CRYSON or other programs. Thus, in order to obtain the protein volume V we have to know the value of $S(q=0)$, which depends on the salt and the protein concentrations, as discussed above. For spherical particles the values of $S(q)$ and in particular $S(q=0)$ can be calculated using the RMSA model, and these are given in Figs. 5 and 6 for different protein and salt concentrations. The value of the corrected scattering cross section can then be calculated using the values of $S(q=0)$ obtained from RMSA: $(d\Sigma_S^{\text{corr}}/d\Omega)(q=0) = (d\Sigma_S^{\text{exp}}/d\Omega)(q=0)/S(q=0)$.

The values of $S(q=0)$, the scattering cross sections $(d\Sigma_S^{\text{exp}}/d\Omega)(q=0)$ and $(d\Sigma_S^{\text{corr}}/d\Omega)(q=0)$, and the corresponding volumes calculated using equation (5) are given in Table 1.

As follows from Table 1, the use of the experimental (not corrected) values of $(d\Sigma_S^{\text{exp}}/d\Omega)(q=0)$ results in values of the protein volume that are too low and which, additionally, depend on the protein concentration. When the corrected values $(d\Sigma_S^{\text{corr}}/d\Omega)(q=0)$ are used, the correct, almost constant values of the volume are obtained.

We can furthermore take advantage of the fact that the measurements were performed at different protein concentrations. This will lead to an increase in statistical accuracy compared with that obtained with only one data set. In order to do so, a convenient way to analyse the intensity data in terms of equation (5) is to plot $(d\Sigma_S^{\text{corr}}/d\Omega)(q=0)$ versus the protein concentration c (Fig. 8). The slope of this line, for c expressed in mg cm⁻³, is

$$\frac{(d\Sigma_S^{\text{corr}}/d\Omega)(q=0)}{c} = \frac{N_A}{M_w} (b - \rho_0 V)^2 \quad (6)$$

and does not depend on the protein concentration (straight line). It allows a reliable determination of the protein volume.

The scattering length of the protein estimated using CRYSON is $b = 6.73 \times 10^{-9}$ cm, the scattering length density of the solvent is $\rho_0 = 6.40 \times 10^{10}$ cm⁻² and the molecular weight of the protein $M_w = 173\,200$ D. The only adjustable parameter in equation (6) is the protein volume V . Using the

experimentally obtained slope, standard water calibration (Fig. 8) of $0.145 \text{ cm}^2 \text{ mg}^{-1}$ and the protein parameters listed above, we obtain the protein volume $V = 206 \text{ nm}^3$, and thus the scattering length density $b/V = 3.27 \times 10^{10} \text{ cm}^{-2}$ and the contrast $\Delta\rho = b/V - \rho_0 = -3.13 \times 10^{10} \text{ cm}^{-2}$. This experimentally obtained protein volume is in good agreement with the dry protein volume calculated from the crystal structure using *CRYSON*, which amounts to 211 nm^3 . Such a good agreement (within $\pm 2\%$) of the value of the protein volume obtained from our SANS data using water calibration with the dry volume of the crystal structure indicates that the overall isomerase structures in solution and in the crystal are very similar (or identical). Additionally, this agreement confirms the correctness of our data correction procedures and the water calibration.

3.4. Absolute calibration of the scattering cross section of a sample

Using equations (1) and (6) we have

$$\begin{aligned} \text{slope(R)} &= \frac{(d\Sigma_R^{\text{corr}}/d\Omega)(q=0)}{c_R} \\ &= \frac{(d\Sigma_R^{\text{exp}}/d\Omega)(q=0)/S_R(q=0)}{c_R} = \frac{I_R^{\text{exp}}(q=0)}{S_R(q=0)c_R} K_N, \end{aligned} \quad (7)$$

where the left-hand side of equation (7) is the slope of the line in Fig. 8, which for isomerase amounts to $\text{slope(R)} = 0.145 \text{ cm}^2 \text{ mg}^{-1}$. The subscript 'R' denotes the reference, *i.e.* the isomerase. Thus, the calibration constant K_N can be calculated as

$$K_N = \frac{0.1595 S_R(q=0) c_R}{I_R^{\text{exp}}(q=0)}. \quad (8)$$

Using this calibration constant, the absolute scattering cross section (in cm^{-1}) of a new sample (S) can be easily calculated.

Figure 8
Absolute scattered intensity extrapolated to $q = 0$ ($d\Sigma_S^{\text{corr}}/d\Omega)(q=0)$ plotted versus protein concentration.

4. Isomerase under compression

In order to check whether the isomerase can also be a good standard sample in pressure-dependent studies, the pressure dependence of $I(q)$ at a concentration of 2.54 mg cm^{-3} and a constant temperature of 293 K was measured in the pressure range from 1 to 150 MPa using SANS (Fig. 9). The raw curves showed a systematic increase in the intensity with increasing pressure. This observation can be explained by the fact that the increase in the scattering length density of the solvent due to the compression of the solution is to a good approximation given by the pressure-induced change in the density of heavy water, which amounts to a 4.3% change per 100 MPa . As can be seen from equation (5) this effect enters quadratically in the expression for the contrast and is thus important. We find from our experimental data that the increase in the forward scattering is 17% per 100 MPa . On the other hand, using the 4.3% density change per 100 MPa the contrast variation with pressure was calculated to be about 18% per 100 MPa , which accounts quantitatively for the observed effect. For clarity, we have superimposed these curves in order to find out whether a change in the shape occurs under the action of pressure (see Fig. 9).

The $I(q)$ curves at all pressures have the same shape and indicate no dissociation or conformational change of the isomerase in this pressure range. This finding also supports our previous assumption that the increase in the forward scattering intensity is only due to the pressure-induced change in the scattering length density of water and is not related to changes in the protein structure, which would give rise to a different b/V value. We have thus to conclude that the structure of the isomerase in solution is very dense, as was already suggested by the fitting of $P(q)$ with *CRYSON*, leading to structural parameters very close to those obtained for the conformation in the crystal. This means that the structure does not contain any loose or markedly less dense regions, which in turn would act as targets for pressure. Glucose isomerase was

Figure 9
(In colour online.) Experimental $I(q)$ curves measured for the isomerase solution of concentration 2.54 mg ml^{-1} at 293 K (25 mM Tris in D_2O , 75 mM NaCl, $\text{pH}7.6$) for pressures indicated in the figure.

found to be conformationally stable up to 150 MPa and thus has the potential to be used as a scattering standard even under conditions of high pressure. Furthermore, it is well known that other biological molecules exhibit no change in structure even at higher pressures as a result of the stabilizing effect of electrostatic interactions in combination with hydrogen bonding. Changes in structure are typically observed for $P > 500$ MPa for a number of monomeric biopolymers. In a preliminary test we have found no changes in the scattering curve for egg white lysozyme up to 350 MPa. Likewise, bovine serum albumin was found to be stable up to 450 MPa. A secondary standard, besides having high pressure stability, must exhibit the following features: high monodispersity, no aggregation, well characterized crystallographic structure and nearly spherical shape, so that $S(q)$ can be calculated using the RMSA model. All these conditions are fulfilled by the isomerase. Therefore, we propose the use of the isomerase as a secondary scattering standard.

The practically identical shape of the scattering curves $I(q)$ obtained for the isomerase for all pressures also indicates that not only is the protein structure insensitive to pressure in this pressure range but also there are no visible changes in hydration that can be detected by SANS. This finding further supports the idea of using this protein as a secondary standard in SANS experiments.

5. Conclusions

The pressure dependence of SANS was measured for solutions of glucose/xylose isomerase from *Streptomyces rubiginosus* of different concentrations in the pressure range 0.1–150 MPa. It was found that the experimental form factor of the protein was practically identical to that calculated using the crystallographic structure and did not change with pressure. This indicates that the structure of the enzyme in solution is the same as that in the crystal and does not depend on pressure in the pressure range studied. Additionally, no changes in hydration with pressure were detected for this protein using SANS.

The deviation of the intensity curve $I(q)$ from the form factor $P(q)$ in the low- q range is quantitatively explained using the RMSA model as resulting from the structure factor $S(q) \neq 1$, owing to hard-sphere and electrostatic interactions.

The scattered intensity extrapolated to $q = 0$ and corrected for $S(q) \neq 1$ can be used to calculate the protein volume and results in practically concentration independent volume values, which are in agreement with the volume calculated for the crystalline protein structure. We find no indication for aggregation behaviour in the concentration range studied.

Taking into account its very high structural stability, glucose/xylose isomerase from *Streptomyces rubiginosus* can be used as a secondary standard in pressure-dependent SANS experiments.

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