Repulsive interparticle interactions in a denatured protein solution revealed by small angle neutron scattering

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Abstract In order to investigate the effect of concentration in biological processes such as protein folding, small angle neutron scattering measurements were used to determine the second virial coefficient of solutions of both native and strongly denatured phosphoglycerate kinase and the radius of gyration of the protein at zero concentration. The value of the second virial coefficient is a good probe of the non-ideality of a solution. The present results show that the unfolding of the protein leads to a drastic change in the repulsive intermolecular interactions. We conclude that these interactions are due mainly to the behaviour of the denatured polypeptide chain as an excluded volume polymer.

Key words: Denatured state of protein interactions; Interparticle; Second virial coefficient; Small angle neutron scattering; Phosphoglycerate kinase

1. Introduction

The understanding of protein folding remains one of the major goals of contemporary structural biology. This requires a detailed characterisation of both the folded and the unfolded states. While X-ray crystallography and nuclear magnetic resonance yield exponentially growing data on native proteins, only a few biophysical techniques can provide structural information about the denatured state considered as unstructured. Among these techniques, small angle scattering (SAS) of either neutrons (SANS) or X-rays (SAXS) is a very powerful tool that yields low- and medium-resolution information about the structure of macromolecules in solution [1]. Recently it has been used increasingly in the field of protein folding [2–4]. This technique provides a direct measurement of the radius of gyration of a molecule, and is therefore very sensitive to the molecule's compactness which is a key parameter characterising the degree of denaturation of a protein. Furthermore, SAS can also give a description of the overall shape of a compact macromolecule and the distribution of configuration of an unfolded chain [5,6].

However, SANS and SAXS experiments require high protein concentrations for accurate measurements. Moreover, the apparent values of the forward scattered intensity $I(0)$ and of the radius of gyration obtained by SAS spectrum analysis depend on the concentration of scattering particles. This arises from the non-ideality of solutions caused by the finite size of the particles, the volume of which would be equal to zero in an ideal solution, and by interactions existing between them, such as van der Waals, hydrophobic or electrostatic interactions due to the surface charge of the particles or to the ionic properties of the solvent. Non-ideality of biomolecule solutions affects experimental measurements and, above all, processes in the cell, where protein concentrations are far higher than those commonly used for experiments [7,8]. In spite of this, this point remains too much neglected.

In order to assess the importance of this phenomenon, we present here a small angle neutron scattering study of the non-ideality of a yeast phosphoglycerate kinase (PGK) solution and of the effect of a drastic unfolding on this non-ideality. The structure and dynamics of native and strongly denatured PGK have been previously studied using neutron and X-ray scattering [9–12]. We reconsider here previous measurements of the apparent radius of gyration and now give values extrapolated to zero concentration. They reflect the actual size of native and totally unfolded PGK. We also determine the second virial coefficient of solutions of both native and completely denatured PGK. Thus we show that non-ideality of these solutions should not be neglected and that the magnitude of the concentration effect is quite different for the native and the unfolded states.

2. Materials and methods

2.1. Experimental

2.1.1. Sample preparation. Recombinant PGK was prepared as described by Minard et al. [13]. The enzyme activity was verified using a coupled assay with glyceraldehyde-3-phosphate dehydrogenase, adapted from Bucher [14], as described by Betton et al. [15]. The experiments were performed in deuterated buffer so as to allow the highest contrast between the protein and its solvent and to minimise incoherent scattering arising from hydrogen atoms. Besides, the scattering-length density of deuterated guanidinium chloride (Gdm-Cl) is almost the same as that of heavy water, and therefore excess coherent scattering arises mainly from the protein. Deuteration of guanidinium chloride was performed as described in [10]. The purified protein was extensively dialysed against D_2O Tris-DCl 20 mM pD 7.0 buffer. The denatured samples were prepared by dissolution in Gdm-Cl to 4 M concentration. All experiments were conducted at 4°C.

The protein concentration of the samples was determined both by absorbance at 280 nm ($\varepsilon = 0.49$ ml/mg cm) and by the bicinchonic acid assay [16]. The molecular weight of PGK in deuterated solution is close to 45.3 kDa.

2.1.2. SANS experiments. The SANS measurements were carried out at the Laboratoire Léon Brillouin, using the PACE instrument. The neutron wavelengths λ were 5 Å and 11 Å and the sample-to-detector distances 0.80 m and 2.80 m respectively. This gives access to scattering vectors ranging from 0.006 to 0.5 $Å^{-1}$. The scattering vector is defined as $q = 4\pi\lambda^{-1} \sin\theta$, where 2θ is the scattering angle.

The samples were contained in fused silica cells of 5 mm inner path length. The scattering data were corrected for the solvent contribution and for the excess incoherent scattering as described in [10]. Detector

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Abbreviations: SANS, small angle neutron scattering; SAXS, small angle X-ray scattering; SAS, small angle scattering; PGK, yeast phosphoglycerate kinase; Gdm-Cl, guanidinium chloride

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non-uniformity was corrected by normalisation to the scattering from a 1 mm thick light water sample.

The duration of data collection was chosen according to the protein concentration, in order to get the same statistical accuracy for all measurements. For instance, 4 h was needed when the protein concentration was 5 mg/ml. The final spectrum of each sample corresponds to the average of all the spectra obtained during consecutive half-hour runs. This enabled us to check that the forward scattered intensity $I(0)$ stayed constant and therefore that no aggregation occurred during the course of the experiment.

2.2. Theoretical analysis

2.2.1. Zimm approximation. For a finite concentration of protein, the scattering function may be altered compared to the signal of a molecule in dilute solution. The Zimm approximation [17] takes into account the concentration effects on the scattering function. It can be written as follows:

$$\frac{I(0,0)}{I(q,c)} \cong \frac{1}{P(q)} + 2A_2Mc + 3A_3Mc^2 + ... \quad (1)$$

where $I(q,c)$ is the apparent scattering function at concentration c (g/cm^3), $P(q)$ is the structure factor of a single macromolecule, i.e. the scattering function in the molecule would give in an ideal solution, M (g/mol) is the molecular weight of the molecule, A_2, A_3, ... are the second, third, ... virial coefficients. A_2 describes interactions between pairs of molecules, A_3 is related to interactions between three molecules, etc. A determination of A_2 can then provide a good estimate of the non-ideality of the relatively dilute solution and of the extent of intermolecular interactions. It can be evaluated by performing experiments at different protein concentrations.

2.2.2. Determination of the second virial coefficient. For an ideal protein solution, the forward scattered intensity can be written [18]:

$$I(0,0) = \frac{cM}{N_A}[(\rho_p - \rho_s)\bar{v}_p]^2 \quad (2)$$

where N_A is Avogadro's number, ρ_p and ρ_s are the scattering length densities (cm^{-2}) of the protein and of the solvent respectively and $\bar{v}_p$ the partial specific volume (cm^3/g) of the protein.

Combining Eqs. 1 and 2 leads to:

$$\frac{c}{I(0,c)} = \frac{N_A}{M}[(\rho_p - \rho_s)\bar{v}_p]^{-2}(1 + 2A_2Mc + ...) \quad (3)$$

At lower concentrations, the third term $3A_3Mc^2$ and the following can be neglected. In this case, a plot of $c/I(0,c)$ vs c should be linear, with a slope proportional to A_2. For repulsive intermolecular interactions ($A_2 > 0$), Eq. 3 shows that the apparent scattered intensity $I(0,c)$ is lower than $I(0,0)$.

2.2.3. Determination of the radius of gyration. The apparent value of the radius of gyration $R_g(c)$ of a compact particle can be inferred from its scattering spectra by means of the Guinier approximation [19]:

$$I(q,c) \cong I(0,c)\exp\left(-\frac{R_g^2 q^2}{3}\right) \quad (4)$$

For a spherical particle this approximation is valid up to $qR_g \cong 1.3$ Å^{-1} [10]. It was used to obtain the apparent values of the radius of gyration and of the forward scattered intensity $I(0,c)$ for native PGK.

In the small q region ($qR_g \leq 3$), the scattering profile of an expanded chain is better described by the Debye function [20]:

$$P(x) = \frac{I(q)}{I(0)} = \frac{2}{x^2}(x - 1 + e^{-x}) \quad (5)$$

where $x = (qR_g)^2$

This function can be approximated as [10]:

$$I(q,c)^{-1} = I(0,c)^{-1}[1 + 0.359(qR_g(c))^{2.206}] \quad (6)$$

In this expression the figures 0.359 and 2.206 allow the accuracy of this approximation to remain within less than $\pm 0.4\%$ for $1 \leq x \leq 13$. The scattering spectra of unfolded PGK were fitted to this expression. A plot of $I(q,c)^{-1}$ vs. $q^{2.206}$ gives a straight line and allows the apparent radius of gyration $R_g(c)$ and the apparent scattered intensity $I(0,c)$ to be inferred from its slope and intercept.

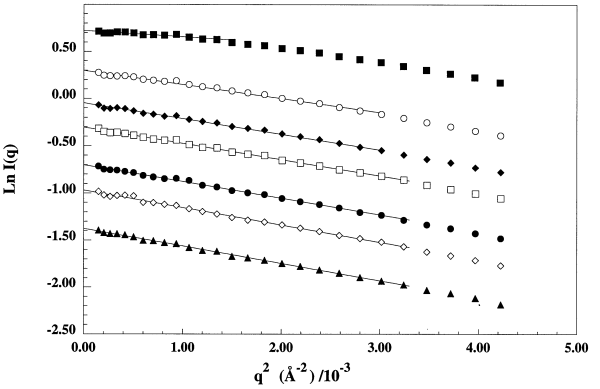

Fig. 1. Guinier plot of the scattering profiles of native PGK. The protein concentrations are: ▲ 5 mg/ml; ◇ 7.5 mg/ml; ● 10 mg/ml; □ 15 mg/ml; ◆ 20 mg/ml; ○ 32 mg/ml; ■ 60 mg/ml.

The Debye law is basically valid for a gaussian chain [20] but it can also be used for excluded volume chains [21,22].

It is noteworthy that Eqs. 4 and 6 are well established for $c = 0$. Inserting them in Eq. 1 for $P(q)$ and rearranging demonstrates that they remain very good approximations for finite, but not too high concentrations. In particular Eq. 4 leads to the following results:

$$[R_g(c)]^2 \cong [R_g(0)]^2(1 + 2A_2Mc)^{-1} \quad (7)$$

This equation enables us to determine the radius of gyration extrapolated at zero concentration for native PGK.

Similarly Eq. 6 leads to:

$$[R_g(c)]^{2.206} \cong [R_g(0)]^{2.206}(1 + 2A_2Mc]^{-1} \quad (8)$$

We can thus determine the radius of gyration extrapolated to zero concentration for the denatured PGK as well. The values of the second virial coefficient obtained from Eqs. 7 and 8 are less reliable than those given by Eq. 3, because the former hold only if the pair correlation function does not depend on the concentration.

If this is true, Eqs. 7 and 8 indicate that an increase of repulsive interactions leads to a decrease of the apparent values of the radius of gyration.

3. Results and discussion

3.1. Native PGK

The scattering experiments were performed for seven different concentrations of protein, ranging from 5 to 60 mg/ml. No aggregation was detected in the sample during data collection, even at the highest concentration.

Fig. 1 exhibits the scattering profiles of native PGK for each protein concentration. The Guinier approximation describes the scattering intensity in the appropriate q-range very well. For $c \leq 20$ mg/ml the linear fits are parallel, suggesting that the radius of gyration of native PGK does not depend strongly on the concentration.

In order to determine the second virial coefficient, we plotted $c/I(0,c)$ vs the protein concentration (Fig. 2a). For concentrations below 30 mg/ml, the dependence is linear and the slope of the straight line gives: $A_2 = (0.4 \pm 0.1) \times 10^{-4}$ ml mol g^{-2}.

For $c \geq 30$ mg/ml, the scattered intensity $I(0,c)$ is weaker than expected. This cannot be due to any aggregation, because aggregation would have led to an increase of $I(0,c)$. This decrease of the intensity reveals higher order contributions from repulsive interactions. In this case, the third term involving the third virial coefficient is not negligible any more.

We also determined the radius of gyration of the native

protein extrapolated to zero concentration (Fig. 2b). We found: $R_g(0) = 23.8 \pm 0.3$ Å.

The value of A_2 inferred from this extrapolation is less accurate but is similar to the value previously obtained.

We have calculated the radius of gyration of native PGK from the atomic co-ordinates of the crystal structure and to the scattering density lengths and taking into account the contrast with the solvent. The theoretical value is $R_g = 23.6$ Å, in very good agreement with our measurements.

Previous work determined the radius of gyration of native PGK using either SANS or SAXS [12,23–26]. Whereas some performed measurements at different concentration, none tried to estimate the second virial coefficient. Henderson et al. [23] did it only for PGK in the presence of substrates. For instance, they found $A_2 = 0.67 \times 10^{-4}$ ml mol g^{-2} for the binary complex PGK+β,γ-bidendate $Cr(H_2O)_4ATP$. This difference with our result may be interpreted in terms of either a lower charge screening due to substrate binding or of higher electrostatic interactions due to a lower pD= 5.9.

The second virial coefficient of a macromolecules solution can be written [27,28]:

$$A_2 = 4\pi^{3/2}\Psi N_A \frac{R_g^3(0)}{M^2} \quad (9)$$

Ψ is a universal constant whose value depends on the shape or the conformation of the molecule. For hard spheres Ψ = 1.619 [29]. Assuming that native PGK may be regarded as a hard sphere, allowing no interpenetration between particles, the second virial coefficient would then be: $A_2 = 1.4 \times 10^{-4}$ ml mol g^{-2}. This calculated value is about 4 times higher than the experimental one. However, the assumption that native PGK is a hard sphere is not correct. First, the geometry of native PGK is not exactly spherical. Secondly, because of the low distribution of charges of PGK in our experimental conditions, the interaction potential between molecules should be attractive. The second virial coefficient should then be smaller. This was indeed observed.

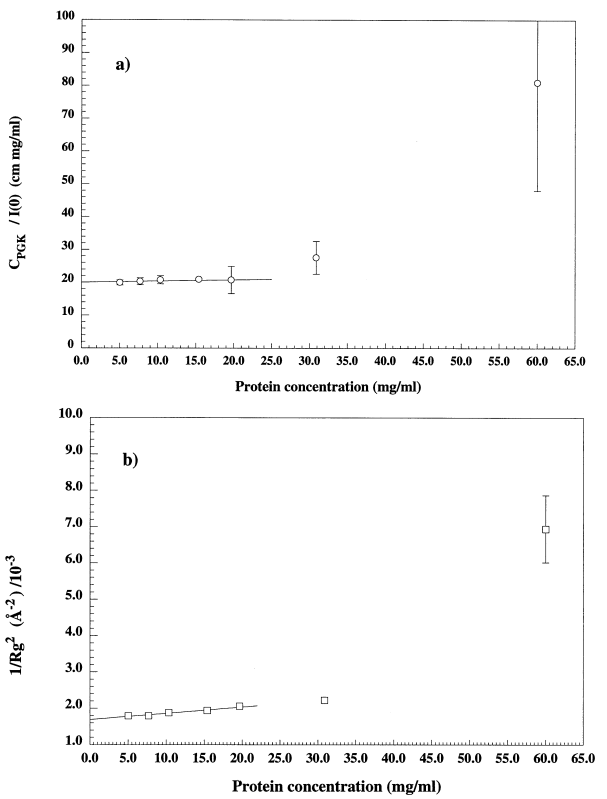

Fig. 2. Dependence on protein concentration of $c/I(0,c)$ (a) and $[R_g(c)]^2$ (b) for native PGK in 20 mM Tris-DCl pD 7.0. The slope of the straight line in a gives the value of the second virial coefficient and the intercept in b the value of the radius of gyration at zero concentration.

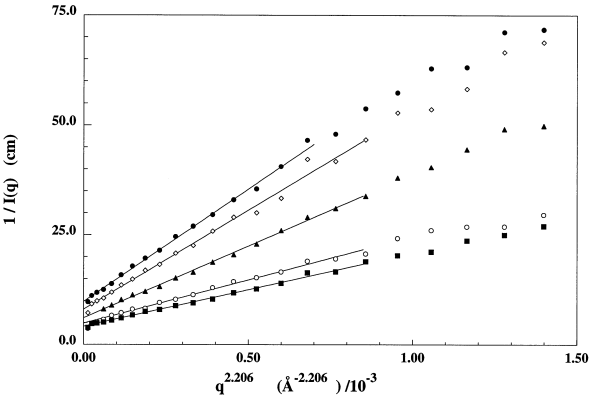

Fig. 3. Plots of $[I(0,c)]^{-1}$ vs $q^{2.206}$ for PGK denatured by 4 M Gdm-Cl. The protein concentrations are: ■ 2.5 mg/ml; ○ 3.5 mg/ml; ▲ 5 mg/ml; ◇ 7 mg/ml; ● 10 mg/ml.

3.2. Denatured PGK

PGK was strongly denatured with 4 M Gdm-Cl. Previous works [11,30,31] have demonstrated that in the presence of such a concentration of denaturant no structure remains and the protein is completely unfolded. Furthermore, Calmettes et al. [10,11] showed that strongly denatured PGK behaves as an excluded volume polymer, where every amino acid is fully solvated.

The scattering experiments were performed at five different protein concentrations ranging between 2.5 and 10 mg/ml. The denaturation was very quick and the system reached equilibrium within a few minutes. A set of consecutive runs revealed that the protein started to aggregate a few hours after addition of Gdm-Cl, depending on the protein concentration. In the data analysis we only kept the first spectra for which no aggregation was detected.

In Fig. 3 we plot the reciprocal of the intensity $I(q,c)$ vs $q^{2.206}$ for denatured PGK and the line fits to Eq. 6. This plot shows that Eq. 6 correctly describes the scattering profiles of denatured PGK. As shown in Fig. 4 the decrease of both the apparent forward intensity and the apparent radius of gyration with the concentration is now substantial. In the concentration range of our experiments, $c/I(0,c)$ varies linearly with c and we can deduce the value of A_2 from the slope of the straight line (Fig. 4a). We obtain: $A_2 = (10 \pm 2) \times 10^{-4}$ ml mol g^{-2}.

By plotting $1/R_g(c)^{2.206}$ vs c we get the value of the radius of gyration of denatured PGK extrapolated to zero concentration (Fig. 4b): $R_g(0) = 89.0 \pm 4.0$ Å.

Previous reports have given values lower than this: 71.5 ± 2.0 Å at 5 mg/ml [10] and 66 ± 2.0 Å at 10 mg/ml [12]. This results from the very significant concentration effect. Now the comparison between native and denatured PGK reveals a far more dramatic increase of the radius of gyration

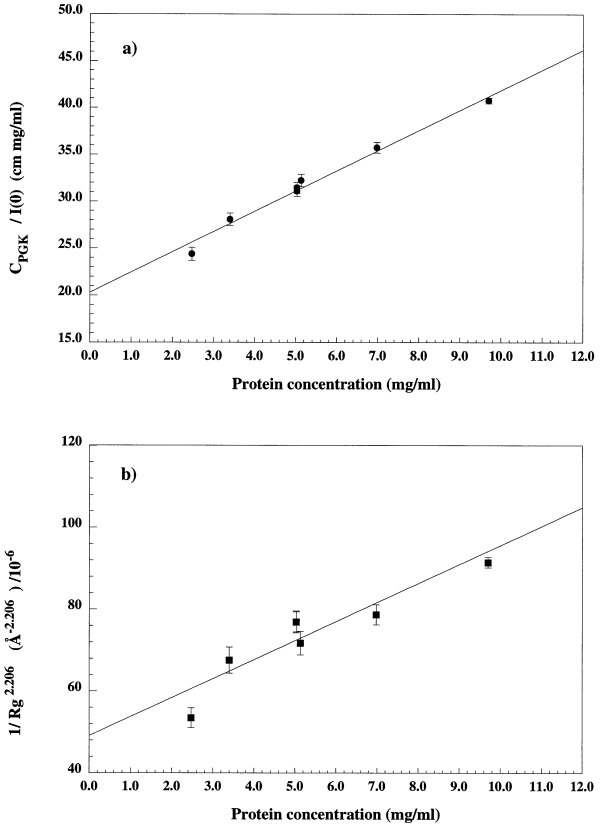

Fig. 4. Dependence on protein concentration of $c/I(0,c)$ (a) and $[R_g(c)]^{2.206}$ (b) for PGK denatured by 4 M Gdm-Cl. The slope of the straight line gives the value of the second virial coefficient and the intercept in b the radius of gyration at zero concentration.

when the protein becomes unfolded: almost a 4-fold expansion.

This strong concentration dependence indicates that unfolded PGK behaves as an excluded volume chain, confirming our previous findings. In fact, the second virial coefficient of a gaussian chain in a θ-solvent is equal to zero and there is no concentration dependence of the radius of gyration nor of the forward scattered intensity. The second virial coefficient of an expanded chain can be calculated using Eq. 9, with $\Psi = 0$ for a gaussian chain and $\Psi = 0.233$ for an excluded volume chain [28,32]. In the last case, the second virial coefficient of denatured PGK should be: $A_2 = 11.5 \times 10^{-4}$ ml mol g^{-2}, according to the value of $R_g(0)$. This is in excellent agreement with our measurements and confirms that PGK behaves as an excluded volume polymer. The excluded volume for each molecule can be estimated to be $u = 2A_2M/N_A = 6.8 \times 10^6$ Å^3.

From osmotic pressure measurements Lapanje and Tanford [33] determined the second virial coefficient of several proteins denatured in 6 M Gdm-Cl. They established the variation of A_2 with the molecular weight. According to their results, the second virial coefficient of denatured PGK would be $A_2 = 8.6 \times 10^{-4}$ ml mol g^{-2}. This value is slightly lower than ours, which is obtained for a deuterated solution, but is still within the error bars of our measurements.

The second virial coefficient of denatured PGK is about 25 times larger than for native PGK. The dramatic increase in the volume of denatured PGK caused by unfolding is not the only reason explaining this deviation from ideality of the solution. The main reason is the drastic change in interparticle interactions. It does not concern electrostatic interactions which are completely screened because of the high ionic concentration in the denaturing solution (4 M Gdm-Cl). In the native protein, hydrophobic and hydrophilic residues were rather segregated. In contrast, in the fully denatured protein every kind of residue is exposed to the solvent and not to other monomers of the chain. Therefore, denatured PGK behaves as a polymer in good solvent. This means that any segment of the unfolded chains repel each other because of preferential interaction of the different residues with the solvent.

4. Conclusion

We have shown here that, whereas there are very few repulsive interactions in the native PGK solution, the strongly unfolded polypeptide chains repel each other significantly. In this case, it seems that the second virial coefficient does not depend on the composition of amino acids in the protein, as it corresponds to the values expected for an excluded volume chain or a protein of the same size. However, this does not mean that the intermolecular interactions are the same for every unfolded protein whatever its amino acid composition: Tanford's results indicate that the second virial coefficient of denatured pepsinogen did not fit with his other results. Moreover, the second virial coefficient of acid-denatured cytochrome *c* measured by Damaschun et al. [34] is also too large compared to the predictions. Therefore, the overall interparticle interactions may strongly depend on many different parameters, among them the conformation of the molecule is very important but also the way the protein is denatured and the polyelectrolyte nature of the polypeptide chain.

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References

[1] Jacrot, B. (1976) Rep. Prog. Phys. 39, 911–953.
[2] Trewhella, J. (1997) Curr. Struct. Biol. 7, 702–708.
[3] Kamatari, Y.O., Konno, T., Kataoka, M. and Akasaka, K. (1996) J. Mol. Biol. 259, 512–523.
[4] Chen, L., Hodgson, K.O. and Doniach, S. (1996) J. Mol. Biol. 261, 658–671.
[5] Calmettes, P., Roux, B., Durand, D., Desmadril, M. and Smith, J.C. (1993) J. Mol. Biol. 231, 840–848.
[6] Petrescu, A.J., Receveur, V., Calmettes, P., Durand, D., Desmadril, M., Roux, B. and Smith, J. (1997) Biophys. J. 72, 335–342.
[7] Martin, J. and Hartl, F.U. (1997) Proc. Natl. Acad. Sci. USA 94, 1107–1112.
[8] Zimmerman, S.B. and Minton, A.P. (1993) Annu. Rev. Biophys. Biomol. Struct. 22, 27–65.
[9] Receveur, V., Durand, D., Calmettes, P., Smith, J.C., Desmadril, M. and Coddens, G. (1997) Proteins Struct. Funct. Genet. 28, 380–387.
[10] Calmettes, P., Durand, D., Desmadril, M., Minard, P., Receveur, V. and Smith, J.C. (1994) Biophys. Chem. 53, 105–114.
[11] Calmettes, P., Durand, D., Receveur, V., Desmadril, M., Minard, P. and Douillard, R. (1995) Physica B 213+214, 754–756.
[12] Semisotnov, G.V., Kihara, H., Kotova, N., Kimura, K., Amemiya, Y., Wakabayashi, K., Serdyuk, I.N., Timchenko, A.A., Chiba, K., Nikaido, K., Ikura, T. and Kuwajima, K. (1996) J. Mol. Biol. 262, 559–574.

[13] Minard, P., Desmadril, M., Ballery, N., Perahia, D., Mouawad, L., Hall, L. and Yon, J.M. (1989) Eur. J. Biochem. 185, 419–489.
[14] Bücher, T. (1955) Methods Enzymol. 1, 412–422.
[15] Betton, J.M., Desmadril, M., Minard, P. and Yon, J.M. (1985) Biochemistry 24, 4570–4576.
[16] Smith, P.K., Krohm, R.I., Hermanson, G.T., Mallia, A.K., Gatner, M.O., Provenzo, E.K., Fujimoto, E.K., Goebe, H.M., Olson, B.J. and Clerc, D.C. (1985) Anal. Biochem. 150, 76–85.
[17] Zimm, B.H. (1948) J. Chem. Phys. 16, 1093–1099.
[18] Zaccaï, G. and Jacrot, B. (1983) Annu. Rev. Biophys. Bioeng. 12, 139–157.
[19] Guinier, A. and Fournet, G. (1955) Small Angle Scattering of X-Rays, Wiley Interscience, New York.
[20] Debye (1944) J. Appl. Phys. 15, 338–342.
[21] Ohta, T., Oono, Y. and Freed, K.F. (1982) Phys. Rev. A 25, 2801–2811.
[22] Rawiso, M., Duplessix, R. and Picot, C. (1987) Macromolecules 20, 630–648.
[23] Henderson, S.J., Serpersu, E.H., Gerhardt, B.S. and Bunick, G.J. (1994) Biophys. Chem. 53, 95–104.
[24] Damaschun, G., Damaschun, H., Gast, K., Misselwitz, R., Muller, J.J., Pfeil, W. and Zirwer, D. (1993) Biochemistry 32, 7739–7746.
[25] Timchenko, A.A. and Tsyuryupa, S.N. (1982) Biophysics 27, 1065–1069.
[26] Pickover, C.A., McKay, D.B., Engelman, D.M. and Steitz, T.A. (1979) J. Biol. Chem. 254, 11323–11329.
[27] Douglas, J.F. and Freed, K.F. (1985) Macromolecules 18, 201–211.
[28] des Cloizeaux, J. and Jannink, G. (1990) Polymers in Solution: Their Modelling and Their Structure, Clarendon Press, Oxford.
[29] Douglas, J.F. and Freed, K.F. (1984) Macromolecules 17, 1854–1870.
[30] Ritco-Vonsovici, M., Mouratou, B., Minard, P., Desmadril, M., Yon, J.M., Andrieux, M., Leroy, E. and Guittet, E. (1995) Biochemistry 34, 833–841.
[31] Garcia, P., Desmadril, M., Minard, M. and Yon, J.M. (1995) Biochemistry 34, 397–404.
[32] des Cloizeaux, J. (1981) J. Physique 42, 635–652.
[33] Lapanje, S. and Tanford, C. (1967) J. Am. Chem. Soc. 89, 5030–5033.
[34] Damaschun, G., Damaschun, H., Gast, K., Gernat, C. and Zirwer, D. (1991) Biochim. Biophys. Acta 1078, 289–295.