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Chemical Physics 292 (2003) 383–387
Chemical Physics
www.elsevier.com/locate/chemphys

High pressure – unfolding of myoglobin studied by dynamic neutron scattering

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Received 2 December 2002

Abstract

Globular proteins tend to unfold in response to the application of hydrostatic pressure typically above 3 kbar. This process is driven by a decrease in volume, which may occur either by releasing intra-molecular voids or by contraction of the solvent near the newly exposed protein surface. The latter involves changes in structure of the protein–solvent network. By dynamic neutron scattering we probe the pressure evolution of protein–solvent bonds. At the unfolding transition, we observe a reduction of the structural inter-conversion rates, while the fluctuation amplitudes remain essentially unaffected. This result suggests that enhanced protein–solvent interactions in the unfolded form may destabilize the native state at high pressure.

1. Introduction

Bridgman [1] discovered in 1914 that hydrostatic pressure has a similar coagulating effect on egg-white as high temperature. Nowadays pressure is appreciated increasingly as a valuable tool in food science and molecular biology to control biochemical processes [2]. While enzymes, which resist high temperature treatment, are routinely extracted from thermophilic organisms, corresponding barophilic bacteria do not exist, except possibly in deep see environments near 1 kbar. Recently it was proposed that bacteria are to resist pressure treatment beyond 10 kbar [3]. For basic research and technical applications, it is important to unravel the common features and differences of pressure- and heat-denaturation.

The latter is driven by a positive entropy change, while a negative volume change is required to shift the equilibrium towards the pressure-unfolded state. In principle the contraction may either come from a reduction of the protein volume or from a more efficient packing of the solvent near the newly exposed protein surface.

Intra-molecular voids, packing deficiencies in the protein structure, are considered to be a major driving force of the pressure instability [4]. The observed (negative) unfolding volumes are small, however, typically 0.5% of the total volume, significantly smaller than the numbers derived from calculations based on the X-ray structure and volume changes observed with model compounds [5,6].

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0301-0104/03/$ - see front matter
doi:10.1016/S0301-0104(03)00064-8

The volume of intra-molecular cavities in myoglobin amounts to 340 ml/mol, while unfolding volumes of 60–100 ml/mol have been observed experimentally [7]. This discrepancy suggests that the second factor, the solvent, needs to be taken into account.

The strength of hydrogen and hydrophobic bonds generally increases with pressure. Stronger protein–solvent interactions will stabilize both the native and the unfolded state. However, the larger interaction surface (20%) in the denatured form will tend to lower its free energy.

Proteins generally exhibit a reentrant pressure–temperature phase diagram, which allows for heat- and cold-denaturation, as shown in Fig. 1. Its elliptical shape can be deduced from the Clausius–Claperon equation using a general expansion of the free energy up second order [8]:

$$\mathrm{d}P/\mathrm{d}T = \Delta S/\Delta V. \quad (1)$$

The phase boundary emerges with a positive slope from the 1 bar line at high temperature. This implies not only positive values of the entropy change but also of the unfolding volume: ΔV, $\Delta S > 0$. The application of hydrostatic pressure thus stabilizes the native state in the vicinity of the heat denaturation temperature. At higher pressures, however, the unfolding volume turns negative, stabilizing the unfolded form. The unfolding entropy also becomes negative. At low temperatures and high pressures protein unfolding is driven by a volume decrease, which overrules the negative entropy change of the system: ΔV, $\Delta S < 0$. Thus cold-denaturation and low-temperature pressure-denaturation proceed with a release of heat, while heat denaturation requires absorption of entropy [10].

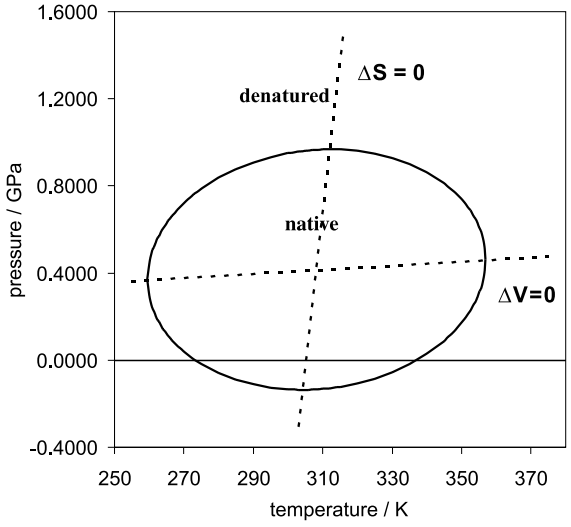

Fig. 1. Phase diagram of cytochrome C adapted from [9].

Since the structural entropy of the protein increases, compensating ordering effects in the exposed protein–water interface have to take place. The strong temperature dependence of the unfolding volume and of the entropy change suggest that solvent effects play a major role in destabilizing the native state. Weber [11] proposed some time ago, that pressure-enhanced protein–solvent bonds should be the main driving force of inducing dissociation and unfolding of proteins.

In this contribution we use a dynamic probe to measure the strength of protein–solvent bonds versus pressure. Dynamic neutron scattering experiments monitor fast molecular motions of the protein–water system on the time scale of bond fluctuations [12]. The difference in the neutron scattering cross-section between hydrogen and deuterium (a factor of ten) is used to emphasize the non-exchangeable protein–hydrogen atoms relative to the solvent deuterons. Increase in strength of the interfacial hydrogen bond network will slow down structural fluctuations or reduce their amplitude.

Myoglobin is soluble even at extreme concentrations for the physiological reason of oxygen transport in a crowded environment [15]. At 0.7 g D_2O/g protein, about 80% of the scattered intensity derives from the non-exchangeable hydrogen atoms of the protein. The remaining 20% are scattered by the hydration shell. Most of this water is in dynamical contact with the protein surface. Since we are interested in the protein–water dynamics as a coupled system, we do not decompose the scattering function into solvent and protein fractions.

2. Materials and methods

The neutron time-of-flight spectrometer IN6 at the Institut Laue–Langevin in Grenoble was

operated at a wavelength of 5.1 Å to obtain maximal flux. The resulting instrumental energy resolution was 100 μeV, which is the full-width at half-maximum. The relevant time window ranges from 0.1 to 15 ps. The pressure cell consists of a spindle-operated piston in a cylinder, which exerts pressure on two small aluminum bars, which were wedge-shaped at one end such that they could fit together. The sample was compressed between the wedges. An expansion strip was employed to determine the pressure. The pressure could be reproducibly adjusted between 1 and 7 kbar to an accuracy of 0.2 kbar. The scattering of the cell (5%) was subtracted from the data. Its scattering power is below the 10% scattering power of the sample. In this contribution we discuss only the large "q" regime, where the spectral contribution of the sample is much stronger than the background. Solvated myoglobin samples (SIGMA, horse muscle) were prepared at a concentration of 0.7 g D_2O/g protein. At this concentration the sample resembles a viscous liquid, which can transmit hydrostatic pressure. From low-angle neutron scattering experiments on this sample we derive an average radius of gyration of 1.6 nm, compatible with values obtained in dilute solution [15].

For the analysis of the scattering function we employ a three-component model: (1) an elastic fraction $A(q)$, which has the shape of the resolution function $R(q,\omega,\Delta\omega)$, (2) a quasi-elastic fraction due to diffusive motions, which is our main interest. Its line-shape is defined by $L(q,\omega,\beta)$, convoluted with the resolution function and (3) a vibrational component, which is approximated by a Debye vibrational density of states g_{vib}. $\hbar\omega$ designates the energy exchange between the neutron and the scattering center and $\hbar q$ is the magnitude of the corresponding momentum exchange. W denotes the Debye–Waller factor. $\Delta\omega$ is the width of the resolution and β denotes deviations from a Lorentzian spectrum. For the scattering function $S(q,\omega)$ we finally write [13]

$$S(q,\omega) = \mathrm{e}^{-2W}\left[A(q)\,R(\omega,\Delta\omega) + (1-A(q)) \times R(\omega,\Delta\omega)\otimes L(q,\omega) + q^2 g_{\mathrm{vib}}\right]. \quad (2)$$

Fits to the monotonic decaying scattering function, $S(q,\omega)$, will emphasize the strong central region of the spectrum, which is dominated by the elastic component. Since most of the relevant dynamic information resides in the wings of the spectrum, we use instead a frequency-weighted quantity, the dynamical susceptibility, $\chi(q,\omega)$. The imaginary part of the susceptibility, $\chi''(q,\omega)$, is related to the scattering function, $S(q,\omega)$ by

$$\chi''(q,\omega) = k_{\mathrm{B}} T\,\omega S(q,\omega). \quad (3)$$

The fits of the data to Eq. (3) exhibit superior sensitivity to parameter variations in comparison with Eq. (2). Such fits force us to discard the simplest model of the quasi-elastic line-shape, the Lorentzian model with $\beta = 1$. Instead we employ the Cole–Davidson function, which is equivalent to a stretched exponential function in the time domain

$$L(\omega,\tau,\beta) = Re\left\{(1-\mathrm{i}\omega\tau)^{-\beta}\right\}, \quad (4)$$

where τ denotes the characteristic time, $\beta \leqslant 1$ is the stretching parameter. The resulting β-values 0.7 (± 0.05) suggest contributions from several components.

A further decomposition into Lorentzian spectra is not attempted because of the limited frequency window. The stretching parameter does not vary systematically with pressure, indicating that the dynamic mechanism.

3. Results

Fig. 2 shows the central peak of the neutron scattering spectrum of myoglobin in D_2O, the so-called elastic component, at various pressure values. The elastic fraction of the spectrum results from scattering centers which do not move within the accessible time window of 15 ps. The elastic intensity increases discontinuously with pressure. Myoglobin denatures at pH 7 in the range between 3 and 4.5 kbar, as judged from the optical absorption spectrum of the hem group. This suggests that the discontinuous increase of the elastic intensity above 3 kbar reflects the pressure-induced unfolding of the molecule. The observed intensity

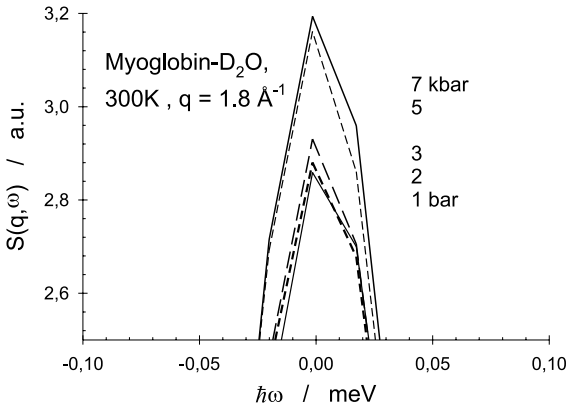

Fig. 2. The elastic spectrum near $\omega = 0$ versus pressure adapted from [14]. Pressure: 1 bar and 2, 3, 5 and 7 kbar.

changes are reversible within 10%. The enhanced elastic scattering may originate either, from a slowing down of structural fluctuations (slower than 15 ps) in the unfolded form, or from geometrical constraints.

The latter seems unlikely in view of the less compact denatured state. Fig. 3 shows the evolution of the susceptibility-spectrum (Eq. (3)) with pressure. The maximum on the low-energy side represents the elastic component (dotted line). The vibrational contribution leads to a maximum at about 6–7 meV. The quasi-elastic fraction in the intermediate energy range decreases with pressure, compensating the increase in the elastic fraction of Fig. 2.

The gap in the quasi-elastic spectra between 3 and 5 kbar reflects again the unfolding transition of myoglobin. Fits to Eqs. (2)–(4) are shown for all pressures, while data are displayed at 1 bar, 3 and 7 kbar for clarity.

To minimize the number of adjustable parameters, several fitting scenarios were tested. It was assumed that the main effect of pressure is to reduce the amplitude of molecular motions. To achieve reasonable fits required the adjustment of further parameters. In contrast, the assumption that essentially the rates of molecular transitions change with pressure, lead to fits of comparable quality (Fig. 2) without the need to adjust the quasi-elastic amplitudes as a function of pressure.

Thus, the simplest model involves only the rates of molecular motions, consistent with the general increase of the hydrogen bond strength and viscosity of water at high pressures. The resulting correlation times are shown in Fig. 4.

The increase of the correlation times below 3 kbar, reflect the change in viscosity with pressure. The librational motions of water at protein surface are somewhat faster than those of the protein side chains. In the unfolding regime, however, we observe an extra slowing down suggesting that the energy barriers, which control the fast structural

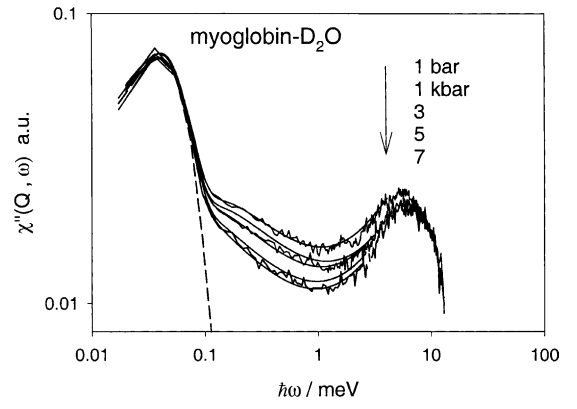

Fig. 3. Susceptibility spectra versus pressure and fits to Eqs. (2)–(4) at 1 bar and 1, 3, 5 and 7 kbar. The dashed line represents the resolution function.

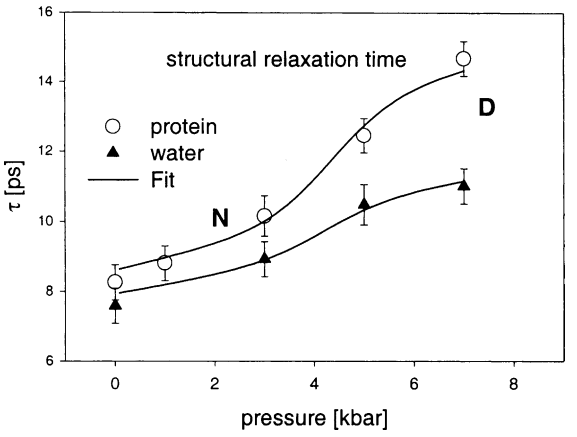

Fig. 4. Correlation times derived from the fits to the data of Fig. 3 and theoretical curves, based on a two-state model, **N** (native) and **D** (denatured), assuming an unfolding volume of 60 ml/mol.

transitions are enhanced in the unfolded form. The effect is somewhat less pronounced with water. Only 50% of the water molecules are in direct contact with the protein surface. To parameterize the transition we assume a thermodynamic model involving only two states, **N** (native) and **D** (denatured).

$$\mathbf{N} \leftrightarrows \mathbf{D}$$

The equilibrium constant is defined as usual by $K_{eq} = [\mathbf{D}]/[\mathbf{N}]$.

From fits to Eq. (5)

$$\mathrm{d}(\ln K_{eq})/\mathrm{d}P = -\Delta V/RT \tag{5}$$

we estimate an unfolding volume of 60 ml/mol as a lower limit. This number is consistent with previous results on myoglobin using optical spectroscopy [7].

4. Discussion and conclusion

These data demonstrate that the method of dynamic neutron scattering can be applied to study pressure effects on dynamics and stability of proteins. In particular, pressure-induced unfolding leads to a step-like change in the inter-conversion rates of molecular substates. In contrast, one should expect that a transfer of side-chains from a tightly packed molecular interior to water should enhance the fluctuation amplitudes without affecting the rates (see article by J. Fitter in this volume). Furthermore, considering the high concentration of the solvated myoglobin, about 1 g/g, one should take into account aggregation, which often occurs with denatured proteins. But again, removal of side-chains from the solvent by protein–protein contacts should affect the amplitudes and to a lesser extent the rates. Furthermore, we also observe an increase in the correlation time of water. Release of water by protein aggregation would speed up its fluctuation dynamics. In a preliminary structural study of pressure denaturation by neutron diffraction, we observe that about 40% of the helical structure is preserved in the pressure-unfolded state. Also, the FT-IR spectrum of the pressure-treated sample does not display the characteristic features of intermolecular β-sheets. The radius of gyration of the molecule increases from 16 to 18 Å, suggesting expansion but also possibly some aggregation. In any case we conclude from our analysis that the energy barriers, which control the transition rates, increase on the average by about 3 kJ/mol. This result may indicate, that the effective strength of protein–solvent bonds increases with increasing interfacial area. Stronger protein–solvent interactions will depress the enthalpy of the denatured form relative to the native state consistent with propositions of Weber [11].

Acknowledgements

The project is supported by a grant of the DFG Forschergruppe Hochdruck (FOR 358) and by a grant of the Bundesministerium für Forschung und Technologie (03 DOE2M1). Technical support by and discussion with M. Diehl, W. Petry, L. Melesi and H. Schober (ILL) are gratefully acknowledged.

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