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The Effect of Crowding on Protein Stability, Rigidity, and High Pressure Sensitivity in Whole Cells

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Abstract :

In live cells, high concentrations up to 300–400 mg/mL, as in Eschericia coli (Ellis, R. J. Curr. Opin. Struct. Biol. 2001, 11, 114) are achieved which have effects on their proper functioning. However, in many experiments only individual parts of the cells as proteins or membranes are studied in order to get insight into these specific components and to avoid the high complexity of whole cells, neglecting by the way the influence of crowding. In the present study, we investigated cells of the order of Thermococcales, which are known to live under extreme conditions, in their intact form and after cell lysis to extract the effect of crowding on the molecular dynamics of the proteome and of water molecules. We found that some parameters characterizing the dynamics within the cells seem to be intrinsic to the cell type, as flexibility typical for the proteome, others are more specific to the cellular environment, as bulk water's residence time and some fractions of particles participating to the different motions, which make the lysed cells' dynamics similar to the one of another Thermococcale adapted to live under high hydrostatic pressure. In contrast to studies on the impact of crowding on pure proteins we show here that the release of crowding constraints on proteins leads to an increase in the rigidity and a decrease in the high pressure sensitivity. In a way similar to high pressure adaptation in piezophiles, the hydration water layer is decreased for the lysed cells, demonstrating a first link between protein adaptation and the impact of crowding or osmolytes on proteins.

Introduction

Most of Earth biotopes experience high hydrostatic pressure (HHP) and high temperatures (HT), and compose the so-called Deep-Biosphere. In the context of biomolecules, high pressure designates rather arbitrarily values above 10 MPa and up to 110 MPa, the highest values which can be found in the trenches. Inhabiting these habitats are Eukaryotes (cells having a nucleus harboring the genome DNA); Bacteria or Archaea, two domains of Prokaryotes (cells lacking a nucleus). It is amongst this latter domain that we find the organisms adapted to the most extreme environmental conditions, although they do not exclusively belong to the Archaea. In Archaea, this ability to live under extreme conditions comes in a large part from the lipids composing their membranes. Archaeal membrane lipids are formed by ether bonds instead of ester bounds, linking the polar headgroups to isoprenoid chains instead of fatty acids [2]. Ether bonds have a higher chemical resistance than ester bonds, while isoprenoid chains have a tighter packing than aliphatic chains, ensuring the stability and function of the membrane under extreme conditions. However, structural adaptation to extreme conditions in Archaea is not restricted to membranes and membrane lipids.

Recently, we found indications for a novel adaptation strategy to HHP in the order of *Thermococcales*[3-4]. *Thermococcales* are hyperthermophiles isolated from hydrothermal vent systems which can be found from the surface to the deepest part of the oceans (4500 m below sea level) [5] and grow optimally at temperatures ranging from 75 to 105°C. In our study we used *Thermococcus kodakarensis*, a piezosensitive (sensitive to HHP) Archaeon isolated from a surface solfatara [6], and *Thermococcus barophilus*, a piezophile (requiring HHP), isolated from a deep-sea hydrothermal vent[7]. Both species grow under identical optimal conditions with the notable exception of their relative HHP optima which are 0.1 MPa for *T. kodakarensis* and 40 MPa for *T.*

barophilus. Although genomic studies have so far been unable to identify the genetic basis of the structural adaptation to HHP in these organisms, using incoherent neutron scattering techniques we succeeded recently to demonstrate the molecular basis of their adaptation to HHP at the whole cell level which occurs through variations in the molecular dynamics and in the hydration shell[3].

Since most investigations only consider parts of cells separately as, for instance, proteins or membrane lipids, important effects resulting from the crowding of the cellular milieu influencing the adaptation mechanisms might be overseen. Such effects are related to self-crowding or to the interplay between several intracellular constituents including co-solutes and osmolytes. In order to reproduce these intracellular conditions, two strategies are usually employed. The first one is self-crowding, i.e. the protein under study is the crowding agent. Numerous studies using this approach can be found in the literature, for example on β-lactoglobulin [8], or lysozyme [9]. The advantage of this approach is that it mimics the high protein concentrations found in cytoplasm (around 300 mg/mL [10]), but it fails at capturing the diversity of proteins present in the cytoplasmic media, as well as its complexity. A second approach consists in using osmolytes as crowding agents, such as sucrose [11], or even synthetic polymers [12]. The presence of these solutes, apart from stabilizing proteins samples, emulates well the protein interactions in the cytoplasm, at the cost of a less realistic situation. It thus also fails at simulating the complexity of intracellular media. We propose here for the first time to study the effects of crowding by comparing lysed and intact cells, thus providing a third alternative for studying the effect of high concentrations that are encountered within cell cytoplasm. Our approach comes at a cost of a greater complexity, but our data analysis allows us nevertheless to extract relevant parameters, as will be shown below.

Indeed, the concentration of the proteins inside cells is in the range 200 – 300 mg/ml (corresponding to the self-crowding), whereas that of RNA is in the range 75 – 150 mg/ml. So, for instance, the total concentration of protein and RNA inside *Escherichia coli* is in the range 300–400 mg/ml[1], which is extremely high even compared to concentrations typically requested for neutron scattering experiments to get sufficient signal-to-noise ratio[13]. It is already established that diffusion rates [14], but also activities, dynamics, aggregation or protein folding are influenced by crowding [1], but much less is known about the correlation between crowding and pressure effects. In a recent investigation by small angle X-ray scattering (SAXS) on structural effects in highly concentrated lysozyme in solution and under HHP, Grobelny et al. [15] extracted the attractive part J of the intermolecular interaction potential using a modified DLVO (Derjaguin-Landau-Verwey-Overbeek)-model. They showed that in the concentration region below 200 mg/ml, the parameter was decreasing as function of pressure in a nonlinear way, which was probably due to significant changes in the structural properties of bulk water, whereas for higher concentrated protein solutions up to 360 mg/ml, where hydration layers below ~4 water molecules were achieved, the interaction potential reached a plateau which was almost independent on pressure.

Further studies by elastic incoherent neutron scattering (EINS) of the crowding effect on molecular dynamics in lysozyme under HHP confirmed that the protein responded differently to pressure according to its concentration[16]. The atomic mean square displacements (MSD) of lysozyme at a concentration of 80 mg/ml in D_2O decreased up to 200 MPa and stayed then constant up to 400 MPa, whereas at a concentration of 160 mg/ml a strong restriction of about 60 % of the dynamics of protein motions took place, rendering at the same time its pressure dependence almost negligible. The reduction of flexibility under pressure was expected as the behavior of all systems under high pressure is governed by Le Châtelier's principle[17], which states that the application of

pressure shifts equilibria towards the state that occupies the smallest volume and damps therefore motions. However, self-crowding does not represent a real cytosol milieu, where many different molecules interact, but the high density indicated that crowding might have a protective effect against pressure. Such experimental situation is thus certainly closer to reality inside cells than a low concentrated solution.

In summary, crowding influences structure and dynamics in high density protein solutions and modifies HHP pressure effects on them. Moreover, we found hints in prokaryotes from the deep sea that dynamics can be changed under, and be part of the adaption strategy to HHP[3]. As it is difficult to mimic the cellular environment realistically, we adopted another approach in the present investigation to imitate a reduction of crowding: we compared the dynamics of proteins in whole cells of the hyperthermophiles *T. barophilus* and of *T. kodakarensis*, before and after cell lysis. When cells are lysed, e.g. their membranes are broken down, mainly the cell content is released in the whole available volume and the concentration of the cytoplasmic components is significantly diminished. In such a way, we modified the crowding while conserving the full molecular content of the cells. The comparison of the dynamics should therefore give access to the pure effect of crowding. As long as the cell is intact, we will speak about "crowding".

Experimental section

Sample preparation. In the present study we used the hyperthermophilic Archaeon *T. barophilus*, a piezophile, and *T. kodakarensis*, a piezosensitive, the latter as intact or lyzed cells. Cells of *T. barophilus* and *T. kodakarensis* were cultivated in TRM medium [18] under anoxic conditions at atmospheric pressure and 85°C until late exponential phase at the LM2E laboratory in Brest

(France). Cells were washed once in isotonic solution under anoxic atmosphere, pelleted in a high pressure aluminum capsule of a diameter of 6 mm and a height of 3 cm, frozen at -80 °C and transferred in dry ice to the Institut Laue Langevin (ILL) in Grenoble (France), in order to avoid damages due to oxygen contamination during transport. Lysis was performed by sonication on ice at 60% amplitude using 10 s pulses in an Ultrasonic Processor (Misonix) for 20 pulses. Upon lysis the cells, the intracellular water and the protoplasm are released and diluted in the extracellular environment, which amounts to approximately the same volume as the volume of cells, e.g. the intracellular concentration is artificially divided by a factor 2 while keeping its compositional complexity.

For the neutron experiments at ILL, the HHP aluminum capsules were loaded into the cylindrical high pressure container [19-20] under anaerobic conditions at room temperature to limit the contact of the samples with oxygen. The sample volume (1 ml) was further filled with D_2O as a pressure transmission medium. All experiments were done at 293K. As neutrons are incoherently scattered essentially by hydrogen atoms[21], this allows to probe mainly the cells and the H_2O molecules originating from inside the cells or bound to their surface. Under the experimental conditions, the samples were stable for several days at atmospheric pressure.

Incoherent neutron scattering experiments. Neutrons scatter always coherently and incoherently, but the coherent part can be neglected by choosing an appropriate Q-range and when using non ordered samples in solution or hydrated powders[22]. Therefore, when using whole cells in solution at room temperature, the significant contribution comes from the incoherent part. Moreover, the incoherent neutron scattering cross section of hydrogen is much higher than that of any other atomic nucleus present in biological samples[21], so that the motions of hydrogens or of the molecular subgroups to which they are bound are probed. When comparing intact and lysed

cells in a D_2O buffer, H_2O is however present as we are using cells that have been cultured in standard light water. Therefore, the existing populations of free or bound water is in part inside the cells, but likewise outside of the cells, in the interstitial space between them. The samples are quite viscous, due to the presence of extracellular material, which reduces water diffusion.

We used EINS on the backscattering spectrometer IN13 [23] and quasi-elastic neutron scattering (QENS) on the disk-chopper time-of-flight spectrometer IN5 [24] at the ILL to compare *T. barophilus* cells with *T. kodakarensis* intact and lysed cells. Elastic scattering takes place without any exchange of energy between the neutrons and the atomic nuclei in the sample. The corresponding atomic mean square displacements (MSD) can be calculated from the Q-dependence of the elastic part of the dynamic structure factor, $S(Q, 0 \pm \Delta E)$, where Q is the momentum transfer between the neutron and the nucleus in units of ħ and ΔE the half-width half maximum (HWHM) of the instrumental energy resolution which is related to the instrumental time window through Heisenberg's uncertainty principle. Using the Gaussian approximation [25] which assumes a Gaussian distribution of the motions around their equilibrium position, the dynamic structure factor reads

$$S_{el}(Q,0 \pm \Delta E) \approx S_0 \exp\left(-\frac{1}{3}\langle u^2 \rangle Q^2\right), \quad (1)$$

where $\langle u^2 \rangle$ is the MSD. For $Q \rightarrow 0$, the approximation is strictly valid, and it holds up to $\langle u^2 \rangle Q^2 \approx 1$[26]. The MSD can thus be obtained for each pressure value by the slope of the semi-logarithmic plot of the incoherent scattering function through

$$\langle u^2 \rangle \approx -3\frac{d \ln S_{el}(Q,0 \pm \Delta E)}{dQ^2}. \quad (2)$$

QENS is the part of scattering where small amounts of energy are exchanged between the neutrons and the sample, giving rise to a broadening of the elastic peak[22]. The structure factor

$$S(Q,\omega) = \left[A_0(Q)\delta(\omega) + \sum_i A_i(Q) L_i(\Gamma_i, \omega) + B(Q) \right] \tag{3}$$

contains an elastic part, proportional to a delta-function in ω, the energy transfer in units of ħ, representing the particles which motions are not resolved within the instrumental setup, and a sum over Lorentzians L_i which describe different motional contributions included in the QENS part. The amplitudes $A_0(Q)$ and $A_i(Q)$ depend on the momentum transfer Q. B(Q) accounts for a background. For data analysis the structure factor has to be convoluted with the instrumental energy resolution, which can be mimicked by e.g. vanadium or the sample at 20 K:

$$S_{\exp}(Q,\omega) = S(Q,\omega) \otimes S_{res}(Q,\omega) . \tag{4}$$

Following the method described by Martinez et al.[3], we did not fit the data according to the most general expression given by eq. (1), which contains an infinite sum over Lorentzian functions. Rather, we used the fact that the proportions of water and biomolecules in bacterial cell pellets, with amount to ca. 70-80 % of total water content[27], are well documented and that we successfully applied a four component-model to analyze QENS data on neurological tissues which has similar molecular compositions[28]. The model consists of four different components and a background, convolved with the instrumental resolution function:

$$S(Q,\omega) \approx \left[\begin{array}{l} p_{el} \times \delta(\omega) + p_{bulk} \times S_1(Q,\omega, D_{Tbulk}, \tau_{bulk}) + \\ p_{hydr} \times S_2(Q,\omega, D_{Thydr}) + \\ p_{prot} \times S_3(Q,\omega,\Gamma_{prot}) + B(Q) \end{array} \right] \otimes S_{res}(Q,\omega), \tag{5}$$

where p_{el} is the elastic fraction describing protons that perform only atomic vibrations. $S_1(Q, \omega)$ and $S_2(Q, \omega)$ are the scattering intensities corresponding to the contributions of free or bulk water, and of water molecules bound to the inner or outer surface of the cell. These two components are characterized by a translational diffusion constant D_T, a rotational diffusion constant D_R and a residence time τ[29], which describes the typical time a water molecule performs oscillatory motions around its equilibrium position before diffusing continuously. Within the model, the typical motions of molecules are taken into account, in particular through an analytical expression of the HWHM Γ_i of the corresponding Lorentzians.

An additional contribution, $S_3(Q, \omega, \Gamma_{prot})$, is needed to optimally reproduce the data. It is related to a faster relaxation described by a large Lorentzian with a constant HWHM Γ_{prot}, which we associated with all possible motions within the proteome of the biological system. p_{bulk}, p_{hydr}, p_{prot} (with $p_{el} + p_{bulk} + p_{hydr} + p_{prot} = 1$) are, respectively, the fractions of atoms corresponding to the various components. These fractions permit to adjust the absolute height of the scattering intensities $S_1(Q, \omega)$, $S_2(Q, \omega)$ and $S_3(Q, \omega)$ in eq. (5).

Two remarks are in order here: 1) We chose voluntarily here to normalize each spectrum neglecting by the way the global Debye-Waller-factor in eq. (5), because we considered that through the fits it could not be determined precisely. However, we measured it separately for the three samples on IN13 by using EINS. 2) It is known that water molecules perform translational diffusion and rotational diffusion simultaneously and the correct description of such superposed motions is a convolution of two Lorentzians. It was done in this way for the two water populations, but the rotational diffusion coefficient was small and constant for all samples (see [3]), so that we do not give the results here. Moreover, the residence time τ can only be extracted when the HWHM of the corresponding Lorentzian tends to a constant value for high Q-values. It was not possible to

get it here with a sufficient precision for the hydration water population due to the limited Q-range of IN5 and was thus neglected.

Results and Discussion

EINS. The three samples were measured by EINS on IN13 at room temperature and pressure values within the physiological range for the cells, e.g. between 2 and 16 MPa. Experiments were performed in December 2012 on IN13 and in March 2013 on IN5, therefore separate sets of samples were produced. The results established the reproducibility of the outcome from various samples. Moreover, at the beginning of the project we were able to measure the same samples several times, yielding comparable results (see figure in [4]).

The MSD were extracted in the Q-range from 0.5 to 1.9 $Å^{-1}$ according to eq. (2) and are presented in Figure 1.

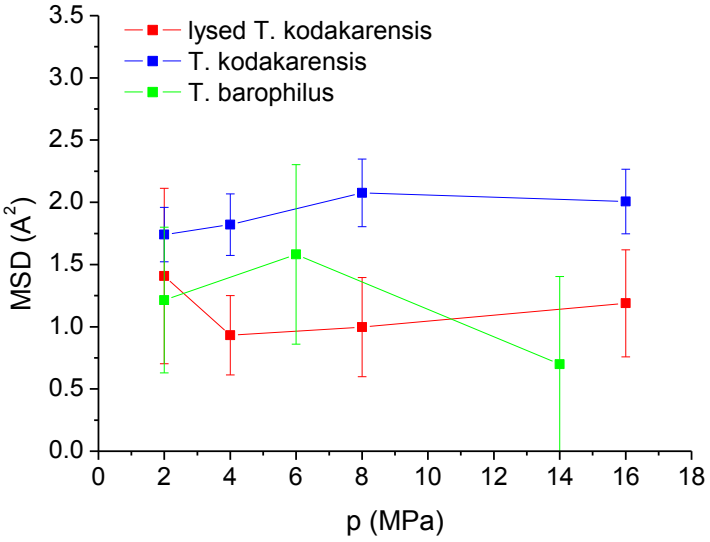

Figure 1. MSD extracted from IN13 data in the Q-range of 0.5 – 1.9 $Å^{-1}$ for the three samples. The lines are guides to the eyes.

The results are somewhat noisy due to the high absorption of the HHP cell[16], however, the curves belonging to the *T. kodakarensis* samples containing intact or lysed cells are well outside the error bars indicating a significant variation due to lysis. Since MSD corresponds to average vibrational motions of the protons around their equilibrium positions, obviously, cell lysis decreases the MSD of *T. kodakarensis*, bringing them close to those of *T. barophilus*. The global confinement and crowding have thus an influence on the local motions, but as shown in [9] it is not trivial to predict the sense of the variation. Recent results have shown that self-crowding can increase protein stability as a function of HHP[16]. In our case it is the first observation of this relationship for a natural, complex system. We therefore search to further characterize the respective contributions of crowding and cell lysis to protein behavior under HHP using QENS.

QENS. QENS data were acquired at room temperature and 0.1 and 40 MPa on IN5 at ILL. The intensities summed over all available scattering angles revealed no changes due to pressure application for the intact *T. kodakarensis* cell sample, but that the curves became significantly narrower and the dynamics significantly reduced (Figure 2) for the lysed *T. kodakarensis* cells and the *T. barophilus* cell samples indicating that both samples were sensitive to HHP.

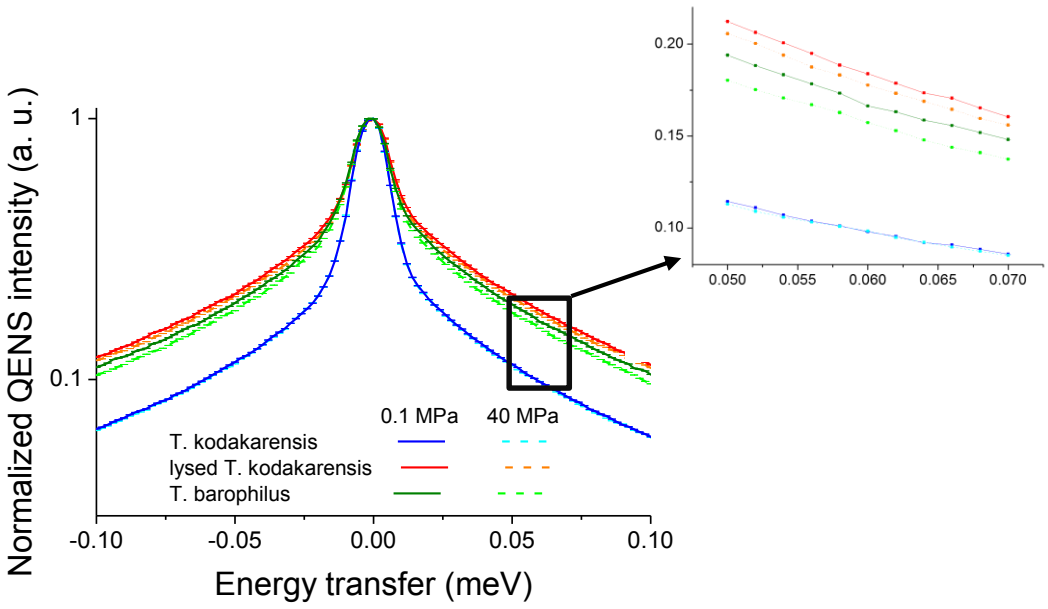

Figure 2. Normalized QENS intensities summed over all accessible scattering angles for the three samples under ambient pressure and at 40 MPa. The insert is a magnification of the curves in between 0.05 and 0.07 meV. The points correspond to errors, which are of the order of the per mill. The solid lines correspond to 0.1 MPa, the dashed lines to HHP.

The dynamical parameters which can be extracted through QENS data treatment are all related to (translational or rotational) diffusion and relaxation processes going beyond mean square displacements. For a better comparability of the samples, all spectra at a given Q-value were normalized to unity at the maximum of the elastic peak (see Figure 2), as already explained in section 2.2. Such procedure does not change the widths of the Lorentz curves, but reveals that the spectra of *T. barophilus* and of the lysed cells of *T. kodakarensis* are much closer to each other than the one of intact *T. kodakarensis* cells. The normalized QENS data were then analyzed following the protocol explained in section 2.2 and according to eq. (5). The best fit values together with errors obtained through error propagation are given in Table 1 and an example of the experimental data compared with a fit is given in Figure 3.

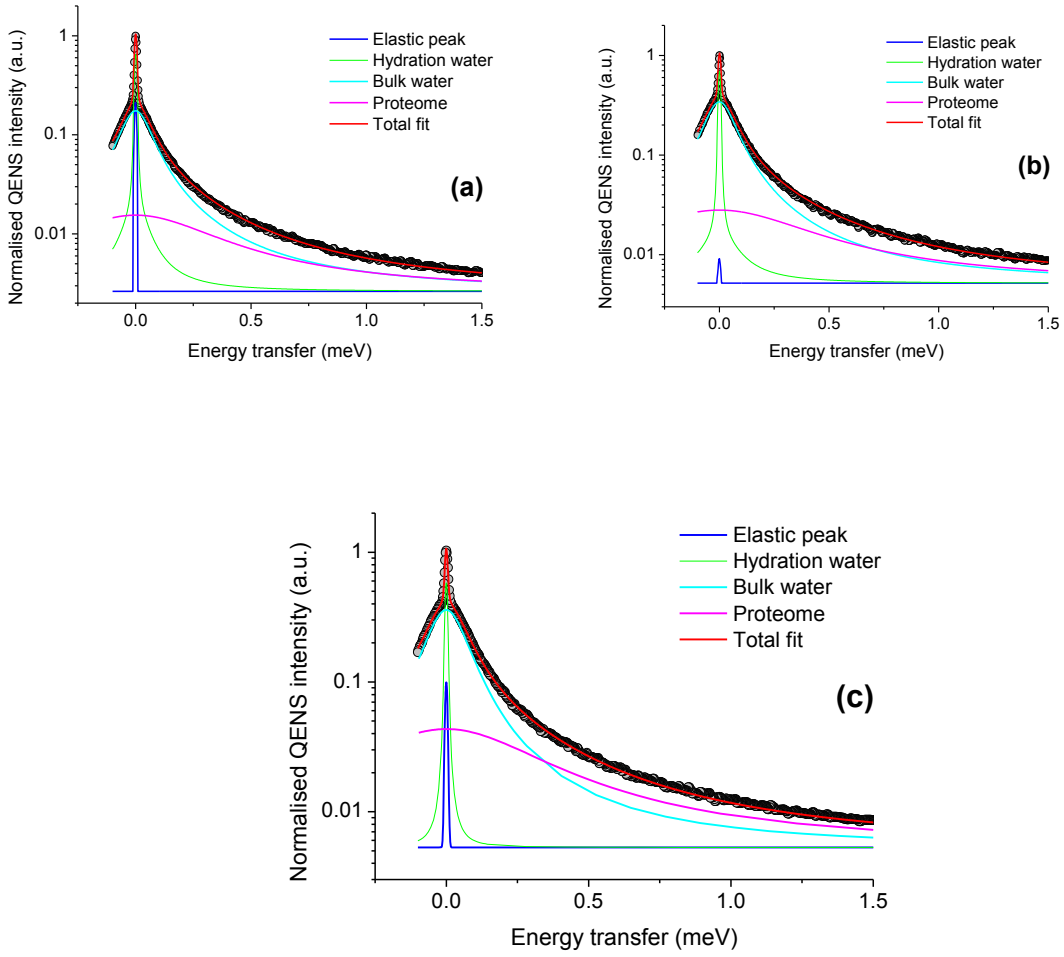

Figure 3. Experimental data and fit of a spectrum of cells from *T. kodakarensis* (a), *T. barophilus* (b) and of lysed cells from *T. kodakarensis* (c) at room temperature and 0.1 MPa at Q = 0.81 $Å^{-1}$ on a semi-logarithmic scale. The reduced χ^2-value of each fit was < 3 10^{-5}, thus very small.

Table 1. Fit parameter values for the three samples at 0.1 and 40 MPa and statistical errors. The values in the first four columns are reproduced from[3]. The fields in orange show similarities between *T. barophilus* and the lysed cells of *T. kodakarensis*, the fields in green indicate similarities between the intact and lysed cells of *T. kodakarensis*, the fields in blue correspond to the only parameter which seems sensitive to both crowding and pressure. The numbers in red are only characteristic for crowding.

	T. barophilus		*T. kodakarensis*		Lysed *T. kodakarensis*	
	0.1 MPa	40 MPa	0.1 MPa	40 MPa	0.1 MPa	40 MPa
p_{el}	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.015 ± 0.005	0.015 ± 0.005
p_{hyd}	0.10 ± 0.01	0.10 ± 0.01	0.17 ± 0.01	0.18 ± 0.02	0.06 ± 0.03	0.06 ± 0.03
p_{bulk}	0.67 ± 0.04	0.66 ± 0.03	0.60 ± 0.03	0.60 ± 0.03	0.63 ± 0.03	0.63 ± 0.03
p_{prot}	0.23 ± 0.03	0.23 ± 0.03	0.23 ± 0.03	0.22 ± 0.03	**0.29 ± 0.03**	**0.29 ± 0.03**
D_{Tbulk} (10^{-5} $cm^2.s^{-1}$)	1.98 ± 0.02	1.98 ± 0.02	1.98 ± 0.02	1.98 ± 0.02	**2.105 ± 0.05**	**2.105 ± 0.05**
τ_{bulk} (ps)	1.05 ± 0.11	1.55 ± 0.16	1.28 ± 0.13	1.15 ± 0.12	0.9 ± 0.1	0.9 ± 0.1
D_{Thyd} (10^{-7} $cm^2.s^{-1}$)	5.17 ± 0.12	3.34 ± 0.07	4.86 ± 0.10	4.41 ± 0.09	4.3 ± 0.5	3.8 ± 0.5
Γ_{prot} (meV)	0.431 ± 0.004	0.451 ± 0.003	0.363 ± 0.005	0.347 ± 0.005	0.350 ± 0.010	0.350 ± 0.010

Effect of crowding. The objective of the presented investigation was to extract the effect of crowding on the molecular dynamics of proteins in a natural and complex environment. Using intact or lysed whole cells, we could compare the dynamics of a complex, realistic, biological system. We could model our data with only four populations, which describe the different dynamical subgroups in percentage. The relative proportions of the four populations are not affected by HHP. However, our results show that decreasing crowding significantly affected them. p_{el} are the particles within the elastic peak. They can only perform vibrational motions as probed by EINS, but no diffusional motion can be resolved for them. It is the highest for intact *T.*

kodakarensis cells and similar for *T. barophilus* cells and lysed *T. kodakarensis* cells (Table 1). These results confirm the EINS results from Figure 1, demonstrating the reproducibility of the results for different sample sets obtained at different times and measured on two different spectrometers. The fraction of bulk water, p_{bulk}, for the lysed cells (0.63 ± 0.03) is intermediate between those of *T. barophilus* (0.66 ± 0.03) and *T. kodakarensis* (0.60 ± 0.03), but within error bars. The proportion of hydration water p_{hyd} is strongly reduced for the lysed cells (0.06 ± 0.03 vs. 0.10 ± 0.01 and 0.17 ± 0.03 for intact *T. barophilus* and *T. kodakarensis* cells, respectively), which is in line with the fact that the constraint of confinement is abolished in the lysed cells and some water molecules are released in the environment whereas others might still be bound to the molecules' surface. The fraction identified to belong to the proteome, p_{prot}, is clearly increased for the lysed cells (0.29 ± 0.03 vs. 0.23 ± 0.03), as their motions are now less constraint and get more importance. None of these fractions is affected by pressure as they characterize the composition within one and the same sample.

One of the main impact of the reduction of crowding is visible in the parameters of the bulk water. While the translational diffusion coefficient D_{Tbulk} was unchanged for intact cells (1.98 ± 0.02), for lysed cells it increased to a value close to that of free water, whatever the pressure, translating the higher freedom of motions of the water molecules not bound to other molecules. The latter one (2.1 10^{-5} cm^2/s at 298 K) is still below the one of pure bulk water, which is 2.3 10^{-5} cm^2/s at 298 K[30], which is understood as the upper limit for diffusional motions of free water molecules. As found also earlier[3], bulk water dynamics is unchanged by pressure application. Following the same trend, the residence time τ_{bulk}, which is related to interactions between molecules[31], and enhanced due to exchange mechanisms of biomolecules with hydration water, e.g. in presence of confinement, is slightly reduced for the lysed cells, to reach values close to that

observed for intact *T. barophilus* cells at ambient pressure. In contrast, the values of the diffusion coefficient corresponding to hydration water, D_{Thyd}, and the HWHM Γ_{prot} of the lysed *T. kodakarensis* cells are rather similar to that found for the intact *T. kodakarensis* cells and are smaller than those of intact *T. barophilus* cells. It is very interesting to note that Γ_{prot} which reflects the motions of biomolecules is slightly increased by cell lysis. This was expected since it reflects an intrinsic property of the biomolecules. To summarise the effect of crowding, one remarks that the populations are changed, as water is released from the hydration shell to the bulk, the part of immobile particles is reduced and more atoms of the proteome become mobile. The bulk water diffusion coefficient is slightly increased due to less constraints and the residence time τ_{bulk} accounting for interactions with other particles is reduced in the more dilute environment.

Effect of pressure. The only parameters clearly sensitive to pressure application are the residence time τ_{bulk} in the intact cells, the translational diffusion coefficient D_{Thyd} of hydration water and the flexibility of the cells' proteome Γ_{prot}. The residence time is reduced in the lysed cells and is no longer sensitive to HHP due to disappearance of the crowding and the constraints resulting from it. The diffusion coefficient of hydration water D_{Thyd} is always decreased under HHP application regardless the system. Finally, the flexibility of the cells' proteome Γ_{prot} is slightly enhanced in *T. barophilus* under HHP, decreased in the intact cells of *T. kodakarensis* and is constant in the lysed cells of the same species. This is in line with the findings of our previous study[3], i.e. that these parameters seem to be the most sensitive to pressure application, which points towards the fact that they participate actively to pressure adaptation.

Our analysis indicates that only some cell parameters, such as the proportion of hydration water molecules and of the proteome itself seem intrinsic properties of each sample type and are

essentially insensitive to HHP. This confirms our previous observations [3] which showed that both quantities were higher for the piezophile, from which we concluded that they were characteristic for the adaptation mechanism of this organism to HHP and HT conditions. Whereas Γ_{prot} was even enhanced under HHP, indicating a higher flexibility of the proteome of the pressure adapted cell at native conditions, the hydration water diffusion coefficient was more reduced for *T. barophilus* under pressure than for *T. kodakarensis*. It demonstrated a high stability of *T. kodakarensis* against pressure, and the reduction of the flexibility for *T. barophilus* as a function of pressure. The high stability of the *T. kodakarensis* components seemed to be slightly enhanced upon cell lysis.

Summary and Conclusions

Recent studies on the impact of self-crowding [16] or of osmolytes [9] have demonstrated an increase in protein stability, decrease protein flexibility and a strong decrease of their sensitivity to high pressure when studied as pure proteins and solution. Here we investigated the impact of crowding on a complex biological system by lysing cells to release crowding constraints. Our results showed in contrast to expectations a decrease in protein sensitivity to high pressure and a decrease in flexibility concomitant with a decrease of crowding.

In summary, some characteristic parameters of motions seem to be intrinsic to the different samples, being all hyperthermophiles, but one being in addition piezophile and the others not, as the diffusion coefficient corresponding to hydration water, D_{Thyd}, and the HWHM Γ_{prot} typical for the proteome of each cell. An additional proof could be provided by adding osmolytes as crowding agents to proteins to increase the total concentration and to reproduce by the way the dynamics found in life cells. Unfortunately, the osmolytes intrinsic to *T. kodakarensis* or *T. barophilus* cells are not available so far, but their extraction is a project for the future.

The exact reasons for the dynamical pressure adaptation techniques in piezophiles are not yet clear. We speculate that the presence and accumulation of osmolytes inside extremophiles are at least partly responsible for it. The osmolyte trimethylamine-N-oxide proved already to change the molecular dynamics[9], specifically of eukaryotes, but the effect of other organic co-solutes is largely unknown. Recent studies demonstrated an accumulation of particular osmolytes under salt and thermal stress conditions[32]. Specifically, a higher concentration of mannosylglycerate and di-myo-inositol phosphate was found in hyperthermophilic Archaea. Crowding and osmolyte concentration might also not be independent, therefore we intend to study now their synergetic effect on the functioning of proteins extracted from *Thermococcales*.

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Notes

The authors declare no competing financial interest.

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