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Adaptation to extreme environments: macromolecular dynamics in bacteria compared *in vivo* by neutron scattering

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Mean macromolecular dynamics was quantified *in vivo* by neutron scattering in psychrophile, mesophile, thermophile and hyperthermophile bacteria. Root mean square atomic fluctuation amplitudes determining macromolecular flexibility were found to be similar for each organism at its physiological temperature (~1 Å in the 0.1 ns timescale). Effective force constants determining the mean macromolecular resilience were found to increase with physiological temperature from 0.2 N/m for the psychrophiles, which grow at 4 °C, to 0.6 N/m for the hyperthermophiles (85 °C), indicating that the increase in stabilization free energy is dominated by enthalpic rather than entropic terms. Larger resilience allows macromolecular stability at high temperatures, while maintaining flexibility within acceptable limits for biological activity.

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INTRODUCTION

Microbial life has adapted to temperatures below 0 °C in glacial waters to above 100 °C and pressures of hundreds of atmospheres in deep ocean hot springs (Price, 2000). What are the mechanisms that allow proteins and nucleic acids to be stable and active under extreme conditions? A comparison of three-dimensional structures revealed no major differences in the folds of homologous psychrophilic, mesophilic, thermophilic and hyperthermophilic proteins (e.g. Aghajari *et al*, 1998; Auerbach *et al*, 1998; Bell *et al*, 2002). A consensus has arisen that thermal adaptation is associated with amino-acid substitutions modifying the balance of stabilization forces, and the concept of resilience as a key factor in thermostability has been introduced by Aguilar *et al* (1997). A hypothesis has been formulated that thermoadaptation is associated with protein dynamics (e.g. Zavodszky *et al*, 1998), in the sense that the higher thermal stability of the thermophile proteins would arise from increased rigidity and lower flexibility (Jaenicke, 2000), while increased flexibility in psychrophile protein molecules would allow activity at low temperature (Lonhienne *et al*, 2000; Petrescu *et al*, 2000; Russell, 2000; Arnold *et al*, 2001). A number of considerations come in the way of fully testing by experiment the link between thermal adaptation and macromolecular dynamics, mainly because there exists a spectrum of adaptive strategies (Jaenicke, 2000), and because *in vitro* protein dynamics strongly depends on solvent conditions (Tehei *et al*, 2001). It is of particular interest, therefore, to develop methods to characterize the mean thermal motions of the entire macromolecular population in a cell, and we propose here a novel neutron scattering approach. The forces (salt bridges, hydrogen bonds, hydration, van der Waals, hydrophobic interactions) that maintain active structures and govern atomic motions in macromolecules are 'weak' forces because their associated energies are close to thermal energy. Fast atomic thermal motions on the picosecond to nanosecond timescale act as a 'lubricant' for larger conformational changes, such as those associated with enzyme or ion pump activity, for example, occurring on slower, millisecond, timescales (Brooks *et al*, 1988; Lehnert *et al*, 1998). Atomic motions are characterized by amplitudes (how far does an atom move?) and frequencies (how often does it move?). Neutron spectroscopy provides a unique tool to study atomic thermal motions in macromolecules, because neutron wavelengths and energies, respectively, match motion amplitudes and frequencies (Brooks *et al*, 1988; Smith, 1991; Gabel *et al*, 2002). Measured variables are the mean square amplitudes in a given timescale, as a function of temperature, from which an effective mean force constant, determining macromolecular resilience, can be calculated (Zaccai, 2000). We applied the method to compare the mean dynamics of macromolecules, *in vivo*, in psychrophile, mesophile, thermophile and hyperthermophile bacteria.

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RESULTS AND DISCUSSION

Macromolecular flexibility and resilience in cells

Neutron scattering experiments were performed on washed pellet samples of whole live cells of the psychrophile *Aquaspirillum arcticum*, the mesophiles *Escherichia coli* and *Proteus mirabilis*, the thermophile *Thermus thermophilus* and the hyperthermophile *Aquifex pyrofilus*. Cells were harvested in the late mid-log phase, just before the neutron experiments. The spectrometer was designed to examine a space–time window of a few Ångstroms in 0.1 ns. At a given temperature, the incoherent scattering from individual atoms moving inside this window was observed and analysed to yield a value for their mean square fluctuation amplitude. Previous neutron experiments on protein dynamics have essentially been performed in D_2O, which scatters neutrons much more weakly than H_2O, to avoid contamination by water scattering (Doster *et al*, 1989; Fitter & Heberle, 2000; Zaccai *et al*, 2000). We avoided the use of D_2O, because it is known to have an influence on protein stability and dynamics (Bonneté *et al*, 1994; Tehei *et al*, 2001). It was possible to collect data in light water solutions with negligible scattering from the water component, because the space–time window essentially selected motions of atoms that are anchored to macromolecules (proteins, nucleic acids and their complexes), and was not sensitive to cytoplasmic bulk water (Trantham *et al*, 1984), small peptides or the smaller membrane components, for example, which diffuse out of the window during the timescale of the experiment. Strongly bound water will contribute as an internal part of the macromolecules (Bon *et al*, 1999). The overall macromolecular composition of the bacterial cells examined is not expected to vary significantly from one cell type to another. Macromolecules constitute 96% of the total dry weight of an *E. coli* cell. DNA represents 3%, and lipids and polysaccharides about 17%; the majority, more than 75% of the dry weight, consists of proteins and ribosomes, themselves made up of about 50% protein and 50% RNA by mass (Bicout & Field, 1996). Within a given bacterium, differences in protein expression due to metabolic modifications in unstressed cells affect a few hundred proteins out of about 5,000 (Rosen & Ron, 2002). It is reasonable to assume, therefore, that the neutron scattering data described in this paper are dominated by the dynamics of the proteins, making up the cellular proteome, in association with their natural environment.

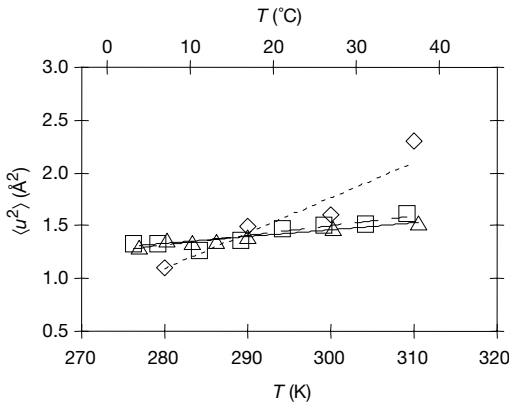

Fig 1 | Mean square amplitudes $\langle u^2 \rangle$ were plotted against temperature T for *E. coli* unstressed cells (triangles), cells heated ('cooked') to 80 °C (diamonds) and cells that were heat shocked at 47 °C (squares). The greater dispersion of data points for the 'cooked' cells reflects larger errors due to lower signal to noise. This is because there is less scattering material in the instrumental space–time window under these conditions. Effective mean force constants $\langle k' \rangle$, describing mean macromolecular resilience, were calculated from the slopes of the straight-line fits as described in Methods. They are 0.42 ± 0.01, 0.08 ± 0.03 and 0.30 ± 0.01 N/m for the unstressed, 'cooked' and heat-shocked cells, respectively.

The mean square fluctuation values $\langle u^2 \rangle$ calculated as described in Methods were plotted as a function of temperature, T (Figs 1,2). The $\langle u^2 \rangle$ values are on an absolute scale and refer to thermal motions in the 0.1 ns time domain defined by the energy resolution of the spectrometer. In order to validate the comparison among different samples, intensity data were collected, normalized and analysed in an identical manner. Effective mean force constants, $\langle k' \rangle$, defining mean macromolecular resilience (Zaccai, 2000; Bicout & Zaccai, 2001), were calculated from $\langle u^2 \rangle$ versus T, as described in Methods. A smaller slope corresponds to a larger resilience and vice versa. The force constants, which are of the order of 0.1 N/m, have also been related to measurements on single molecules by using near-field microscopy or laser tweezers (Linke & Granzier, 1998; Oesterhelt *et al*, 2000).

Dynamic fluctuations and force constants

Different mean square amplitudes are expected for folded and unfolded proteins (Receveur *et al*, 1997; Bu *et al*, 2000, 2001). In order to check that measurements do in fact refer to macromolecular dynamics in cells, we compared data from native samples and from samples where various degrees of denaturation were expected. Experiments were performed on three *E. coli* samples: freshly harvested live cells, freshly harvested live cells heated at 80 °C in the sample holder for 2 h before data collection (the 'cooked' sample), and cells that had been heat-shocked for 1.5 h at 47 °C, then harvested and placed in the sample holder for data collection (the 'stressed' sample). The $\langle u^2 \rangle$ values for the *E. coli* samples plotted in Fig 1 increase linearly over the entire temperature range. The significantly larger mean square amplitudes and lower macromolecular resilience for the 'cooked' sample are indicative of denatured protein systems, in agreement with previous neutron scattering experiments on pure protein samples (the larger scatter of the data for the denatured system reflects lower signal to noise due to very large fluctuation amplitudes exiting in part from the measurement window). Interestingly, a small difference also appeared for the heat-stressed sample. We concluded that the method allows the measurement of values that are truly representative of the folding status of macromolecules and therefore relevant to address the question of thermoadaptation.

The $\langle u^2 \rangle$ values for the unstressed mesophile, thermophile and hyperthermophile cell samples increased linearly with temperature, while the plot for *A. arcticum* showed a striking transition above 20 °C (Fig 2). We note that 17 °C is the maximum temperature at which *A. arcticum* can maintain net growth (Butler *et al*, 1989). Following the observations on denatured protein and 'stressed' and 'cooked' *E. coli* cells, it is reasonable to assume that the transition from linear behaviour in the *A. arcticum* data reflects macromolecular denaturation occurring during the time of data collection.

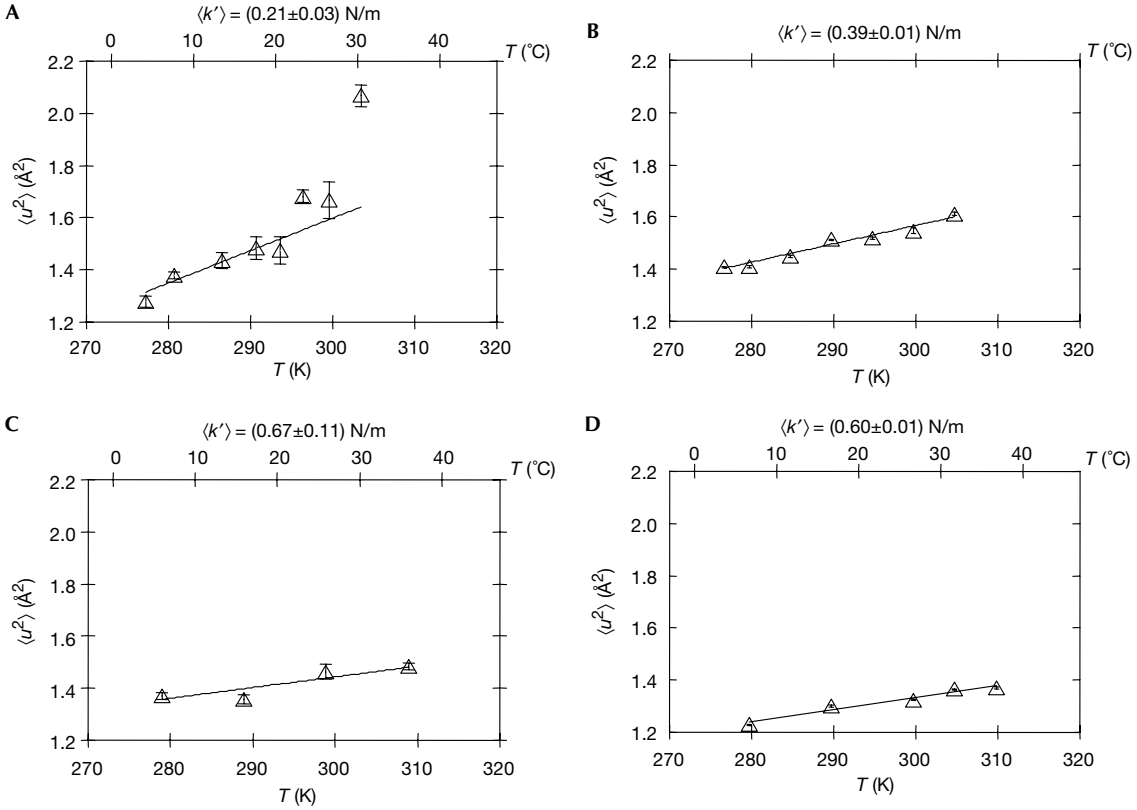

Fig 2 | Mean square amplitudes $\langle u^2 \rangle$ were plotted against absolute temperature T for *A. arcticum* (**A**), *P. mirabilis* (**B**), *T. thermophilus* (**C**) and *A. pyrofilus* (**D**) samples. Effective mean force constants $\langle k' \rangle$, describing mean macromolecular resilience, were calculated from the slopes of the straight-line fits in the temperature region where the bacteria proteins are stable, as described in Methods.

The same scan temperature range was examined for all the samples. Lower temperatures were not measured because of ice formation and in order to avoid the use of cryo-solvents. Due to technical limitations, it was not possible to collect data at the physiological temperatures of the thermophiles and hyperthermophiles. Based on the assumption supported by the mesophile and psychrophile data that stable native systems show straight-line behaviour, the amplitudes for the thermophiles and hyperthermophiles at their respective physiological temperatures were calculated by extrapolation of the straight lines fitted to the data. Similar values were found for the respective $\sqrt{\langle u^2 \rangle}$ values of the bacteria at their physiological temperatures (Fig 3). Note that these values are significantly smaller than the values associated with protein denaturation seen in Figs 1,2A. Functional root mean square fluctuations, therefore, appear to be maintained within narrow limits around 1.2 Å, independent of the adaptation temperature (from 4 to 85 °C) (Fig 3). This experimental finding may be useful as a guide to force field calculations in molecular dynamics simulations of proteins.

Effective force constants ($\langle k' \rangle$) were calculated from the temperature range where the macromolecules from the five organisms remained native (Figs 1,2). We named the $\langle k' \rangle$ value 'mean resilience' in order to avoid 'rigidity', which has been used broadly and in qualitative terms. The straight-line approximation for $\langle u^2 \rangle$ versus T was adequately supported by the data with significantly different slopes for the different bacteria. Using

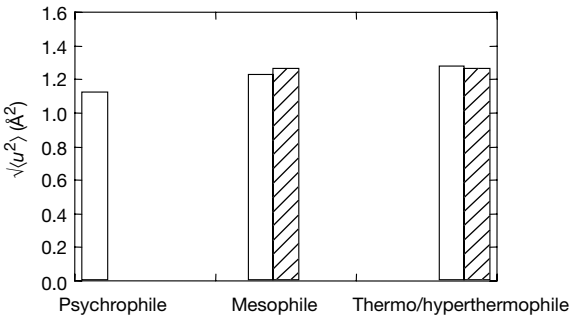

Fig 3 | Root mean square fluctuation $\sqrt{\langle u^2 \rangle}$ values (flexibility) were calculated at each adaptation temperature (from Figs 1,2; by extrapolation for *T. thermophilus* and *A. pyrofilus*) and plotted versus adaptation temperature T for the three bacterial types: 4 °C for *A. arcticum* (blank), 37 °C for *E. coli* (blank) and *P. mirabilis* (hatched), 75 °C for *T. thermophilus* (blank) and 85 °C for *A. pyrofilus* (hatched). The calculated uncertainty for the extrapolated values is ±0.04 Å for the thermophile and ±0.01 Å for the hyperthermophile.

equation (2) (see Methods), $\langle k' \rangle$ values of 0.21±0.03, 0.42±0.01, 0.39±0.01, 0.67±0.11 and 0.60±0.01 N/m were calculated for *A. arcticum*, *E. coli*, *P. mirabilis*, *T. thermophilus* and *A. pyrofilus* cells, respectively. For comparison, the resilience values of hydrated myoglobin and myoglobon trapped in a hard

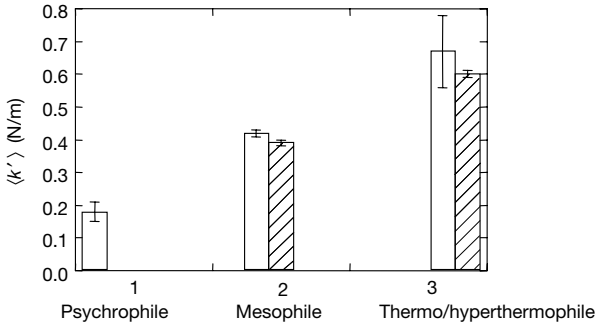

Fig 4 | Mean macromolecular resilience $\langle k' \rangle$ for each bacterial type, plotted versus adaptation temperature (histograms are as for Fig 3).

trehalose glass are 0.3 and 3 N/m, respectively (Zaccai *et al*, 2000). The correlation between mean resilience and physiological growth temperature is shown in Fig 4. To the best of our knowledge, the experiments described here provided the only quantification, so far, of mean macromolecular resilience in a cellular environment.

Protein and cellular adaptation

Our results suggested that macromolecular resilience is an important parameter in adaptation to extreme temperatures in order to maintain functional stability at physiological temperatures. The resilience values, which increased with stabilization temperature, indicated the dominance of enthalpy terms in the stabilization free energy differences. For proteins in which entropy terms (such as the hydrophobic interaction) are dominant, a more flexible and less resilient macromolecule will be more stable (Zidek *et al*, 1999; Fitter & Heberle, 2000; Hernandez *et al*, 2000; Arnold *et al*, 2001; Tehei *et al*, 2001).

We note that the force constants analysed are mean values for all the macromolecules in the cell. Can the molecular determinants associated to these forces be identified by genomic or structural analysis? Haney *et al* (1999) compared protein sequences from mesophilic and extremely thermophilic *Methanococcus* species. The observed replacements decrease the content of uncharged polar residues, increase the content of charged residues, increase residue hydrophobicity and increase residue volume in the extremely thermophilic proteins. Cambillau & Claverie (2000) have published a correlation between adaptation to high temperature and the average charged minus polar amino-acid percentage (Ch-Pol) in mesophile and hyperthermophile genomes. In general, an increase in the Ch-Pol percentage would lead mainly to enthalpic (hydrogen bonds, hydration interactions, salt bridges) contributions to the free energy landscape, as is reflected in our data by the increase in $\langle k' \rangle$ values. A number of studies have been published analysing structural differences among homologous psychrophile, mesophile and thermophile proteins (Aghajari *et al*, 1998; Bell *et al*, 2002; Gianese *et al*, 2002); common trends include a decrease in the number of salt bridges and of surface-exposed side chains in the psychrophiles as well as decreased protein–protein and interdomain interactions within proteins. All these effects would contribute to the decrease in resilience observed in the data presented in this paper. Protein dynamics is strongly affected by solvent effects (Tehei *et al*, 2001), however, and adaptation of cytoplasm composition (e.g. through the presence of salt or compatible solutes) may also contribute to the observed macromolecular resilience differences.

Conclusion

The neutron experiments presented quantified the extent to which macromolecular dynamics in bacterial cells is affected by adaptation to extreme temperatures. The results support the hypothesis that a contribution to thermal adaptation is through the evolutionary selection of appropriate resilience in order to maintain macromolecular structure and flexibility within the narrow limits required by biological activity.

METHODS

Sample preparation. *A. arcticum* (ATCC 49402), *E. coli* (MRE600), *P. mirabilis* and *T. thermophilus* (ATCC 579) were grown aerobically in their respective complex media at 4, 37 and 75 °C, respectively, until the late mid-log phase. *A. pyrofilus* (DSM 6858) were grown at 85 °C in 2 l bottles under 1.4 bar of H_2/CO_2 in SME medium modified at pH 6.8. The cells were collected by centrifugation and washed twice by suspending the pellets in a 50 mM Tris-HCl pH 8 buffer containing 150 mM NaCl and 5 mM KCl. In all, 420 mg of the washed cell paste was immediately sealed in the sample holder. Control experiments were carried out in order to ensure that the cells were not damaged and were still viable after about 20 h of total measuring time in the beam. To avoid cell damage, a limited temperature range, 4–37 °C (277–310 K), was examined in the neutron scattering experiments.

Neutron scattering experiments. Experiments were performed on the IN13 spectrometer at the Institut Laue Langevin (information on the Institut Laue Langevin in Grenoble and its neutron spectrometers is available at http://www.ill.fr). Aluminium sample holders had a 0.3 mm path length. The samples showed a transmission of about 90% for the 2.23 Å neutron beam. Elastic incoherent scattering data were collected with an energy resolution of 8 μeV in a scattering vector range of $1.2\ \text{Å}^{-1} \leqslant Q \leqslant 2.2\ \text{Å}^{-1}$, corresponding to a space–time measurement 'window' of a few Ångstroms in 0.1 ns. Intensity data were corrected for sample holder and buffer scattering and normalized by vanadium scattering to yield I (Q, *elastic*) as a function of temperature for each sample, where *elastic* corresponds to zero energy transfer. The scattering vector Q is given by $4\pi\sin\theta/\lambda$, where 2θ is the scattering angle and λ is the incident neutron wavelength. For each sample and temperature point, $\ln\{I\ (Q, elastic)\}$ was plotted against Q^2; the mean square fluctuation $\langle u^2 \rangle$ was calculated from the slope of the straight-line fit to the experimental data according to the Gaussian approximation (Zaccai, 2000):

$$I(Q, elastic) = \text{constant.}\ \exp[-(1/6)(\langle u^2 \rangle Q^2)]. \qquad (1)$$

The approximation is valid for Q values satisfying $Q^2 \langle u^2 \rangle \approx 2$.

The mean square fluctuations were plotted as a function of absolute temperature; effective force constants $\langle k' \rangle$ and their errors were calculated from the slopes of the weighted straight-line fits (using the Levenberg–Marquardt algorithm) to the data (Zaccai, 2000; Bicout & Zaccai, 2001) from

$$\langle k \rangle = 0.00276/(\mathrm{d}\langle u^2 \rangle/\mathrm{d}T). \qquad (2)$$

The numerical constants are to express $\langle k' \rangle$ in N/m when $\langle u^2 \rangle$ is in Ångstrom squared and T is in Kelvin.

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