To explore protein adaptation to extremely high temperatures, two parameters related to macromolecular dynamics, the mean square atomic fluctuation and structural resilience, expressed as a mean force constant, were measured by neutron scattering for hyperthermophilic malate dehydrogenase from Methanococcus jannaschii and a mesophilic homologue, lactate dehydrogenase from Oryctolagus cuniculus (rabbit) muscle. The root mean square fluctuations, defining flexibility, were found to be similar for both enzymes (1.5 Å) at their optimal activity temperature. Resilience values, defining structural rigidity, are higher by an order of magnitude for the high temperature-adapted protein (0.15 Newtons/meter) for O. cuniculus lactate dehydrogenase and 1.5 Newtons/meter for M. jannaschii malate dehydrogenase). Thermoadaptation appears to have been achieved by evolution through selection of appropriate structural rigidity in order to preserve specific protein structure while allowing the conformational flexibility required for activity.

Hyperthermophilic organisms grow at temperatures above 80°C. Proteins from these organisms are themselves optimally active between 60 and 125°C and serve as paradigms for the characterization of factors responsible for protein fold stability and flexibility. Hyperthermophilic enzymes have also attracted considerable attention because of a range of biotechnological applications (1, 2).

Sequence comparison studies and structural analyses carried out on hyperthermophilic proteins and their mesophilic homologues have shown that thermal stability is associated with multiple factors, including an increase in hydrogen bonding, complex salt bridge formation, and helix stabilization by acidic residues. The commonly accepted hypothesis is that increased thermal stability is due to enhanced conformational rigidity of the molecular structure (3). Hyperthermophilic enzymes are also characterized by a higher temperature of maximum activity (3, 4). The more rigid hyperthermophilic enzyme would then require higher temperatures to achieve the requisite conformational flexibility for activity.

Experiments have shown that thermostable enzymes exhibit reduced structural flexibility at room temperature with respect to their mesophilic homologues (4, 5), whereas others, on α-amylase (6) and on rubredoxin (7, 8), have shown the opposite effect, i.e. the thermostable homologues were found to be more flexible, suggesting stabilization through entropic effects. Relations between flexibility and stability are, therefore, complex. Atomic fluctuations only were measured in these experiments and interpreted in terms of flexibility.

It is important to point out that reduced structural “flexibility” does not necessarily imply a more “rigid” structure. Atoms are maintained in a structure by forces that link them to their neighbors. In terms of a force field, the width of the potential well in which an atom moves is a measure of its flexibility in terms of a root mean square fluctuation amplitude (\( \sqrt{\langle u^2 \rangle} \)), whereas the detailed shape of the well reflects the rigidity of the structure, in terms of an effective force constant (\( \langle k \rangle \)). In this picture, the stability would be given by the depth of the well (9). Two types of potential wells are illustrated in Fig. 1. In the case of harmonic motion (Fig. 1A), flexibility and rigidity are related, as expected intuitively. The potential is given by \( V(u) = \frac{k}{2} u^2 \), and the atomic mean square fluctuation is related to the force constant by \( \langle u^2 \rangle(T) = \frac{k_B T}{\langle k \rangle} \) (10, 11); a less rigid harmonic structure is indeed more flexible.

Protein structures, however, are not harmonic at physiological temperature, and atoms move in different types of potential. Fig. 1B illustrates a simplified, extreme case in which an atom can move quite freely in a “box” formed by its neighbors but cannot go out of the box. Mathematically this can be described by the square well potential shown. The flexibility, in this case, is a temperature-independent constant value, while the effective force constant stopping the atom from leaving the box is infinitely high. Flexibility and rigidity are independent parameters therefore that, as shown below, can be obtained separately from neutron scattering data.

We proposed a novel neutron scattering approach that provides independent measurements of the atomic mean fluctuations in a protein structure (the global thermal flexibility) and of the mean force constant maintaining the structure, which we called resilience because “rigidity” has been used in a broadly qualitative way (11, 12). In the present work, the combined analysis of neutron data on dynamics, on the one hand, with activity and stability data, on the other, for hyperthermophilic and mesophilic enzymes of the malate lactate dehydrogenase family revealed a strong adaptation of resilience and mean square fluctuations to physiological temperature. By performing comparative analysis using the sequences and the three-dimensional crystal structures, we suggested mechanisms that govern the high thermal stability of Methanococcus jannaschii malate dehydrogenase (Mj MalDH) through increased resilience.
Protein Adaptation via Macromolecular Dynamics

Oryctolagus cuniculus lactate dehydrogenase (Oc LDH) was from Sigma. Mj MalDH was prepared as described in Ref. 13.

Activity—The measurements of the rate of NADH oxidation at 340 nm were carried out in a 1.00-cm thermostated cuvette containing 0.2 mM NADH and 0.6 mM appropriate substrate buffered with 50 mM Tris-HCl, pH 8. Pyruvate and oxaloacetate were used with Oc LDH and Mj MalDH, respectively. The optimal activity was expressed as the percentage of the maximal activity relative for each enzyme.

Stability—Samples of Oc LDH and Mj MalDH were incubated in 50 mM Tris-HCl, pH 8, for 30 min at the specified temperature, and their residual activities were measured. The samples were covered with paraffin oil to avoid evaporation at high temperature.

The guanidinium hydrochloride-induced unfolding of Oc LDH and Mj MalDH was monitored using circular dichroism spectroscopy. Samples (0.5 mg/ml) were incubated in 50 mM Tris-HCl, pH 8, for 24 h at the specified GdnHCl concentration. Far-UV circular dichroism spectra were recorded between 190 and 260 nm with an interval of 1 nm and an integration time of 15 s.

Neutron Scattering Experiments—Samples for neutron scattering were concentrated protein solutions (~200 mg/ml) in 20 mM KCl, 20 mM Tris-HCl, 0.5 mM buffer. Experiments were performed on the backscattering spectrometer IN13 at the Institut Laue Langevin, Grenoble, France (information on the institute and the instrument is available at www.ill.fr). The incident neutron wavelength used was \( \lambda = 2.23 \) Å. Neutron spectrometers are characterized by their energy resolution, \( \Delta \omega \), and scattering vector, \( Q \), range, corresponding to time and space windows related to \( 1/\Delta \omega \) and \( 1/Q \), respectively. Elastic incoherent scattering data were collected with an energy resolution of 8 \( \mu \)eV in a scattering vector range of \( 1.2 \, \text{Å}^{-1} \leq Q \leq 2.2 \, \text{Å}^{-1} \), corresponding to a space-time measurement window of \( \sim 1 \, \text{Å} \) in 0.1 ns. Samples were contained in aluminum sample holders with a 0.3-mm path length. Correction for self absorption using Paalman-Pings coefficients were carried out with standard programs. All samples, including the vanadium and empty aluminum can, were oriented at 135° with respect to the incident neutron beam direction. The scattering of the buffer alone was much lower than the protein solution scattering, barely above the scattering of the aluminum container. It (the scattering of the buffer) was subtracted from the data with no correction for protein-excluded volume. The data were normalized by the vanadium scattering to correct for detector response. In the instrument space-time window and according to a Gaussian approximation, the incoherent elastic scattered intensity can be analyzed as (14) \( I(Q, \omega) = \text{constant} \exp((1/6) (<u^2 > Q^2)) \), where \( Q = 4 \sin \theta/\lambda, 2 \theta \) is the scattering angle, and \( \lambda \) the incident neutron wavelength; \(<u^2 >\) values include all contributions to motions in the accessible space and time windows from vibrational fluctuations (usually expressed as a Debye-Waller factor) as well as from diffusional motions. The validity of the Gaussian approximation for the mean square fluctuation \(<u^2 >\) and its analogy to the Guinier formalism for small angle scattering by particles in solution has been discussed by Rét et al. (15) and more recently by Gabel (16). In the Guinier formalism a radius of gyration \( R_g^2 \) of particles in solution is calculated (17). The particle equivalent is the volume swept out by a single proton during the time scale of the experiment (~100 ps). The analogy holds if the motion is localized well within the space-time window defined by the \( Q \) and energy transfer ranges, respectively. The Guinier approximation is valid if \( \sqrt{R_g^2}Q^2 \approx 1 \). Following our definition of \(<u^2 >\), \( R_g^2 = 1/2 <u^2 > \).

As a consequence, the Gaussian approximation is valid in the domain where \( \sqrt{<u^2 >} Q^2 \approx \sqrt{2} \). The mean square fluctuations \(<u^2 >\) at a given temperature \( T \) were calculated according to the Gaussian approximation as (Fig. 2): \( \ln[1/(Q, 0 \pm d\omega)] = \text{constant} + A^2 Q^2 \). The mean square fluctuations were therefore calculated as: \(<u^2 > = -6 \, \text{Å}^2 \).

The \(<u^2 >\) values were then plotted as a function of absolute temperature \( T \) (Fig. 3). The value of the root mean square fluctuation \( \sqrt{<u^2 >} \) in absolute Å units quantifies the global flexibility of the system studied. An effective mean force constant \(<k'>\), defining mean resilience, can be calculated from the derivative of \(<u^2 >\) plotted versus temperature \( T \) (10, 11) (Fig. 3): \( <k'> = 0.00276/(d <u^2 >/dT) \).
Protein Adaptation via Macromolecular Dynamics

The numerical constants allow the expression of $<k'>$ in N/m with $<u'^2>$ in Å² and $T$ in Kelvin. The mean square fluctuations $<u'^2>$, the effective force constants $<k'>$, and their respective errors were calculated from the slopes of weighted straight line fits using the Marquart-Levenberg algorithm.

Sequence and Structural Comparison—The sequence comparison was performed using the program BLAST (18). The structural alignment was performed using the program VAST (19) and rendered graphically with Cn3D (20). Hydrogen bond interactions were listed using the program HBPlut (21). Ion-pair analysis was performed using X-PLOR (22) with distance cutoffs of 4 Å. Water-accessible surface area and water-enzyme hydrogen bond interactions were calculated and listed using DSSP (23). Molecular protein volume and cavities were calculated with the program VOIDOO (24). A probe radius of 1.4 Å was used for cavity calculations. Secondary structure assignment was calculated using Cn3D (20).

RESULTS AND DISCUSSION

Malate dehydrogenase from the hyperthermophile archaeon *Mj* MalDH was compared with its mesophilic homologue, the lactate dehydrogenase from *O. cuniculus* muscle (*Oc* LDH). *Mj* MalDH is a member of the LDH-like family of malate dehydrogenases, which are tetrameric and have similar structures to the LDH (13, 25, 26).

Activity and Stability—The instantaneous activities of the enzymes were measured as a function of temperature (Fig. 4A). The temperatures of optimal activity are 37 °C for *Oc* LDH and 90 °C for *Mj* MalDH. The relative stabilities of the enzymes were assessed by residual activity measurements and CD using guanidinium hydrochloride as denaturant (Fig. 4, B and C). The residual activity is abolished in *Oc* LDH after incubation at ~50 °C and in *Mj* MalDH after incubation above 90 °C. The guanidinium chloride unfolding transition concentrations for *Oc* LDH and *Mj* MalDH are 1.3 and 2.1 M, respectively. Taken together, these data show that the hyperthermophilic protein is significantly more stable.

Macromolecular Dynamics Study—Various experimental methods have been applied to explore protein dynamics adaptation to high temperature. Hydrogen/deuterium exchange (4), fluorescence quenching (27), high-resolution NMR (28), and neutron scattering (29) are probably the major ones. The flexibility of protein molecules is reflected in conformational fluctuations. These reversible local rearrangements expose buried segments of the polypeptide chain to solvent. In D₂O, hydrogen/deuterium exchange occurs during such exposure, and the probability distribution of the accessibility of protein hydrogens can be determined by hydrogen/deuterium exchange experiments providing a measure of protein fluctuations. These experiments do not provide total protein dynamics and must be performed in D₂O, which may affect the activity, the stability, and the dynamics of proteins (9, 30). Fluorescence quenching experiments measure structural flexibility and integrity for protein structures. They require the use of a quencher, for example acrylamide, for the quenching of tryptophan fluorescence. In general, however, different tryptophan residues have a different accessibility to the quencher, and the quenching behavior is only weakly coupled to structural fluctuations. NMR permits the study of backbone dynamics in proteins. The method usually requires isotope enrichment of the protein by ¹⁵N labeling and provides information essentially in terms of order parameters and entropies. There is an overlap between the time scales of neutron scattering experiments and those of NMR. Neutron scattering experiments can be performed under close to physiological condition on proteins of any size and do not require isotope labeling, although D₂O solvent or deuterium labeling can be used to explore...
specific effects. The method provides direct information on atomic fluctuation amplitudes in a given time scale in absolute units.

Neutron scattering experiments were performed on the IN13 spectrometer at the Institut Laue Langevin (Grenoble). We recall that the space-time window of the experiments corresponds to ~1 Å in 0.1 ns. Motions outside the window, such as the diffusion of bulk water (~10 Å in 0.1 ns), for example, did not contribute to the scattering signal, so that experiments could be performed in H2O solvent, close to physiological conditions (we recall that many of the previous neutron scattering experiments were performed in heavy water). In this space-time window, H atoms reflect the motions of the side chains and backbone atom groups to which they are bound and thus provide information on protein dynamics (31). The mean square fluctuation <u²> of the sample atoms was measured as a function of temperature. Two parameters providing information on dynamics were analyzed: the value of the root mean square fluctuation √<u²> in absolute Å units, which quantifies the global flexibility of the protein, and the mean resilience <k> in absolute N/m units, calculated from the slope of <u²> versus T (11).

The global mean square fluctuations <u²> in the proteins measured by neutron scattering as a function of temperature are shown in Fig. 3. In the explored temperature range, the <u²> of the hyperthermophilic protein lie above those of its mesophilic homologue. This result is in agreement with neutron studies on α-amylases that showed larger fluctuation amplitudes for the thermophilic protein (6). It should not be concluded, however, that this higher flexibility implies a lower rigidity (see above). Because of technical constraints, the mean square fluctuation at 90 °C for Mt MalDH, the temperature of its optimal activity, was obtained by linear extrapolation to 2.3 Å, corresponding to a root mean square fluctuation (flexibility) of 1.5 Å. This value for Mt MalDH is essentially identical to that for Oc LDH at 37 °C. The observation suggests that the enzymes have conformational flexibility adjusted to the optimum working temperature in accordance with the hypothesis that adaptation of proteins to different physiological temperatures tends to maintain enzymes in “corresponding states” characterized by similar conformational flexibility (32).

The mean resilience values of the protein structures were calculated as effective mean force constants from the slopes of <u²> versus T in Fig. 3. A smaller slope indicates a more resilient (rigid) protein structure and vice versa. The mean resilience is an order of magnitude larger for Mt MalDH (1.50 N/m) than for Oc LDH (0.15 N/m). For comparison, we recall that the resilience of MalDH from Haloarcula marismortui measured in 2 M NaCl H2O solution is 0.11 N/m (9). The resilience of myoglobin as a D2O-hydrated powder is 2 N/m below 200 K (minus 73 °C) and drops to ~0.3 N/m at physiological temperature (11). The higher stability of Mt MalDH is, therefore, correlated with resilience ~10-fold higher than the resilience of mesophilic protein. What are the sequence and structural mechanisms that govern the higher stability and resilience of Mt MalDH?

**Sequence and Structural Determinants Associated with Thermal Adaptation**—The Mt MalDH enzyme subunit is made up of 30% charged residues (lysine, aspartate, arginine, and glutamate), which is by far larger than the corresponding fraction (~21.7%) found in the mesophile Oc LDH. The fraction of lysine residues (8.6% of the total number of residues) in Mt MalDH is comparable with that found in Oc LDH (8.1%). The fractions of arginine, aspartate, and glutamate in Mt MalDH representing 5.8, 7, and 8.6% are much larger than the corresponding fractions (3, 5.1, and 5.5%) found in Oc LDH. A correlation has been published between adaptation to high temperature and the average charged minus noncharged polar (asparagine, glutamine, serine, and threonine) amino acid percentage (Ch-Pol) in protein structures (33). The fraction of polar residues is 13.7 and 18.7% in Mt MalDH and Oc LDH, respectively. The Ch-Pol value is therefore much higher for Mt MalDH (16.3%) than for Oc LDH (3%). The obvious strong correlation between the effective resilience and the average percentage (Ch-Pol) provided a hint as to which protein stabilization forces are affected by thermoadaptation. The increase of the Ch-Pol value for the Mt MalDH, compared with Oc LDH, is largely due to an increase in charged residues (62.4%) that favors hydrogen bonds, hydration interactions (34), and ion pairs and therefore leads to a dominance of the enthalpic (H-bonds, hydration interactions, ion pairs) contributions to the free energy landscape.

The three-dimensional crystal structure of the enzyme Oc LDH is not yet determined. However, to address the structural implications of the correlation between resilience and adaptation to high temperature, we selected a mesophilic homologue of Oc LDH whose structure has been solved. Lactate dehydrogenase from Sus scrofa (pig) muscle (Ss LDH) belongs to the same phylogenetic domain and has the same optimum physiological temperature and number of amino acids as Oc LDH. Its denaturation temperature also is about the same as that of Oc LDH. Moreover, the two proteins display a strong sequence identity (TABLE ONE).
Protein Adaptation via Macromolecular Dynamics

The structural alignment of the Ss LDH monomer with Mj MalDH structure (TABLE ONE and Fig. 5) shows a high degree of conservation of the overall three-dimensional fold. The total volume occupied by the Mj MalDH tetramer is smaller (by 8%) than that occupied by the tetramer of Ss LDH. Improvement in packing density can be obtained also by a reduction in the number and total volume of internal cavities (35). Two cavities are present in the Mj MalDH monomer, and they constitute a total volume of 64 Å³. The number and the total volume (by ~91%) of internal cavities of Mj MalDH monomer are smaller than that present in the Ss LDH monomer (TABLE TWO). Cavities are energetically unfavorable due to a loss of van der Waals contacts (36). Filling or creating cavities by site-directed mutagenesis can increase or decrease the thermostability of proteins (37, 38), and the most destabilizing replacements tend to occur in the most rigid parts of a protein structure (39). The water-accessible surface area (WASA) allows quantification of the extent to which atoms on the protein surface can form contacts with water. The WASA of Mj MalDH is slightly smaller (by 1%) than that of Ss LDH (TABLE TWO). The WASA has energetic significance in that it is directly related to hydrophobic free energies. The reduction of each square Angstrom of WASA yields a gain in free energy of 25 cal mol¹⁻¹ (40). There is an improvement of the packing density for Mj MalDH, which favors the resilience increasing through a dominance of the enthalpic (increase of van der Waals contacts, small decrease of hydrophobic free energies) contributions to the free energy landscape.

The Ch-Pol value is much higher for Mj MalDH (16.3%) than for Ss LDH (3.3%). The charged residues are likely to favor ion pairs, hydration interactions, and hydrogen bonds. Ion pairs have been proposed to play a key role in the maintenance of enzyme stability at high temperature (41, 42). The number of intrasubunit ion pairs in Mj MalDH is increased significantly (50%) compared to its mesophilic counterpart (TABLE TWO), confirming the proposed stabilizing effect of ion pairs. Because the majority of charged residues are located on the protein surface, interaction with the solvent is also an important factor for stability. Neutron scattering experiments on the halophilic malate dehydrogenase from Haloarcula marismortui show that the enzyme is more resilient under solvent conditions where it is more stable (9). The number of potential water-enzyme hydrogen bonds/residue is higher for Mj MalDH dimer (by 9%) than that of the equivalent dimeric unit of Ss LDH (TABLE TWO).

Mj MalDH has fewer amino acid residues than its mesophilic homologues. In Mj MalDH, 39% of the amino acid residues are located in α helices and 25% in β strands. The difference with Ss LDH is small but significant, as the α helix and β strand contents of the mesophilic homologues are 36 and 26%, respectively. Therefore, the fraction of residues belonging to secondary structure elements in Mj MalDH is slightly higher (by 3%) than what is seen in Ss LDH (TABLE TWO). The increase in structure leads to a reduction in the lengths of the external loops and to an increase in the number of hydrogen bonds. Effectively, the number of hydrogen bonds/residue is slightly higher for Mj MalDH (TABLE TWO). We note, however, that the higher resolution of the Ss LDH structure (2 Å compared with 2.8 Å for Mj MalDH structure) probably led to the proper identification of more hydrogen bonding interactions, suggesting the difference may be significantly higher. All these Mj MalDH structural features contribute to a global increase in protein resilience, through enthalpic (ion pairs, hydration interactions, and hydrogen bond) contributions to the free energy landscape.

CONCLUSION

Our results emphasized how the distinction between protein stability and enzyme activity is reflected in a corresponding distinction between resilience and flexibility. The structural bases of thermophilic stability in the (LDH-like) malate dehydrogenase group have been discussed (35, 43). Enhanced stability arises from a combination of different mechanisms. This study indicates that several factors, such as increased (Ch-Pol) value of protein sequence and increased packing density, as well as a reduction of number and total volume of internal cavities, increased ion pairs, and increased hydrogen bond interactions, are responsible for a more stable protein and contribute to an observed increase in protein resilience, suggesting the dominance of enthalpic contributions to the

| TABLE TWO |
| Structural comparison observations |
| Structural comparison observations of each of the listed factors: + observed. |
| | Mj MalDH | Ss LDH | Mj MalDH structure comparisons performed with Ss LDH |
| Total volume decreased (10⁶ Å³) | 1.164 | 1.261 | + |
| Number of internal cavities decreased (per monomer) | 2 | 6 | + |
| Total cavity volume decreased (per monomer) (Å³) | 64 | 122.5 | + |
| Total water-accessible surface area decreased (per dimer) (Å²) | 26204 | 26499 | + |
| Increased number of intrasubunit ion pairs (per monomer/residue) | 15 / 0.048 | 10 / 0.03 | + |
| Increased number of hydrogen bonds water-enzyme/residue (dimer) | 1.54 | 1.41 | + |
| Increased secondary structure content(%) | 64 | 62 | + |
| Increased number of hydrogen bonds/ residue (dimer) | 0.893 | 0.889 | + |
free energy landscape in the thermoadaptation. For proteins in which entropic effects are dominant, a less resilient macromolecule will be more thermostable.

To what extent can the results on the malate lactate dehydrogenases be generalized to other protein families? Neutron scattering experiments similar to the ones described here, on whole bacterial cells adapted to various physiological temperatures, have permitted the measurement of the mean dynamics of the entire macromolecular populations under in vivo conditions (44). The trend of the results is consistent with the findings on the pure protein samples even though absolute parameter values are different, because the cell values represent averages over a large heterogeneous macromolecular population. A constant root mean square fluctuation of 1.2 Å was found at the corresponding physiological temperature of all the bacterial cells studied, compared with 1.5 Å for the malate lactate dehydrogenases. The resilience values increased with physiological temperature: from 0.4 N/m for the mesophile bacteria (37 °C) to 0.6 N/m for the hyperthermophiles (85 °C).

We may speculate that mutations accumulated in proteins during the time course of evolution have mainly modified the intra-molecular resilience in order to cope with stability under the physico-chemical constraints of the environment. Modifications were selected that also optimized flexibility for efficient enzyme activity at the physiological temperature.

Acknowledgments–We thank Marc Bee, Christine Ebel, Frank Gabel, Frédéric Vellieux, and Martin Weik for fruitful discussions, advice with the experiments, and critical readings of the manuscript.

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