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Science **288**, 1604 (2000);
DOI: 10.1126/science.288.5471.1604

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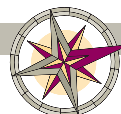

REVIEW: BIOCHEMISTRY

How Soft Is a Protein? A Protein Dynamics Force Constant Measured by Neutron Scattering

Giuseppe Zaccai

An effective environmental force constant is introduced to quantify the molecular resilience (or its opposite, "softness") of a protein structure and relate it to biological function and activity. Specific resilience-function relations were found in neutron-scattering experiments on purple membranes containing bacteriorhodopsin, the light-activated proton pump of halobacteria; the connection between resilience and stability is illustrated by a study of myoglobin in different environments. Important advantages of the neutron method are that it can characterize the dynamics of any type of biological sample—which need not be crystalline or monodisperse—and that it enables researchers to focus on the dynamics of specific parts of a complex structure with deuterium labeling.

It is now well accepted that conformational flexibility is essential for enzyme catalysis and for biological molecular activity in general. But the concept of flexibility refers to molecular motions on a range of time scales, from picosecond-scale thermal fluctuations to millisecond-scale (or slower) conformational changes involved in functional kinematics. The concept of dynamics has a more precise meaning (from the Greek Δυναμις, strength)—it pertains to forces. The forces that maintain biological structure and govern atomic motions in macromolecules are known. They include hydrogen bonding, electrostatic, and van der Waals interactions, as well as pseudo-forces arising from the hydrophobic effect (*1*). They are "weak" forces because their associated energies are similar to thermal energy at usual temperatures. This is why biological matter is "soft" at room temperature. "Strong" forces, such as those exerted by covalent bonds, are less relevant here because these bonds are not usually broken when a protein unfolds, for example, or when a protein–nucleic acid complex dissociates.

Protein flexibility has been described qualitatively by conformational disorder, as observed in crystal structures or with nuclear magnetic resonance (NMR), and by the extent of H-D exchange in D_2O solvents (*2*). Motions are characterized by their amplitudes and frequencies; we basically want to know how far an atom moves and how long this movement takes. Neutron-scattering experiments can provide quantitative measurements of the thermal mean square atomic fluctuations and their frequencies. Thermal fluctuations are important for biological activity because they act as the lubricant that enables conformational changes on a physiological time scale (*3*). In crystal structures, the amplitudes (but not the frequencies) of thermal fluctuations are measured through a parameter named the Debye-Waller factor, which also includes other contributions to displacements from mean atomic positions, such as crystal disorder (*4*). Unlike crystallography, however, the neutron experiments rely on incoherent scattering and can be performed on samples that need not be crystalline or even monodisperse. The scattering of ^1H atoms is dominant, and deuterium labeling enables a focus on specific parts of a complex structure (*5*).

At physiological temperatures, protein flexibility arises from fluctuations between different states. This has been represented in a "conformational substate" model, which describes the dynamical behavior of proteins over a broad temperature range (*6*). At very low temperatures, proteins are usually biologically inactive. They behave like hard, solid materials: Their atoms are held tightly in the structure, trapped in one conformational substate. Thermal motions under these conditions are represented by harmonic vibrations about equilibrium positions. At a higher temperature, activation energy becomes available for the atoms to sample different conformational substates. Thermal motions become anharmonic because atoms are no longer vibrating around single equilibrium positions but can "jump" between positions; the protein is "soft" and active. Dynamical transitions from harmonic to anharmonic regimes have been observed at ~200 K (–73°C) in various proteins by Mössbauer spectroscopy (*7*), neutron scattering (*8–10*), and photon absorption spectroscopy (*11*) and have been simulated successfully in force field calculations (*12*).

Relations among dynamics, stability, and function were established in recent neutron-scattering experiments on myoglobin and bacteriorhodopsin. The extreme stability of myoglobin in trehalose is explained by the observation that the sugar traps it in a hard, harmonic state even at high temperatures (*13*). Because protein activity, which in general requires flexibility (*2*), is usually inhibited in a harmonic regime, it appeared that activity is in fact incompatible with high stability. In another study, neutron-scattering measurements established a strong correlation between the amplitudes of thermal motions and much slower conformational changes associated with the light-driven proton pump activity of the membrane protein bacteriorhodopsin in purple membranes of *Halobacterium salinarum* (*14*). Furthermore, with experiments on deuterium-labeled membranes, a specific dynamics-function correlation was demonstrated. The functional core of bacteriorhodopsin, which acts as a valve in the proton pump mechanism, is more rigid than the membrane globally (*5, 15*).

An analysis of neutron data on thermal fluctuations in terms of mean environmental force constants is introduced in this review and illustrated by results with myoglobin and purple membranes. In the exploration of the relations among dynamics, stability, and biological activity, this approach enables the quantification of molecular "softness" with respect to thermal motions and a comparison of proteins in different environments.

Dynamics and Stability in Myoglobin

The dynamical transition in proteins was revealed by neutron-scattering elastic temperature scans, which plot the mean square thermal fluctuations as a function of absolute temperature. The first such scan, which was performed on a hydrated myoglobin powder (*8*), is shown in Fig. 1 together with the scan for myoglobin in trehalose (*13*). Region A is the harmonic region, where a mean environmental force constant $\langle k \rangle$ can be calculated from the slope of the line, as described below. The deviation from the straight line is called a dynamical transition, and region B corresponds to the anharmonic regime. This portion of the scan describes the physiological dynamics of the

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protein. A force constant, in the strict sense, is not defined in the case of anharmonic motion. The resilience of an anharmonic environment at a temperature T can be quantified by a pseudo-force constant, $\langle k' \rangle$, calculated from the slope of the scan above the dynamical transition (see below for detailed analysis). Trehalose is a disaccharide that allows certain organisms to survive essentially total dehydration at high temperatures. It appears to help proteins avoid denaturation by surrounding them by a continuous vitreous layer. The data for myoglobin in trehalose (Fig. 1) do not show a dynamical transition, and the dynamics of the system remain harmonic to high temperature (*13*). This suggested an explanation in terms of dynamics for the protective effect of the sugar: The protein atoms are trapped within individual conformational substates, precluding the anharmonic motions that ultimately lead to unfolding.

Mean environmental force constants were calculated from the slopes of the different parts of the plots in Fig. 1. Not only is the dynamical transition essentially abolished in trehalose, but also the environment is more resilient than in the low-temperature harmonic regime of the hydrated powder ($\langle k \rangle$ = 3 and 2 N/m, respectively). A threshold force on the order of 100 pN was required to unfold domains of the giant protein titin in atomic force microscopy and laser tweezers experiments (*16*). Calculating force constants from these experiments is not a straightforward process, and, in any case, the response to an external force may not be compared directly with thermal behavior. Nevertheless, an order-of-magnitude analysis is worthwhile. Most of the weak bonds that maintain folded protein structure are short range, and we could consider them as broken if stretched beyond ~1 Å from their equilibrium length. The room-temperature $\langle k' \rangle$ value from neutron scattering for the hydrated myoglobin powder is 0.3 N/m, or 30 pN/Å, with root mean square fluctuations of 0.9 Å with respect to an equilibrium position. It is interesting to note, therefore, that the neutron, AFM, and laser tweezers experiments measure forces of the same order and suggest that very few bonds need to be broken to unfold a domain, in agreement with the relatively low energy of stabilization of folded protein domains found in calorimetric studies (*1*).

Function Is Related to Specific Dynamics in Purple Membrane

Purple membrane is made up of 25% lipids and 75% bacteriorhodopsin, a retinal-binding seven-helix membrane protein that functions as a light-activated proton pump. Its dynamics has been studied extensively by neutron scattering (*5, 9, 14, 15, 17–19*). Elastic temperature scans, under the same hydration conditions, are shown in Fig. 2 for a native (unlabeled) sample and a deuterated (labeled) sample where only the retinal, Trp, and Met residues were hydrogenated (*5*). The $\langle k \rangle$ values at low temperature are close to each other (1.7 and 1.4 N/m, respectively) and slightly lower than for hydrated myoglobin powders (2 N/m). There are significant differences, however, as the temperature increases, with two breaks in the scan for the unlabeled sample with $\langle k' \rangle$ = 0.55 N/m between 150 and 270 K and $\langle k'' \rangle$ = 0.12 N/m at room temperature. This is "softer" than hydrated myoglobin at room temperature and may reflect the dynamics of the bacteriorhodopsin loops on either side of the membrane; the slope of the room-temperature part of the scan in purple membrane is strongly dependent on relative humidity (*14*).

The scan of the labeled sample reflects the dynamics of the retinal, Trp, and Met groups, which are located predominantly in the extracellular half of bacteriorhodopsin. The scan shows one break at ~200 K with a $\langle k' \rangle$ value at room temperature of 0.33 N/m, similar to hydrated myoglobin but almost 3 times the room-temperature $\langle k'' \rangle$ of the unlabeled sample. The room-temperature mean square am-

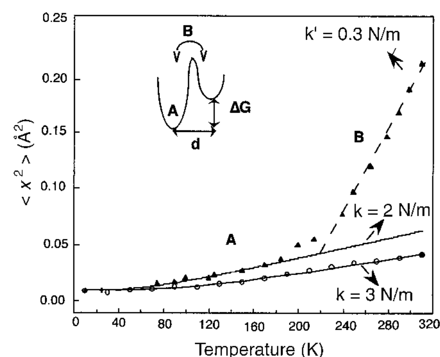

Fig. 1. Neutron elastic temperature scans of myoglobin as a hydrated powder in D_2O [triangles, redrawn from (*8*)] and in trehalose [circles, redrawn from (*13*)]. The different parts of the scans (A and B) and the k and k' values calculated from the slopes of the lines are discussed in the text. The $\langle x^2 \rangle$ values are calculated from the angular dependence of the incoherent scattered elastic intensity (*27*). The inset shows the two–potential well model of (*8*). In the A part of the scan, protein atoms are trapped in the lower harmonic well; in the B part, they can sample both wells. In trehalose, myoglobin atoms are trapped in an A-type potential well in the entire temperature range.

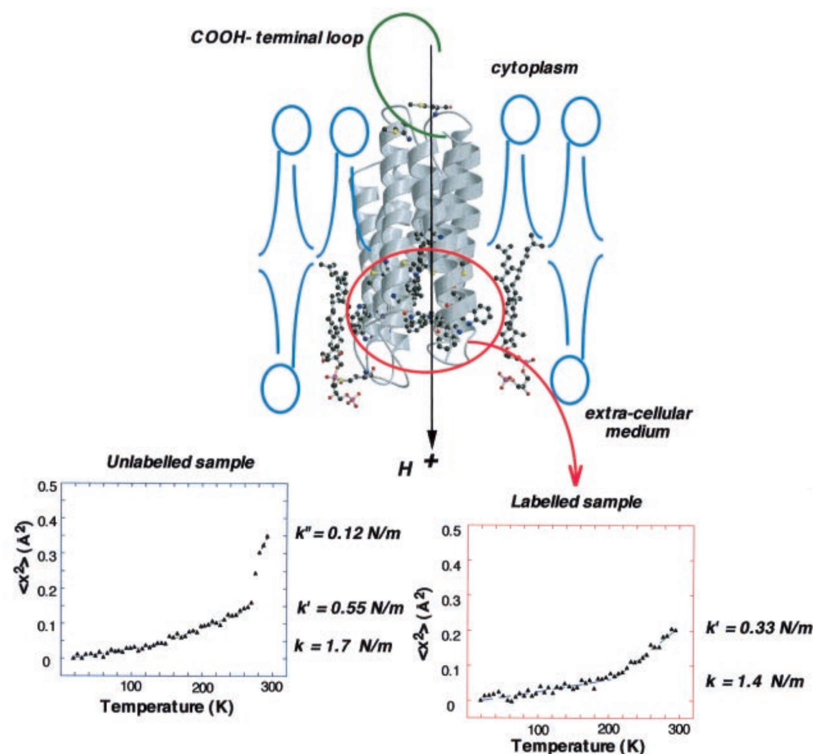

Fig. 2. A schematic diagram of the purple membrane from *H. salinarum*, showing the phospholipid molecules in blue, a bacteriorhodopsin monomer in ribbon mode, the glycolipids localized next to the protein on the extracellular leaflet by neutron diffraction (*24*), and the retinal, Trp, and Met residues that were labeled in the experiments of (*5*), in ball-and-stick mode. The COOH-terminal of the protein, which is not visible in the crystal and electron microscopy structures because of disorder, is drawn schematically in green. The elastic scans are replotted from (*5*). In the unlabeled sample, the mean fluctuations within the system globally were measured, whereas data from the labeled sample were dominated by motions in the extracellular half of bacteriorhodopsin, circled in red. The k, k', and k'' values were calculated from the slopes of the lines, as discussed in the text. Structural data are from (*20*), deposited in the Protein Data Bank and drawn with Molscript (*31*).

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plitude of the labeled sample is about half that of the unlabeled sample. Debye-Waller factors from electron diffraction of purple membranes (*20*) and from x-ray diffraction of bacteriorhodopsin crystals (*21*) also revealed smaller fluctuations in the extracellular half of bacteriorhodopsin than in the cytoplasmic half. Conformational changes during the bacteriorhodopsin photocycle occur essentially in the cytoplasmic half of bacteriorhodopsin (*22*). The relative humidity dependence of these conformational changes (*23*) is strongly correlated with that of the thermal fluctuations measured by neutron scattering (*14*), further supporting the concept of a strong relation between picosecond thermal motions and the capability of a protein to undergo much slower conformational changes of larger amplitude (*3*). Also shown in Fig. 2 are the glycolipid molecules, which were localized by neutron diffraction and were found to be in close interaction with bacteriorhodopsin (*24*). They appear to mediate protein-protein interactions, anchoring bacteriorhodopsin on the extracellular leaflet of the membrane, and may contribute to the stiffer environment observed for that side.

In summary, the thermally soft cytoplasmic half of bacteriorhodopsin allows the conformational changes associated with proton transfer along its pathway, whereas a stiffer core and extracellular half harness the isomerization of retinal to provide the valve function for vectorial proton transfer. This suggests an evolutionary selection of specific dynamics as well as structure with relation to function. This hypothesis could be tested in purple membrane, for example, with bacteriorhodopsin mutants that modify the dynamics but not the structure of the proton pathway. There are now more than 12,000 protein structures forming about 1000 families in data banks, and dynamics data cannot become an element of phylogenetic studies unless they are measured for many more different proteins.

Neutron Scattering to Measure Force Constants

Force (F) represents the variation of potential energy (ΔU) over a length Δl ($F\Delta l = -\Delta U$). Thus, more energy is stored in a spring when it is stretched further. The resilience of a given environment is reflected in the force exerted on an atom when it is "plucked" away from its equilibrium position; the smaller the force, the less resilient or softer the environment. This can be quantified by a force constant k, the force exerted per unit displacement ($k = |F|\Delta l$). In the spring example, the energy stored (ΔU) is related to the force constant by

$$\Delta U = \tfrac{1}{2}k(\Delta l)^2 \quad (1)$$

Because of the temperature, atoms in a protein structure move about their average positions. The resultant force acting on an atom when it moves away from its average position can be expressed in terms of a force constant attributable to its environment. Thermal energy is on the order of 1 kcal/mol, whereas atomic fluctuations due to thermal motions are ~1 Å. The mean of the force constants, $\langle k \rangle$, acting on all the protein atoms can therefore be calculated by measuring $\langle(\Delta l)^2\rangle$ and $\langle \Delta U \rangle$ (ensemble average) values in the appropriate respective ranges (~1 Å^2 and ~1 kcal/mol). In the case of electromagnetic radiation, the angstrom wavelength range corresponds to x-rays but the thermal energy range corresponds to infrared radiation, so that structural and spectroscopic experiments with electromagnetic radiation are usually performed under completely different experimental conditions. Because of their mass, neutrons of energy ~1 kcal/mol have a wavelength of ~1 Å, which makes them uniquely suitable for diffraction and spectroscopic measurements in the ranges of interest to provide, simultaneously, amplitudes and related energies of motion, and therefore $\langle k \rangle$ values.

Neutrons are scattered by atomic nuclei, and the incoherent cross section of ^1H is much larger than that of other atoms in proteins (*25*). The incoherent neutron-scattering experiments provide information on protein dynamics because, in the energy range examined, H atoms reflect the motions of the side chains and backbone atoms to which they are bound (*26*).

The cross section of deuterium (^2H) is also weak with respect to that of ^1H, and H-D labeling has been used to strongly enhance or reduce scattering contributions of different parts of a structure (*5*). But the mass of D is twice that of H; does deuteration not affect molecular dynamics? In an important control experiment, the scattering of a fully deuterated purple membrane sample was measured to show that the mean atomic fluctuations are essentially identical to those in the natural-abundance sample (*5*). This observation confirms that it is the dynamics of larger chemical groups (for which the relative change in mass due to deuteration is small), and not the dynamics of the H atoms with respect to these groups, that is probed in such experiments.

In an elastic temperature scan, $\langle x^2 \rangle$ values, which are proportional to mean square displacements and are calculated from the angular dependence of the scattered elastic incoherent intensity (*27*), are plotted as a function of absolute temperature. Typical scans are shown in Fig. 1. The very low temperature part of the scan is dominated by quantum effects, and the fluctuation value at 0 K is due to the zero point energy. Region A is the harmonic region. The straight line should extrapolate to $\langle x^2 \rangle = 0$ at 0 K. A mean force constant $\langle k \rangle$ can be calculated from the derivative of the line:

$$\langle k \rangle = 0.00138/(d\langle 3x^2 \rangle/dT) \quad (2)$$

(The numerical constants enable $\langle k \rangle$ to be expressed in newtons per meter when x is in angstroms and T is absolute temperature.) Beyond the dynamical transition, region B corresponds to an anharmonic regime, where [in the conformational substate model of (*6*)] the protein atoms, after being trapped in harmonic potential wells at low temperature, have sufficient thermal energy to jump between different wells. It is this portion of the scan that describes the physiological dynamics of the protein. At each temperature we can read off a mean square fluctuation and calculate the local slope of the curve. Because a force constant is not defined for anharmonic motion, an operational approach is suggested here in which the resilience of an anharmonic environment at a temperature T is quantified by $\langle k' \rangle$ calculated from the derivative of the scan at T, according to Eq. 2. A two–potential well model is the simplest to describe anharmonic dynamics above the transition (*8*) (Fig. 1). In this case, potential energy should be replaced by the free energy difference between the two wells (ΔG, which includes an entropy term). The $\langle k' \rangle$ value calculated from the slope of the straight-line part of the scan between 240 and 300 K is a very good approximation for $\Delta G/d^2$, where d is the distance between the wells (Fig. 1). Therefore, $\langle k' \rangle$ represents well the resilience of the environment toward the jump from one well to the other (*28*). With the force constant approach, elastic temperature scans that at first appear to be very similar can be seen to provide measurements of significant differences in resilience, as illustrated by the data on myoglobin and purple membranes presented above.

Conclusion and Perspectives

The thermodynamic resilience of a molecular system can be characterized in terms of a mean pseudo-force constant acting on each atom. At very low temperatures, a protein behaves like a harmonic solid with a resilience that depends on the environment, represented by a mean force constant of a few newtons per meter. At physiological temperatures, structures are about 10 times as "soft"; the mean force constant measured in a hydrated myoglobin powder is 0.3 N/m, whereas it is ~0.1 N/m in hydrated purple membranes. Do these values reflect the compact α-helical structure of myoglobin and the higher mobility of the extramembrane loops in bacteriorhodopsin, respectively? The active core of bacteriorhodopsin is stiffer than the membrane globally characterized by a mean force constant of 0.33 N/m, similar to myoglobin. Exploration of dynamics-function relations should be extended to other proteins. In systems involved in electron transfer reactions, for example, it is poorly understood how electrons are always chan-

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neled through one of several apparently symmetric structural pathways. Large fluctuations are likely to be unfavorable for specific electron transfer reactions, and the selection may well be dynamics-based, with a structural rigidity defining the preferred pathway.

An interesting advantage of the neutron-scattering experiments is that they can be performed on samples that need not be either crystalline or monodisperse. Thermal fluctuations can be measured in situ on any type of sample (powders, solutions, pellets from the ultracentrifuge, membranes, or even whole cells). This opens up opportunities to characterize the mean resilience of a variety of biologically relevant but complex environments. Beyond applications to single macromolecular structures such as myoglobin or bacteriorhodopsin, the method can be extended to problems at the cellular level. Recent work comparing elastic scans from various cell types showed that such measurements were possible and that they in fact characterize the mean dynamics of all the proteins within the cellular environment (*29*). The question of dynamics adaptation to extreme temperatures (*30*) should be addressed by comparing the mean molecular resilience within cells of mesophilic, psychrophilic, and thermophilic organisms. If whole cells are to be analyzed, it is very easy to obtain the ~200 mg of material required for a neutron-scattering experiment, an amount that is often prohibitive for pure protein.

Deuterium labeling by biosynthesis or reconstitution from labeled and unlabeled starting material (obtained, for example, from expression in *Escherichia coli*, grown in fully deuterated and normal medium, respectively) is a rapidly developing tool because of its powerful applications in NMR as well as neutron scattering. We have seen, in the bacteriorhodopsin example, that specific amino acids within proteins can be labeled. The method can also be applied at a higher level of complexity—for example, to study the dynamics of different parts of macromolecular machines such as ribosomes, proteasomes, chaperonins, or multienzyme complexes—by using deuterium labeling to focus on specific protein or RNA components of the complex structure.

The experiments on myoglobin and purple membranes were performed at the high-flux reactor of the Institut Laue Langevin, Grenoble, France. Neutron-scattering applications provide unique information in many areas of science, but they are at present limited by the scarcity of appropriate sources and instrumentation. Plans for the improvement of existing neutron-scattering installations and for the construction of new-generation sources in the United States (at Oak Ridge, Tennessee), Europe, and Japan encourage us to look forward to many more experimental results on protein dynamics in the coming years.

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$$I(Q,0\pm\Delta\omega) = (\text{constant})[\exp(-\langle\bar{x}^2\rangle Q^2)]$$
where $Q = 2\pi \sin\theta/\lambda$ (2θ is the scattering angle and λ is the incident neutron wavelength). The mean square fluctuation is $\langle u^2\rangle = 6\langle\bar{x}^2\rangle$, the harmonic mean square fluctuation from equilibrium is $\langle r^2\rangle = 3\langle\bar{x}^2\rangle$, and the Gaussian approximation is valid for Q values satisfying $Q^2\langle r^2\rangle \approx 1$.
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32. I thank D. Bicout and M. Ferrand for enlightening discussions on protein thermodynamics, and B. Z. Ginzberg, M. Ginzburg, U. Lehnert, D. Madern, C. Pfister, J. L. Popot, V. Réat, and M. Weik for critical readings of the manuscript.

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