The ‘glass transition’ in protein dynamics: what it is, why it occurs, and how to exploit it

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Abstract

All proteins undergo a dramatic change in their dynamical properties at approximately 200 K. Above this temperature, their dynamic behavior is dominated by large-scale collective motions of bonded and nonbonded groups of atoms. At lower temperatures, simple harmonic vibrations predominate. The transition has been described as a ‘glass transition’ to emphasize certain similarities between the change in dynamic behavior of individual protein molecules and the changes in viscosity and other properties of liquids when they form a glass. The glass transition may reflect the intrinsic temperature dependence of the motions of atoms in the protein itself, in the bound solvent on the surface of the protein, or it may reflect contributions from both. Protein function is significantly altered below this transition temperature; a fact that can be exploited to trap normally unstable intermediates in enzyme-catalyzed reactions and stabilize them for periods long enough to permit their characterization by high-resolution protein crystallography.

Keywords: Protein dynamics; Glass transition; X-ray crystallography; Protein structure; Protein function; Collective motions

1. Introduction

The functions of proteins require some flexibility because all interactions invariably lead to at least small rearrangements of atoms in response to those events. It is now accepted that one of the unique physical characteristics of proteins as polymers is that they display such flexibility over a vast range of time scales and distances. Motions in proteins cover time scales from femtoseconds (individual bond vibrations) to picoseconds (small group fluctuations) to nano- and microseconds and longer (collective motions of groups of bonded and nonbonded atoms) and corresponding distance scales of fractions of an Angstrom to nanometers. Since all these motions must be thermally driven, they must depend on temperature; since all organisms have an optimum temperature at which they thrive, if protein flexibility is essential for protein function, then one might expect that at this optimum temperature, any protein from that organism will display a flexibility that is appropriate for its function. This is exactly what is observed: if one
measures the flexibility of a homologous protein from two organisms with vastly different optimum growth temperatures, one finds those flexibilities are very different when measured at the same temperature (e.g. room temperature), but become very similar at the respective optimum temperatures [1].

Since protein motions are temperature-dependent, temperature is a potential probe for the functional role of such motions. But if changing the temperature affects all motions at the same time, the value of this probe is less than if it could be used to perturb selectively one class of motions. Over 20 years ago, a series of experiments conducted in different labs with different proteins using different techniques gave results that suggested that not all motions in proteins were sensitive to temperature in the same way.

2. ‘Something happens’ at ~200 K

The earliest evidence for unusual temperature dependence of protein dynamics came from a study in which the atomic motions of the iron atom in the heme group of sperm whale metmyoglobin were measured using Mossbauer spectroscopy [2]. The expectation was that the flexibility of such an atom would decrease linearly with decreasing temperature in a manner not very different from that typically observed in small molecule organometallic crystals. Instead, biphasic behavior was observed with a low-temperature regime whose linear behavior did resemble small molecule compounds and a higher temperature region, which displayed a steep temperature dependence that could not be described with reference to model compounds. The transition between these two regions occurred over a range of temperatures centered at approximately 200–220 K.

These observations called to mind earlier studies of the temperature dependence of the rebinding of flash-photolyzed CO to the heme proteins hemoglobin and myoglobin by Frauenfelder and coworkers [3]. Myoglobin rebinding of carbon monoxide and dioxygen after photodissociation was observed in the temperature range between 40 and 350 K. Four different rebinding processes were found. Between 40 and 160 K, a single process was observed. At approximately 170 K, a second and at 200 K, a third concentration-independent process emerged. At 210 K, a concentration-dependent process set in. If myoglobin is embedded in a solid, only the first three can be seen, and they are all nonexponential. In a liquid glycerol–water solvent, rebinding is exponential. To interpret the data, a model was proposed in which the ligand molecule, on its way from the solvent to the binding site at the ferrous heme iron, encounters four barriers in succession. The barriers were tentatively identified with known features of myoglobin. The nonexponential rebinding observed at low temperatures and in solid samples was interpreted in terms of a spectrum of activation energies for the innermost barrier. The shape of the spectrum was determined and its existence explained by assuming the presence of many conformational states for myoglobin. Above approximately 200 K, these states interconvert and rebinding becomes exponential.

3. Accumulating experimental evidence

Building upon these early observations, new and different experiments by others over the next decade could be interpreted in terms of a similar phenomenon. Alben et al. studied the infrared spectra of the bound and photodissociated states of Mb–CO from 5.2 to 300 K [4]. In the bound state of Mb–CO, the known lines have center frequencies, widths, and absorbances that are independent of temperature between 5.2 and 160 K. Above 160 K, one of these gradually shifts. The low-temperature photodissociated state (Mb) shows three lines, the absorbances of which depend on temperature. One was tentatively assigned to free CO in the heme pocket and the others, to CO weakly bound to the heme or heme pocket wall. The observation of essentially free CO in the photodissociated state at low temperatures implies that the difference between Mb and deoxyMb is not due to an interaction of the flashed-off ligand with the protein but is caused by an incomplete relaxation of the protein structure at low temperatures.

Inelastic neutron scattering experiments on myoglobin by Doster and Cusack probed the motions
of hydrogen atoms in the protein on the 0.1–100-ps time scale [5]. Below approximately 200 K, elastic scattering intensity could be modeled by harmonic behavior. Above 200 K motions occur that could not be modeled as harmonic motions of individual atoms. The simplest model accounting for these observations involves transitions between at least two conformational states, i.e. collective motions of groups of atoms superimposed on simple vibrations. The extra mobility above 200 K was reflected in a strong temperature dependence of the mean-square atomic displacements, a phenomenon previously observed specifically for the heme iron by Mossbauer spectroscopy, but on a much slower time scale \((10^{-7} \text{ s})\). It also correlated with the transition in the hydration shell of myoglobin and with the temperature dependence of ligand-binding rates at the heme iron, as monitored by flash photolysis.

More quantitative infrared spectroscopy (IR) experiments by Champion et al. were aimed at determining the height of the energy barrier to CO rebinding in photolyzed CO myoglobin. Below 180 K, CO could be flashed off but did rebind quickly. Above approximately 200 K, CO begins to escape the protein to the solvent. The interpretation of these data was that fluctuations required for rebinding or the release of CO from the binding pocket are quenched below \(\sim 200\) K. The data were consistent with a ‘phase transition’ of the protein that is coupled to the surrounding matrix [6].

Almost at the same time, very different experiments by Hoffman and coworkers showed a similar peculiarity at the same temperature ([7]). Electron transfer rates between two proteins that form a specific complex were shown to go to 0 at approximately 200 K. One interpretation of these observations was that complex formation had become impossible at the lower temperatures.

The dynamical behavior found for myoglobin and other proteins suggests a coupling of fast local motions to slower collective motions, which is a characteristic feature of dense glass-forming systems.

4. X-ray evidence

In general, X-ray diffraction experiments provide a snapshot of the time- and space-average structure of a crystalline molecule; any dynamic information is contained either in the thermal diffuse scatter or in the spread of electron densities about the average atomic positions. The spatial distribution of atomic motions, even in molecules as large as proteins, can be determined crystallographically by fitting a Debye–Waller expression to this electron density spread, provided the resolution of the data is high enough to furnish a reasonable observations-to-parameters ratio [8]. Of course, the electron density spread will also reflect static disorder in the crystal lattice as well as atomic and overall molecular motion, but the static contribution can be estimated from temperature-dependent studies and the overall motion can be fit to various models.

To validate the extraction of atomic dynamic parameters from temperature-dependent diffraction data, Parak and Ringe examined the X-ray structure of myoglobin at 80 K [9]. (The flash-freezing technique they developed has since become the standard method for cryocrystallography of proteins.) The overall structure at 80 K is similar to that at 300 K except that the volume is smaller. Comparison of the fitted isotropic Debye–Waller parameters at 80 K with the values obtained earlier at 250–300 K indicates that the protein at 80 K is more rigid. The average experimentally determined
Debye–Waller factor, $B$, for the protein is $14 \text{ Å}^2$ at 300 K and $5 \text{ Å}^2$ at 80 K (to a first approximation, $B = 8 \bar{N}^2 \langle \Delta x^2 \rangle$, where $\langle \Delta x^2 \rangle$ is the mean-square atomic displacement from the average position). Plots of backbone mean-square displacement vs. temperature showed a discontinuity of slope at approximately 200 K for at least one-third of all residues. This behavior is in good agreement with the temperature dependence of the mean-square displacement of the heme iron as measured by Mossbauer absorption. The magnitudes of the smallest mean-square displacements observed at 80 K indicated that intramolecular motions can be frozen out to a surprisingly large degree. Even at 80 K, however, some atoms in myoglobin still have mean-square displacements greater than 0.1 Å$^2$, which was interpreted as providing evidence for conformational substates.

This behavior is not unique to myoglobin. Tilton et al. determined structures using X-ray diffraction data collected to 1.5-Å resolution for the protein ribonuclease-A at nine different temperatures ranging from 98 to 320 K [10]. In agreement with the myoglobin observations, the protein molecule expands slightly (0.4% per 100 K) with increasing temperature and this expansion is linear. The expansion was found to be due primarily to subtle repacking of the molecule, with exposed and mobile loop regions exhibiting the largest movements. Individual atomic Debye–Waller factors exhibited predominantly biphasic behavior, with a small positive slope at low temperatures and a larger positive slope at higher temperatures. The break in this curve occurs at a characteristic temperature of 200–220 K, as in the case of myoglobin (Fig. 1). The distribution of protein Debye–Waller factors was observed to broaden as well as shift to higher values as the temperature was increased (Fig. 2). The authors concluded that this effect might be due to fundamental changes in the dynamical structure of the protein and bound solvent.
5. Characteristics of the transition

The nature of this transition could be inferred in part from the change in slope: above the transition temperature, the steep slope of $B$ vs. $T$ suggested dynamic parameters that were large and highly temperature-dependent. Below the transition, the small slope was similar to the temperature dependence of harmonic atomic vibrations in small molecule crystals. Recognizing that this transition was independent of the specific protein, Ringe, Petsko, Frauenfelder and Parak hypothesized that there exists for all proteins a dynamical property that is unique to a peptide-based polymer in water. This property is the existence of a transition in the low frequency, large amplitude collective modes of motion at approximately 200 K such that below this temperature such motions are effectively frozen out while the higher frequency, low amplitude individual atomic fluctuations are unaffected [9].

These workers also noted that the temperature of this transition was observed to be broad and dependent both on cosolvent and on the rate of cooling, shifting to lower temperatures with slower cooling protocols, all consistent with this interpretation. Because these characteristics were reminiscent of the changes in enthalpy, entropy, volume and viscosity that occur when a liquid forms a glass instead of a crystalline solid [11–14], the transition was described as a ‘glass transition’ in protein dynamics [15].

In simple materials, the transition to the glassy state is continuous and reversible, and the glass transition temperature $T_g$ is defined operationally as the intersection of the extrapolated liquid and glassy solid curves. It is the temperature at which the glass would be in metastable equilibrium if it could be brought to $T_g$ instantaneously. Interestingly, Tilton et al. found that if crystals of ribonuclease were flash-frozen to 200 K, they became disordered, but it was possible to preserve order in the lattice on cooling below 200 K if this temperature was passed rapidly [10].

The nature of the classical glass transition is complex and even now is poorly understood. The discontinuous behavior of the heat capacity at the transition temperature is suggestive of a second order phase transition involving more than one ordering parameter. Kauzmann, in a seminal paper in 1948, pointed out that the precipitous decrease in heat capacity at $T_g$ can be used to relate the formation of a glassy state to changes in entropy. The heat capacities for both the crystalline and glassy states of most materials are essentially the same, and arise from vibrational contributions. Above $T_g$, the glass exhibits excess heat capacity, implying the existence of additional configurational degrees of freedom that the material possesses in the supercooled liquid state. Kauzmann suggested that there must be a lowest temperature to which such a liquid could be supercooled while maintaining the large liquid-like heat capacity, an ‘ideal’ $T_g$. The experimental $T_g$ cannot decrease below this value since, if it did, the total entropy of the liquid would fall below that of the crystal, violating the Third Law of Thermodynamics; this is called the ‘Kauzmann Paradox’. For simple glassy materials, experimental values of $T_g$ occur just 20 K above the ideal value.

Kauzmann’s arguments are very important for understanding the glass transition in protein dynamics that occurs approximately 200 K. Above this temperature, protein molecules possess additional configurational degrees of freedom due to anharmonic, collective motions involving groups of bonded and nonbonded atoms (proteins are typically almost as close packed in their interiors as simple solids, so large-scale atomic motions are strongly interdependent). As the temperature is lowered, there is a continuous reduction in the energy available to drive these modes of motion until, as $T_g$ is approached, their time scales become very long compared to the observation time and a broad transition is observed. Below $T_g$, the protein behaves, in dynamic terms, as an amorphous solid, where there is little correlation between the motions of particles (here, atoms in the protein) and individual harmonic vibrations predominate.

6. Evidence from simulations

Further insight into the nature of the transition has come from molecular dynamics simulations. Molecular dynamics was used to probe the atomic motions of carboxy-myoglobin (MbCO) as a func-
tion of temperature. The simulations attempted to mimic the neutron scattering experiments very closely by including a partial hydration shell around the protein. Theoretical elastic, quasi-elastic and inelastic neutron scattering data were derived from the trajectories and directly compared with experiment. At low temperature there is one fast decay process, and at high temperatures there is an additional slow relaxation process that is due to quasi-elastic scattering. The average atomic fluctuations show that the protein behaves harmonically at low temperatures. At approximately 200 K, a glass-like transition in atomic fluctuations was seen. Above the transition temperature, the atomic fluctuations exhibit both harmonic and anharmonic behavior. Heavy-atom dihedral transitions were monitored as a function of temperature. Dihedral transitions involving backbone atoms occur only above the glass transition temperature. The overall results suggest that at low temperatures there is purely vibrational motion with one fast decay process, and above the glass transition temperature there is more anharmonic motion with a fast and a slower relaxation process occurring simultaneously [16].

Independently, another simulation study was also performed on myoglobin to calculate the incoherent neutron scattering spectra between 80 and 325 K for comparison with the experimental data. There was good agreement over the entire temperature range for the elastic, quasi-elastic, and inelastic components of the scattering. Analysis of the simulations showed that at low temperatures a harmonic description of the molecule is appropriate and that the molecule is trapped in localized regions of conformational space. At higher temperatures the scattering arises from a combination of vibrations within wells (substates) and transitions between them; the latter contribute to the quasi-elastic scattering. The simulations yield results for the structural changes between 80 and 325 K that are also in general accord with those from X-ray data. Both the experimental and calculated radii of gyration, distances from the center of mass and main-chain difference distance matrices show that there is a significant but inhomogeneous shrinkage with decreasing temperature [17].

To characterize the functionally important anharmonic motions, simulations of MbCO dynamics have been performed during which dihedral transitions were prohibited. Comparison of torsionally restrained and unrestrained protein dynamics simulated at three levels of hydration and at temperatures ranging from 100 to 400 K suggests that hydration ‘catalyzes’ protein mobility by facilitating collective anharmonic motions that do not require dihedral transitions. When dihedral transitions were prohibited, dehydrated MbCO, to a good approximation, exhibited only harmonic fluctuations, whereas hydrated MbCO exhibited both harmonic and anharmonic motions [18].

7. The mechanism

Proteins exist in a watery environment; their surfaces are covered with a layer of bound solvent, typically 2–3 ordered water molecules per residue. This water can be considered an intrinsic part of the protein structure, as opposed to the disordered bulk solvent. The obvious question raised by observation of a glass transition in protein dynamics is the following: Is the transition due to a transition in the bulk solvent, the bound solvent, the protein alone, or some combination?

Experimental evidence for the involvement of the bound water comes from spectroscopy. Doster studied the water of hydration in myoglobin crystals and solutions at subzero temperatures by calorimetry and IR. The hydration water remained amorphous at low temperatures. He found a broad transition between 180 and 270 K depending on the degree of hydration. The ice component showed a noncolligative melting point depression that was attributed to a finite conformational flexibility. The transition in crystals was qualitatively similar to what is found in amorphous samples at the same water content. These data were compared with the Mossbauer experiments; the similar temperature dependencies suggested a cross correlation between structural fluctuations and the thermal motion of bound water. A hydrogen bond network model was proposed to explain these features. The essential ingredients are cooperativity and a distribution of hydrogen-bonded clusters [19].
Detailed analysis of protein crystal structures, especially of the same protein in multiple cosolvent systems, indicates that bound water molecules on protein surfaces can be considered as belonging to several distinct classes. Some are completely disordered and, therefore, invisible to X-ray diffraction experiments, though they can be observed by NMR. A second class of bound waters are observed in many, but not all, structures of the same protein and have positions and interactions that can differ slightly. As a rule, they may have one or more unsatisfied potential hydrogen bonding interactions. Finally, there are bound water molecules that are always observed in the same positions making the same interactions in nearly all structures of the protein: these waters, which constitute an estimated 20% of the total bound solvent, usually make the full complement of possible hydrogen bonds. These waters have Debye–Waller factors comparable to those of well-ordered protein atoms and are thought to contribute to the stability of the folded protein [20].

The contribution of hydrogen bonds to protein–solvent interactions and their impact on structural flexibility and dynamics were probed using the shift of vibrational peak frequencies with the temperature of myoglobin in sucrose/water and glycerol/water solutions. A characteristic change in the temperature slope of the O–H stretching frequency was observed at the glass transition, which correlates with the discontinuity of the thermal expansion coefficient. The temperature-difference spectra of the amide bands show the same tendency, indicating that stronger hydrogen bonding in the bulk affects the main-chain solvent interactions in parallel. However, the hydrogen bond strength decreases relative to the bulk solvent with increasing cosolvent concentration near the protein surface, which suggests preferential hydration. The central O–H stretching frequency of protein hydration water is red-shifted by 40 cm$^{-1}$ relative to the bulk. The shift increases towards lower temperatures, consistent with contraction and increasing strength of the protein–water bonds. The temperature slope shows a discontinuity near 180 K. The contraction of the network has reached a critical limit, which leads to frozen-in structures. This effect may represent the molecular mechanism underlying the dynamic transition observed for the mean-square displacements of the protein atoms and the heme iron of myoglobin [21].

Normal mode analysis and molecular dynamics simulations of small globular proteins predict delocalized vibrations with frequencies below 20 cm$^{-1}$, which may be overdamped in solution due to solvent friction. In search of these modes, Doster et al. studied deuterium-exchanged myoglobin and lysozyme using inelastic neutron scattering in the low-frequency range at full and low hydration to modify the degree of damping. At room temperature, the hydrated samples exhibit a more pronounced quasi-elastic spectrum than the dehydrated samples. The analysis of the corresponding line shapes suggests that water modifies mainly the amplitude, but not the characteristic time of fast protein motions. At low temperatures, in contrast, the dehydrated samples exhibit larger motional amplitudes than the hydrated ones. The excess scattering, culminating at 16 cm$^{-1}$, is suggested to reflect water-coupled librations of polar side chains that are depressed in the hydrated system by strong intermolecular hydrogen bonding. Both myoglobin and lysozyme exhibit ultra-low-frequency modes below 10 cm$^{-1}$ in the dry state, possibly related to the breathing modes predicted by harmonic analysis [22].

Evidence for both ‘solidification’ of correlated motions in proteins below the transition temperature and participation of the bound solvent in the process was further provided by Teeter et al., who showed by high-resolution crystallography that the radius of gyration ($R_g$) of the small seed protein crambin remains constant below approximately 180 K and that both atom positions and dynamics of protein and solvent are physically coupled, leading to a novel cooperative state. This glassy state could be identified by negative slopes of the Debye–Waller ($B$) factor vs. temperature and, as in the cases of myoglobin and ribonuclease, is composed of multisubstate side chains and solvent. Based on generalization of Adam–Gibbs’ notion of a cooperatively rearranging region and decrease of the total entropy with temperature, these investigators calculated the slope of the Debye–Waller factor; the results were in accord with experiment [23].
The idea that changes in the dynamical properties of bound solvent might be responsible for the observed temperature dependence of protein motions was supported by simulation studies by Vitkup et al. Using a Nose–Hoover thermostat to differentially regulate the temperature of the protein and the solvent, they found that solvent mobility was the dominant factor in determining the atomic fluctuations above 180 K (Fig. 3), although intrinsic protein effects become important at lower temperatures [24]. The simulations thus complement the experimental studies by demonstrating the essential role of solvent in controlling functionally important protein fluctuations.

This conclusion has been challenged by recent studies of Lee and Wand, who carried out a survey of the temperature dependence of the fast dynamics of methyl-bearing side chains in a calmodulin–peptide complex using site-specific deuterium NMR relaxation methods. The amplitudes of
motion, which occur on a sub-nanosecond time scale, had a markedly heterogeneous spectrum and segregated into three largely distinct bands. (Similar data for other proteins obtained at single temperatures indicate that this may be a general property of protein molecules.) The temperature dependence of the dynamics of the calmodulin complex shows large variations, implying a significant and heterogeneous distribution of residual entropy and, through the associated temperature dependence, reveals local contributions to the heat capacity of the protein. These observations also suggested an unexpected explanation for the low-temperature ‘glass transition’ of proteins. Most of sites in the protein were found to have maximal order values broadly centered at approximately 180 K, near the temperature often associated with the protein glass transition. If one averages the site-resolved information obtained using NMR relaxation methods to construct an analogue of the temperature dependence of the average mean-squared atomic displacements obtained from neutron scattering studies, one obtains a similar curve. Typically, such profiles are interpreted to indicate a general transition in the protein–solvent system, as stated earlier, but in the NMR results it is the freezing out of the middle band of motion, corresponding to transitions between different tiers of conformational substates of the protein itself, that produces the apparent dynamical transition at approximately 200 K. Furthermore, the upper band, corresponding to the lowest-amplitude motion at room temperature, tends to its limiting value at temperatures well above \( T_g \). The behavior of the lower band (which corresponds to the highest-amplitude motion at room temperature) as the temperature approaches \( T_g \) is less certain. Some members of this class of motion have temperature dependencies that require significant nonlinear behavior at lower temperatures to converge to maximum order at approximately 200 K. Since a simple interpretation of this temperature dependence, at temperatures far above the apparent \( T_g \) of proteins, is predictive of the glass transition, Lee and Wand conclude that thermal activation of these motional modes is sufficient to explain the temperature dependence observed by neutron scattering and X-ray diffraction methods without the introduction of a global solvent-driven glass transition model [25].

However, since the NMR studies did not probe the behavior of the bound solvent, it is not clear from these results whether this solvent, as opposed to bulk water, may still be a principal contributor. The relative roles of the protein itself, the protein-bound solvent, and the bulk solvent in the determining the glass transition behavior in protein dynamics, thus, remain unresolved.

8. Importance for function and practical applications

The observation that photolyzed oxygen and CO cannot escape from the myoglobin heme pocket nor rebind rapidly to the heme iron at temperatures below the glass transition [3] implies that, at least for this protein, collective motions are important for function. Extrapolation of this idea to any protein, particularly enzymes, suggests that any activity requiring conformational adjustments of protein atoms, either to achieve specific binding or to move substrate and active site functional groups along a reaction coordinate, should be effectively quenched below approximately 200 K. This expectation was dramatically born out in an experiment performed by Rasmussen et al. on ribonuclease A. They showed by high-resolution X-ray diffraction that the crystalline enzyme does not bind substrate or inhibitor at 212 K but will bind either rapidly at 228 K. Once bound at the higher temperature, inhibitor cannot be washed off (using a flow cell) after the enzyme is cooled to below the transition temperature. They proved that small molecules were still able to react with the protein surface nonspecifically below 200 K by exposing the crystal to a platinum compound that covalently modified exposed methionine side-chains [26]. These results suggest that enzyme flexibility is required for at least part of catalytic function, namely specific binding of substrates and substrate-like ligands to the active site.

This dependence of molecular recognition on flexibility is logical. Since nearly all proteins are not absolutely specific, binding sites cannot be perfectly complementary to every ligand; conformational adjustments, sometimes large, at other
times very small, must occur to allow access to the site and to permit the interactions between protein and ligand to develop. Many of the motions required will be collective, and therefore are expected to have a steep temperature dependence and be sensitive to the glass transition. This property can be exploited: by allowing, say, a specific substrate to bind to an enzyme above the glass transition temperature and then rapidly cooling the complex below $T_g$, the substrate can be locked into the active site. If the substrate has time to transform into an intermediate before or during cooling, and if further transformation requires protein motions that are also quenched below $T_g$, then this technique may also be used to trap metastable species and render them accessible to detailed characterization [27].

Exactly this strategy has been employed to observe intermediates in the reaction catalyzed by the serine protease elastase [28]. The structure of a specific acyl-enzyme intermediate in the elastase-catalyzed hydrolysis of the artificial substrate $N$-carbobenzoxy-$l$-alanine $p$-nitrophenyl ester was determined and refined against X-ray diffraction data at 2.3-Å resolution. The acyl-enzyme was formed at $-26^\circ C$ and was stabilized at $-55^\circ C$ during data collection, taking advantage of the glass transition in protein dynamics that occurs at approximately $-50^\circ C$. The difference Fourier electron density map clearly shows electron density for the trapped acyl-enzyme, indicated by covalent attachment of the alanine carbonyl carbon to the side-chain oxygen of Ser 195, the catalytic nucleophile. In a second experiment, $N$-(2-furyl)-acryoyl-$l$-leucine-methyl ester was bound to crystalline elastase at $-26^\circ C$ and the structure stabilized at $-5^\circ C$. The resulting electron density maps again show a covalently bound acyl derivative (Fig. 4). Finally, the authentic elastase substrate, the tripeptide acetyl-$l$-Ala-$l$-Ala-$l$-Ala-methyl ester was incubated with crystalline elastase at $-26^\circ C$ and then rapidly cooled to $-50^\circ C$. This structure also showed a covalently attached acyl derivative, with the alanine tripeptide bound in the active site in the manner expected for polypeptide substrates (data not shown). To prove that the complex was productive, the temperature of this acyl enzyme was then raised to $-20^\circ C$ and another data set collected; the active site showed no electron density for any bound substrate species, indicating that the intermediate had been hydrolyzed as expected (Ding et al., in preparation).

The power of this strategy was demonstrated by its use to obtain structures for all of the kinetically significant intermediates in the reaction catalyzed by cytochrome P450cam. Members of the cytochrome P450 superfamily catalyze the addition of molecular oxygen to nonactivated hydrocarbons at physiological temperature—a reaction that requires high temperature to proceed in the absence of a catalyst. Structures were obtained for three intermediates in the hydroxylation reaction of camphor by P450cam with trapping techniques and cryocrystallography. The structure of the ferrous di-
oxygen adduct of P450cam was determined with 0.91-Å wavelength X-rays; irradiation with 1.5-Å X-rays results in breakdown of the dioxygen molecule to an intermediate that would be consistent with an oxyferryl species. The structures show conformational changes in several important residues and reveal a network of bound water molecules that may provide the protons needed for the reaction [29]. Flash-cooling of protein crystals exposed to a substrate with the objective of trapping a bound species has now become a widespread technique in structural enzymology [27]. Although most such experiments simply cool rapidly to 80 K with liquid nitrogen for convenience, the success of the method relies on the fact that this temperature is below the glass transition, and therefore any species that is formed would be stable, even though it might not be if one were to extrapolate its lifetime from higher temperatures assuming Arrhenius behavior.

9. Future directions

Proteins isolated from organisms with optimal growth temperatures close to the boiling point of water—so-called hyperthermophilic organisms—show very little catalytic activity when assayed at room temperature. As discussed above, their activity becomes respectable at higher temperature when their flexibility approaches values for mesophilic proteins at ordinary temperatures [1]. This observation begs the question of whether hyperthermophilic proteins have a glass transition that is shifted to higher temperatures. If this were true, it would imply that the protein rather than the bound solvent, was the major contributor to the transition. No measurements of the dynamic behavior of a hyperthermophilic protein have yet been made over a temperature range that would answer this question. Of course, it is possible that such proteins could show two transition temperatures, one at 200 K and one closer to room temperature. One experiment that suggests that such a study would be very valuable was carried out by Kohen and Klinman, who investigated the effect of protein dynamics on the contribution of quantum-mechanical tunneling to an enzyme reaction. Hydrogen tunneling has increasingly been found to contribute to enzyme reactions at room temperature. In reactions involving small molecules, the relative importance of tunneling increases as the temperature is reduced. These authors investigated whether hydrogen tunneling occurs at elevated temperatures in a biological system that functions physiologically under such conditions. Using a thermophilic alcohol dehydrogenase (ADH), they find that hydrogen tunneling makes a significant contribution at 65 °C; this is analogous to previous findings with mesophilic ADH at 25 °C. Contrary to predictions for tunneling through a rigid barrier, the tunneling with the thermophilic ADH decreases at and below room temperature. These findings were interpreted as providing experimental evidence for a role of thermally excited enzyme fluctuations in modulating enzyme-catalyzed bond cleavage [30]. By analogy, proteins from psychrophilic organisms, which have optimal growth temperatures close to the freezing point of water, are thought to be more flexible than their mesophilic counterparts at room temperature. If so, they might display a downshifted $T_g$, provided once again that protein contributions dominate. No studies of their dynamic behavior have been made so far.

If bound solvent makes a significant contribution to the glass transition, one might expect that any folded macromolecular polymer such as RNA would be subject to the same dynamical behavior. Crystal structures are now available for many large, folded RNAs, some of which possess catalytic activity. To our knowledge, no study of their activity or dynamical behavior as a function of temperature has been carried out over a range that would reveal such behavior. However, molecular dynamics simulations of the small DNA oligonucleotide duplex d(CGCCGCG)$_2$ in aqueous solution demonstrated an apparent glass transition phenomenon. The simulations were performed at temperatures in the 20–340 K range. The mean-square atomic fluctuations showed that the behavior of the oligonucleotide duplex was harmonic at low temperatures. A glass transition temperature at 223–234 K was inferred for the oligonucleotide duplex, which is in agreement with some experimental observations. The largest number of hydro-
gen bounds between the polar atoms of the oligonucleotide duplex and the water molecules was obtained at the glass transition temperature. With increasing temperature a decrease in the average lifetime of the hydrogen bonds to water molecules was observed [31].

To date, nearly all crystallographic studies of protein dynamics—and all of the ones done over a range of temperatures—have used isotropic Debye–Waller factors, even though it is well-known that protein motions are highly anisotropic. With the advent of third-generation synchrotron sources of X-rays, it is now possible to obtain data to beyond 1-Å resolution for a number of crystalline proteins [32,33]. At this so-called ultrahigh resolution, the ratio of observations to parameters allows fitting of anisotropic thermal ellipsoids to the electron density around every atom. Thus, as well as the amplitudes of atomic motions, protein crystallography can now in principle provide information on the preferred directions of motion. It would be very interesting to observe how this behavior changes as one goes below the glass transition temperature for a number of different proteins.

As a material forms a glass, the configurational changes that cause the relaxation of the supercooled liquid become increasingly slow with decreasing temperature until $T_g$, below which the substance behaves as an amorphous solid. Kauzmann’s discussion of the heat capacity of substances below $T_g$ implies that, even if no other factor contributed to the classical glass transition, the heat capacity should decrease sharply at a certain temperature solely as a result of equilibrium thermodynamic properties. The existence of an ideal glassy state possessing zero residual entropy, having an ideal glass transition temperature in the Kauzmann sense, is probably only hypothetical, since near $T_g$ the probability of crystal nucleation increases rapidly, and thus, for long time scales crystallization is more probable than relaxation to an amorphous state. It has been pointed out, however, that there are certain materials, the so-called atactic polymers, which have a random arrangement of side groups and, therefore, cannot crystallize. These polymers offer the prospect of investigating whether an ideal amorphous solid can exist, but we are unaware of any data on such systems to determine whether excess entropy in the liquid state is observed. It may be that such data are very difficult to obtain because intermolecular interactions between random-coil polymers are likely to become more important at lower temperatures. But a protein molecule, whose surface is surely atactic and which is folded into a compact shape, might offer the possibility of obtaining the necessary data without this complication. It would be fitting if proteins were to provide a way of testing Kauzmann’s ideas about the behavior of glasses, since his own brilliant career has ranged between studies of the fundamental thermodynamic properties of simple materials to the interpretation of protein denaturation.

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