Glassy Behavior of a Protein

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Quasistatic and kinetic studies of the infrared CO stretch bands of carbonmonoxymyoglobin show that proteins and glasses share essential characteristics, in particular metastability below a transition temperature and relaxation processes that are nonexponential in time and non-Arrhenius in temperature.

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Proteins and glasses may appear to have little in common. Proteins are macromolecules with well defined structures[1]; glasses are frozen liquids. Despite this difference, proteins and glasses share one fundamental property: Both can assume a very large number of nearly isoenergetic conformational substates (CS), valleys in the conformational energy landscape. For proteins, the existence of CS followed from the nonexponential time dependence of the binding of small molecules (O_2 and CO) to myoglobin at low temperatures.[2] Supporting evidence came from other experiments[3] and from theory.[4] For glasses, a potential-energy surface with a large number of minima was postulated by Goldstein[5]; for spin glasses, the evidence came from theory.[6] The existence of CS in proteins and glasses raises the question as to whether these systems share other properties. We now describe some attributes of glasses and later show that these are also found in proteins.[7]

Glasses are formed when, on cooling, a liquid becomes a structurally disordered solid.[8] The temperature at which the viscosity reaches 10^{13} poise is called the glass temperature, T_g. The specific heat below 1 K is approximately proportional to the temperature.[9] Glass properties well below T_g depend on history; glasses are in a metastable (nonequilibrium) state. Near and above T_g the response of a glass to a mechanical or electrical perturbation is dominated by the α relaxation. Its relaxation function $\Phi_r(t)$ is usually nonexponential in time and can be parametrized by a stretched exponential, $\Phi_r(t) = \exp[-(kt)^{\beta}]$, or by a power law,

$$\Phi_r(t) = [1 + k_r(T)t]^{-n}. \quad (1)$$

The average rate at temperature T is $\langle k \rangle = nk_r(T)$. The temperature dependence of $k_r(T)$ follows the Arrhenius relation, $k_r(T) = A\exp[-E/k_BT]$, only over small temperature intervals. Typical values near T_g, $E \approx 1.6$ eV, $A = 10^{42}$ s^{-1}, also imply that the Arrhenius relation is inappropriate for glasses. However, $k_r(T)$ can be described over more than 10 orders of magnitude either by the Vogel-Tammann-Fulcher equation,[8]

$$k_r(T) = A_{\mathrm{VTF}}\exp[-E/k_B(T - T_0)] \quad (2)$$

or by the relation[10,11]

$$k_r(T) = k_0\exp[-(T_0/T)^2]. \quad (3)$$

Both relations fit the data for glycerol ($T_q \approx 185$ K) from 190 to 260 K.[10,12]

We now examine the proteins for glasslike properties. Two are well known: Each individual protein is disordered (aperiodic) and the specific heat of proteins below 1 K is glasslike.[13] The other attributes, however, have been less well explored. Here we report experiments that verify the metastability at low temperatures, and the nonexponential time and the non-Arrhenius temperature dependence of the protein relaxations near 200 K in carbonmonoxymyoglobin (MbCO).

The folded polypeptide chain of the oxygen-storage protein myoglobin (Mb) embeds a heme group with a central iron atom which reversibly binds ligands such as O_2 and CO.[1] Our experiments focus on the stretch bands of CO bound to Mb which are very sensitive to external parameters such as solvent, pH, temperature (T), and pressure (P).[14,15] We measure the stretch bands with a Mattson Fourier transform infrared spectrometer. Figure 1 shows that MbCO displays at least three different CO stretch bands, A_0, A_1, and A_3. Fits to Voigtian line shapes[16] yield the areas (A_i), center frequencies (ν_i), and linewidths (Γ_i) of the A bands. We also observe the rate of heat absorption via differential scanning calorimetry (DSC). The experiments fall into two classes, quasistatic and kinetic. Quasistatic indicates that the glasslike behavior of MbCO below a transition temperature T_{sg} prevents attainment of thermodynamic equilibrium. Quasistatic measurements determine the band parameters as functions of solvent, pH, T, and P. In the kinetic studies we observe the relaxation of the protein after a pressure release.

Quasistatic experiments.— Figure 1 shows the ir spectra from 1910 to 1990 cm^{-1} in a 75% glycerol-water solvent (3:1 by volume) at pH 6.8 with potassium phosphate buffer. The sample was brought to a pressure P at 300 K. Data were then taken under constant pressure at successively lower temperatures. The cooling rate of 0.01 K/s and waiting time of about 600 s at each temper-

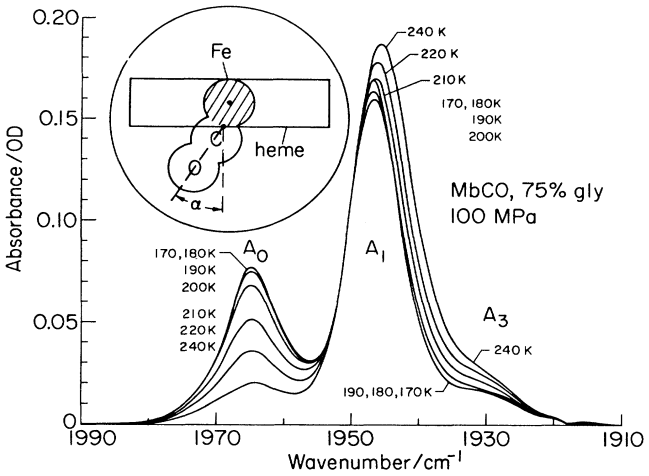

FIG. 1. Absorption spectra of the CO stretch bands of MbCO at 100 MPa. Solvent: 75% glycerol and water mixture, buffered with 100 mM potassium phosphate to pH 6.8. Inset: Cross section through the heme showing the angle α, between the CO dipole and the heme normal.

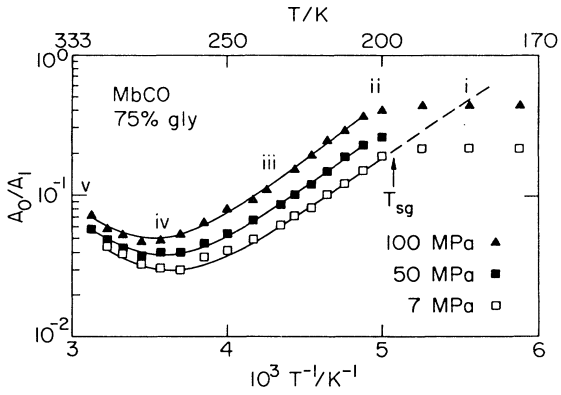

FIG. 2. Plot of $r_0 = A_0/A_1$ vs $10^3/T$ for three pressures. Roman numerals signify the five temperature regions defined in the text. The transition temperature T_{sg} is indicated. The dashed line shows the extrapolation from region (iii) for 7 MPa.

ature were kept constant. Measurement of an ir spectrum took about 250 s. Figure 2 gives the temperature dependence of the ratio $r_0 = A_0/A_1$ at three different pressures. The ratio r_0 exhibits five regions: (i) *Glass region.* The protein is in a metastable state in which large-scale motions are too slow to be observed. We prove metastability by showing that the ir spectrum depends strongly on the path by which a point in the (T,P) plane is reached. (ii) *Slaved glass transition.* The bend at T_{sg} in Fig. 2 reveals the transition from an equilibrium situation well above T_{sg} to a metastable one well below. A similar bend occurs in $\Gamma(T)$,[14] and the transition is also visible in DSC. The glass temperature T_{sg} of the protein depends crucially on the glass temperature T_g of the solvent; so we call the transition "slaved"[14]: DSC data at a heating rate of 0.083 K/s in 75% glycerol and water mixture give $T_g = 175$ K and $T_{sg} = 178$ K; in 60% ethylene-glycol-water mixture $T_g = 149$ K and $T_{sg} = 155$ K; in solid poly(vinyl)alcohol $T_g > 280$ K and $T_{sg} > 280$ K. T_{sg} and T_g depend approximately logarithmically on cooling or heating rate; a factor of 10 increase in heating rate raises T_{sg} by about 5 K. T_{sg} obtained from the bend in r_0 is about 20 K higher than that from DSC. With increasing pressure T_{sg} increases weakly as is apparent from Fig. 2. (iii) *Lower equilibrium region.* Transitions among the A substates occur faster than the time of observation so that the A substates are in thermal equilibrium. The temperature and pressure dependences of the ratios r_i determine the relative energies, entropies, and volumes of the A substates. (iv) *Upper equilibrium region.* From 250–320 K, the ratio r_0 shows a remarkable reversal. (v) *Unfolding.*[17] The protein begins to unfold irreversibly. Since regions (iv) and (v) are not the focus of this Letter we give no further details.

Relaxation experiments.— In a pressure-release experiment, 100 MPa is applied at 240 K, the sample is cooled to the desired temperature and held there for 600 s followed by measurement of an ir spectrum for about 250 s. The pressure is then released to 7 MPa in a few seconds and ir spectra are taken at approximately exponentially increasing times from about 10 to more than 10^4 s. The time-dependent behavior of the A bands after pressure release reveals several relaxation processes. To interpret the relaxation data, we assume that the conformational substates of a protein are arranged in a hierarchy of at least two tiers, CS^0 and CS^1.[14,18] The different stretch bands correspond to substates of tier 0 (CS^0). We characterize each substate A_i by the center frequency ν_i of the CO stretch band (Fig. 1) and by the angle α_i between the CO dipole and the heme normal (inset of Fig. 1).[19] It is likely that the substates A_i differ not only in the angle α_i, but also in the overall structure of the molecule. A protein may move from one substate of tier 0, say A_0, to another, say A_1. We call this motion FIM 0, where FIM is an acronym for "functionally important motion."[18] Rebinding of CO after flash photolysis to each of the A substates is nonexponential in time.[14] This observation implies that each substate of tier 0 is subdivided into substates of tier 1, CS^1. We denote as FIM 1 the nonequilibrium conformational motions between substates of tier 1. With these concepts we explain our experiments.

Below 160 K.— The center frequencies of the A bands shift (0.4 cm^{-1} for A_0) faster than our shortest measurement time (10 s), but no changes in relative intensities occur up to 10^4 s. Since the fast shift occurs even at 15 K, we interpret it as an elastic relaxation.

165–185 K.— The area of A_0 remains constant to within 2% from 10 to more than 10^4 s while the peak narrows and its amplitude increases. The center fre-

quency ν_0 initially shifts rapidly from 1964.5 to 1964.9 cm^{-1} and then moves measurably towards its low-pressure value, 1966.1 cm^{-1}. The shift in ν_0 can be described by a relaxation function $\Phi_1(t)=[\nu_0(t)-\nu_0(\infty)]/[\nu_0(0^+)-\nu_0(\infty)]$, where t is the time after pressure release and $\nu_0(0^+)$ represents the center frequency immediately after the rapid elastic relaxation. For $\nu_0(\infty)$ we use two approximations, the 7 MPa value from a quasistatic experiment at the same temperature and the value obtained by extrapolation from region (iii). Figure 3 shows the relaxation function $\Phi_1(t)$ using the former choice for $\nu_0(\infty)$. $\Phi_1(t)$ is not greatly changed with the other choice. We interpret the relaxation characterized by Φ_1 as FIM 1, a redistribution of substates CS^1 within the substate A_0.

To evaluate the data in Fig. 3, we parametrize $\Phi_1(t)$ by the power-law equation (1).[20] If we further assume that $k_r(T)$ is determined by the Arrhenius law, we obtain $E=1.3$ eV, $A=10^{36}$ s^{-1}. These values are similar to the values quoted above for the α relaxation in glasses near T_g; they imply that the Arrhenius relation is inappropriate for describing FIM 1 and suggest the use of Eq. (2) or (3). We select Eq. (3) to fit $k_r(T)$ because it uses only two parameters and therefore provides less ambiguous extrapolations. A fit with Eqs. (1) and (3), using a Levenberg-Marquardt algorithm to minimize χ^2, is shown as solid lines in Fig. 3 and yields $\log(k_0/s^{-1})=17.0\pm2.5$, $T_0=1130\pm80$ K, $n=0.26\pm0.12$. The errors are conservative. We have also measured the α relaxation in 75% glycerol and water mixture using specific-heat spectroscopy[12] and obtain $\log(k_0/s^{-1})\approx18$ and $T_0\approx1130$ K. The values of k_0 and T_0 for the protein and the solvent are remarkably close and support the notion of a slaved-glass transition: Not only do the solvent and protein exhibit comparable glass temperatures, the α relaxation of the solvent and FIM 1 in the protein obey nearly identical temperature dependences.

A second relaxation, now shown here, occurs between 170 and 190 K: A_1 and A_3 interconvert, with A_1 decreasing and A_3 increasing nonexponentially in time; the barrier between A_1 and A_3 is smaller than that between A_0 and the other A substates.[21]

190–210 K.—FIM 1 and the interconversion between A_1 and A_3 are faster than 10 s so that after pressure release the center frequencies, the linewidths, and the ratio A_3/A_1 have the values measured quasistatically at 7 MPa. A new relaxation process is observed, the exchange of A_0 with A_1+A_3. We interpret this exchange among substates of tier 0 as a conformational change of the entire protein and call it FIM 0. We define a relaxation function for FIM 0 through $\Phi_0(t)=[A_0(t)-A_0(\infty)]/[A_0(0^+)-A_0(\infty)]$. The area $A_0(\infty)$ is found by extrapolation from region (iii) as indicated by the dashed line in Fig. 2.[21] $\Phi_0(t)$, shown in Fig. 4, displays more structure than FIM 1 and a simple power law or stretched exponential does not fit the data well. Nevertheless, insight is gained by fitting the data with Eqs. (1) and (3), with the result $\log(k_0/s^{-1})\approx14$, $T_0\approx1200$ K, $n\approx0.8$. The fit reproduces the general behavior of $\Phi_0(t)$, but the detailed structure will require a more elaborate model.

Our results permit four conclusions: (1) Proteins and glasses indeed share common properties in addition to the existence of many valleys (conformational substates) in the energy landscape. The similarities include metastability below the glass temperature and the presence of a relaxation process governing large-scale motions (α relaxation in glasses, FIM 1 in proteins) which is nonexponential in time and does not follow the Arrhenius law. (2) While glasses usually show only one relaxation process with the general characteristics of the α relaxation, MbCO shows at least two, FIM 0 and FIM 1. FIM 0 is slower than FIM 1 and probably describes overall changes in the entire protein, characterized by different CO stretch frequencies and angles between the bound CO and the heme normal. FIM 1 describes large-scale motions within a given substate CS^0. (3) The protein relaxation process FIM 1 is slaved to the solvent; the transition temperature T_{sg} in the protein is close to the glass transition of the solvent. Moreover, FIM 1 in the protein

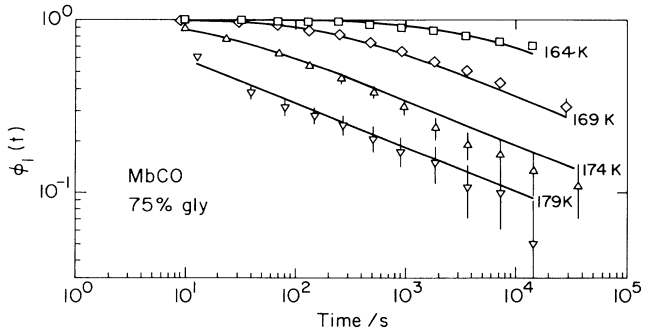

FIG. 3. Relaxation function $\Phi_1(t)$ for the center frequency ν_0 of the A_0 band vs time. Solid lines are fits with Eqs. (1) and (3) with parameters as given in the text.

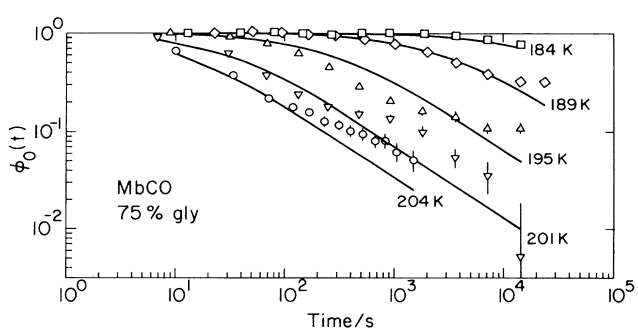

FIG. 4. Relaxation function $\Phi_0(t)$ for the area A_0 vs time. Solid lines are fits with Eqs. (1) and (3) with parameters as given in the text.

and the α relaxation in the solvent, at least in the case of glycerol and water, possess remarkably similar temperature and time dependences. The hydration shell may well play an important role in this coupling between protein and solvent.[22] (4) FIM 0 and FIM 1 can be extrapolated to 300 K with Eq. (3) with the result $\langle k \rangle \approx 10^7$ s^{-1} for FIM 0 and $\langle k \rangle \approx 10^{10}$ s^{-1} for FIM 1. While these extrapolations are speculative, they indicate where to look experimentally and suggest that molecular dynamics[23] may be able to simulate FIM 1 but probably cannot yet reach FIM 0.

The technique and the results presented here may be important for both physics and biology. For physics, proteins may well become paradigms of complex systems. The combination of the pressure jump approach with site-specific spectroscopic observation permits site-specific studies of relaxation phenomena in amorphous systems. When applied to genetically engineered proteins,[24] the pressure jump technique would probe the relaxation of specifically designed systems. For biology, detailed knowledge of protein motions at different time and length scales is necessary for an understanding of protein and enzyme reactions at the molecular level. Slaving may be an efficient control mechanism in cells and membranes.

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