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Terbium luminescence-lifetime heterogeneity and protein equilibrium conformational dynamics

(glass/rubber/phase transition/fluorescence)

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ABSTRACT **The fluorescence decay of the rare earth terbium when bound to the protein calmodulin changes from a simple exponential decay to a complex nonexponential decay as the temperature is lowered below 200 K. We have fit the observed decay curves by assuming that (*i*) the terbium emission is a forced electric dipole transition and (*ii*) proteins have a distribution of continuous conformational states. Quantitative fits to the data indicate that the root-mean-square configurational deviation of the atoms surrounding the terbium ion is 0.2 Å, in good agreement with other measurements. We further point out that because the protein seems to undergo a glass transition yet retains configurational order at room temperature, the proper name for the physical state of a protein at room temperature is the rubber-like state.**

Globular proteins are polymers of amino acids that form a condensed ball due to infolding of the heterogeneous amino acid polymer chain. Although the tertiary conformation of a protein is a well-defined structure in the crystalline state as determined by x-ray crystallography, and in solution as determined by three-dimensional NMR techniques (1), it has become clear that proteins are also capable of considerable micro-conformational flexibility (2). In fact, many important biological processes depend crucially on this conformational flexibility, which allows for switching between different macroscopic states of the enzyme. Frauenfelder and coworkers characterize the transitions between micro-conformational states as *equilibrium* fluctuations, and macroscopic state changes as *nonequilibrium* functionally important motions, or "FIMs" (3). The best studied example of a macroscopic state change is hemoglobin's R→T switch (4); another is the conformational state change in calmodulin triggered by the binding of multivalent ions (ref. 5; R. C. Miake-Lye, C. B. Klee, S. Doniach, and K. O. Hodgson, personal communication). In the experiment described here, we measure the dynamics of the equilibrium fluctuations—in fact we deliberately keep the ion loading low in order to prevent just such macroscopic conformational switches—but in the future our technique could be expanded to study the conformational dynamics of calmodulin as a function of tertiary structure.

While biophysicists and chemists have become aware of the importance of protein conformational flexibility, condensed-matter physicists have become increasingly concerned with understanding highly disordered systems such as polymers and glasses (7). Concepts from the theory of disordered systems may be helpful in understanding structural, thermal, and dynamical properties of compact proteins. In this paper we make a direct measurement of protein conformational heterogeneity through the use of time-resolved rare earth luminescence, a technique that allows direct comparison of the data with theories based on the physics of disordered systems.

Our experiment probes conformational dynamics of calmodulin by measuring time-resolved luminescence of bound terbium(III) ions. If one wishes to focus solely on the conformational dynamics of the protein, this approach offers an advantage over chemical kinetics experiments such as ligand recombination (8), in that the results may be directly interpreted in terms of the time dependence of the crystal field of the local ionic environment; interpretation of chemical kinetics experiments is often less clear due to the complicated nuclear and electronic rearrangements that occur during a chemical reaction (9).

The $^5D_4 \rightarrow {}^7F_6$ electronic transition at 540 nm in terbium is a 4f→4f transition that is parity-forbidden and consequently has a very small oscillator strength and a lifetime of approximately 1 msec in aqueous solution. As we discuss later, a low-symmetry crystal-field environment creates mixed-parity wavefunctions that enhance the electric dipole matrix elements for photon emission. If the crystal field varies among different protein conformations, and the conformational relaxation time is greater than the mean luminescence lifetime of the ion, then the time-resolved emission of photoexcited terbium will be nonexponential, and measurement of the temperature dependence of the emission will contain structural and dynamical information.

MATERIALS AND METHODS

Calmodulin is a protein of known molecular weight (M_r 16,800), amino acid sequence (10), and structure (11) that binds four calcium ions cooperatively. At present there is considerable controversy concerning the relative strengths of the binding constants for calcium (12), due to the difficulty of ascertaining the binding of an ion with few spectroscopic indicators of complex formation. The terbium(III) ion is similar both electronically and chemically to calcium(II) (13) and is known to bind to calmodulin in a cooperative manner at the four binding sites (14). The first two binding sites, termed the high-affinity binding sites, are known to bind terbium(III) with an extremely high binding constant, well under 1 μM, while the two low-affinity binding sites have binding constants on the order of 2 μM (15).

Calmodulin (from bovine brain) was purchased from Sigma. The lyophilized protein was first dissolved in a 75% glycerol/water solvent (50 mM Tris·HCl buffer, pH 7.5) with 10 mM added Na EDTA to remove any calcium or multivalent ions from the protein. The protein was then dialyzed for 48 hr at 4°C with several solvent changes against 2 liters of 75% glycerol/water to remove the EDTA. Protein concentrations were determined by UV absorbance (ε_{277} = 3.3 $mM^{-1} \cdot cm^{-1}$; ref. 16).

We chose to work with glycerol/water mixtures in order to maintain optical clarity at low temperatures and to allow possible comparison to the work of Frauenfelder and coworkers on protein dynamics. As we will discuss later, there

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are several important questions concerning the use of cryoprotectant solvents with low-temperature glass transition temperatures that this experiment may help answer. Terbium(III) chloride (99.9%, Alpha Products) was freshly dissolved in distilled water at 10 mM concentration and then added to the calmodulin solution to the desired final concentration. The final molar ratio of terbium to calmodulin was 1:1, and the final protein concentration was 100 μM. Since the first two terbium ions bind quantitatively but subsequent binding constants relax to a binding constant of 2 μM, the terbium should be predominantly at either of the tight binding sites.

The terbium/calmodulin solution was placed in a 10 mm × 5 mm quartz cuvette and mounted in a Janis Vari-Temp cryostat (Janis Research, Stoneham, MA). The sample temperature was monitored by both a thermocouple and a silicon diode to an accuracy of 0.5 K. The terbium(III) was excited by a tripled Nd:YAG laser (Cooper LaserSonics, Santa Clara, CA) at 355 nm, with a pulse duration of approximately 10 nsec. At this wavelength the transition between the $^5D_4$ and $^7F_6$ levels is not made directly, but instead excitation causes transition to higher level states, which then cascade down to the $^5D_4$ lowest excited state. The subsequent emission was collected by a f/3 lens and focused onto an f/3 grating monochromator (model H-10, Instruments SA, Metuchen, NJ) set at 540 nm with a 10-nm bandpass. A photomultiplier (model 928, Hamamatsu, Bridgewater, NJ) served as the emission detector. Because there was a large scattered light artifact with the pulsed laser, it was necessary to gate the photomultiplier off during the excitation pulse (17).

Although the gated photomultiplier has a time response of about 0.5 μsec, it was necessary at low temperatures (<220 K) to reject about 20 μsec of signal due to long-lived emission from the dewar windows, in particular the epoxy cement used to seal the windows to the metal body. The laser was run at 10 Hz and data were digitized by a Biomation 6500 transient recorder and averaged on a computer. Each trace in the data represents 256 laser shots. Computer fits were done by a steepest-descent least-squares algorithm.

RESULTS

Three basic sets of experiments were done. The control set of experiments measured the temperature dependence of free terbium dissolved in the solvent. The second set involved measurements on slowly cooled samples of terbium–calmodulin in solutions, and the third set also used terbium–calmodulin, but at two widely separated temperatures with maximal cooling rate excursion between them. Table 1 summarizes the results.

Fig. 1 shows the time-resolved emission decay of the slowly cooled terbium–calmodulin at several different temperatures. The sample was slowly cooled at a rate of 0.1 K/sec between measurement temperatures and allowed to equilibrate for approximately 5 min at the desired temperature before data were taken. A considerably larger number of temperatures was measured than is shown in the figure. Table 1 lists the results of all temperatures studied. Fig. 2 combines the results of the control and quenched experiments. The quenching was accomplished by immersing a sample at room temperature into a pool of liquid nitrogen in order to cool the sample as quickly as possible. We estimate that the cooling period in this case was approximately 20 sec (300 K to 77 K). In the control experiments the same concentration of terbium(III) as in the protein experiments was dissolved in 75% glycerol/water and quenched.

Table 1. Distribution parameters

System	Temperature, K	σ, Å	R_0, Å
Terbium/glycerol	293	0.004 ± 0.0015	2.2 ± 0.2
Terbium/glycerol (fast cooling)	100	0.03 ± 0.01	2.2 ± 0.2
Terbium/calmodulin	293	0.005 ± 0.001	2.2 ± 0.2
	240	0.004 ± 0.001	2.2 ± 0.2
	220	0.005 ± 0.001	2.2 ± 0.2
	200	0.065 ± 0.01	2.2 ± 0.2
	190	0.106 ± 0.01	2.2 ± 0.2
	180	0.106 ± 0.01	2.2 ± 0.2
	160	0.170 ± 0.01	2.2 ± 0.2
	140	0.150 ± 0.01	2.2 ± 0.2
Terbium/calmodulin (fast cooling)	120	0.36 ± 0.01	2.0 ± 0.2

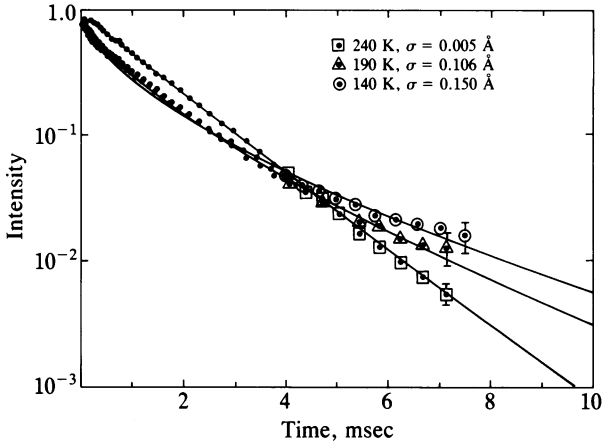

FIG. 1. Semilogarithmic plot of the intensity of terbium(III) luminescence vs. time at three different temperatures (240 K, 190 K, and 140 K). Error bars on the longest time points are representative of error bars for all points. Computer fits to the luminescence decay using a gaussian disorder distribution are also shown (solid curves). The corresponding disorder widths (σ) are given.

Although the protein-free terbium emission remained an adequate fit to a single exponential over all temperatures studied, we observed that the terbium–calmodulin system undergoes a rather abrupt crossover from exponential to nonexponential decay as the temperature is lowered below approximately 210 K. The rapidly cooled terbium–calmodulin exhibited considerably more nonexponential behavior at low temperatures than did the slowly cooled sample. It is well

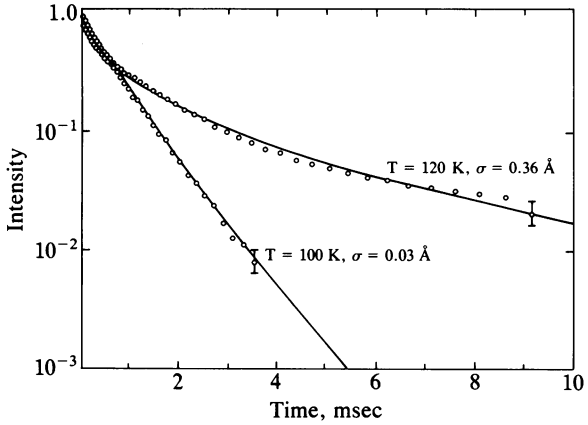

FIG. 2. Semilogarithmic plot of the terbium(III) emission for the free ion in a quench-cooled 75% glycerol/water solution (100 K) and a quench-cooled terbium/calmodulin sample, as described in the text. Error bars shown are representative of error bars for all points. Gaussian fits to the data are also shown (curves) with corresponding disorder widths (σ).

known that a 75% glycerol/water solvent undergoes a liquid→glass phase transition in the vicinity of 200 K (18). It is considerably more controversial as to whether the protein itself can undergo a phase transition from a glass-like object to a flexible object over a narrow temperature range independent of the solvent.

THEORY AND DISCUSSION

We propose a model that quantitatively explains the observed time dependence of the luminescence decay and which incorporates recently proposed ideas of compact proteins as glassy systems.

Our first task is to understand quantitatively the terbium-emission lifetime and how it is affected by the environment. The emitted radiation is known to be electric dipole radiation (19) in spite of the 4f-initial and final states, which should make electric dipole radiation forbidden. Electric dipole transitions do in fact dominate, since the local electric fields from the ionic surroundings can mix even- and odd-parity atomic states, resulting in a "forced" electric dipole transition. The rate of this transition is proportional to the square of the amplitude of the admixture of opposite parity states.

The high-affinity binding site for terbium probably contains three carboxylate amino acids (aspartic or glutamic acid) that act as coordination sites for the ion (11, 20, 21) and which also provide the local field mentioned above. We assume that at high temperatures the protein passes through many conformational substates during the excited-state lifetime of the terbium ion, resulting in an effectively time-averaged field seen by the ion and hence a single lifetime in spite of the heterogeneity of the environment. At low temperatures, however, we believe that the protein has been frozen like a glass into a distribution of configurational states. There is both static and dynamical evidence for this model. Dynamical evidence comes mainly from the recombination kinetics experiments already cited (2) and Mössbauer experiments (22, 23), whereas static evidence comes from specific heat measurements at low temperatures (24, *), from the anomalous line widths of the heme absorption lines as a function of temperature (25, 36), from low-temperature x-ray measurements (26), and from the line widths of rare earth transition metals bound to proteins (27). These experiments led to a theoretical model using ideas from spin glass theory to understand the conformational substructure of the protein (28). We therefore expect that below approximately 210 K in our glycerol/water solvent the surrounding charges are effectively frozen during a typical emission lifetime into fixed positions that vary from protein to protein, resulting in a distribution of emission lifetimes.

The calculation of the emission lifetime for a fixed external electric multipole field follows the procedure outlined by Judd (29) and by Ofelt (30). The time dependence of an atomic excited state with δ-function excitation is (31)

$$I(t) = I(0)\exp(-te^2\omega_{if}^2 F_{if}/6\pi\varepsilon_0 m_e c^3), \quad [1]$$

where $I(t)$ is the intensity of the emitted light from a large ensemble of the rare earth ions; ω_{if} is the angular frequency of the transition between the initial state i and the final state f; F_{if} is the oscillator strength of the transition, a dimensionless number less than or equal to one; e is the electron charge; m_e is the mass of the electron, c is the speed of light, and ε_0 is $8.85 \times 10^{-12}\,C^2{\cdot}N^{-1}{\cdot}m^{-2}$. A quantum mechanical electronic transition has a strength relative to that of the classical atom given by the oscillator strength

$$F_{if} = \sum_{l,m} [2\varepsilon m_e \omega_{if}/3h]\langle i|D_m^l|f\rangle^2, \quad [2]$$

where ε is the dielectric constant of the medium, h is Planck's constant, $\langle i|$ and $|f\rangle$ are the initial and final states, and D_m^l are the electric l-pole operators:

$$D_m^l = R^l C_m^l(\theta,\phi), \quad [3]$$

where R is the distance of the charge distribution center from the origin, m is the azimuthal angular momentum number, and C_m^l are normalized spherical harmonics (l corresponds to the total angular momentum). In the absence of admixtures of even- and odd-parity states the electric dipole oscillator strength (that is, $l = 1$) in Eq. **2** is zero for 4f→4f transitions. The fact that atoms like terbium undergo electric dipole transitions is believed to be due not to higher order multipole emission (that is, use of $l > 1$ in Eq. **2**) but rather from admixtures of mixed-parity eigenstates due to the local crystal field. The local potential V caused by the ionic environment can similarly be expanded via multipole operators:

$$V = \sum_j \sum_{l,m} A(j)_{l,m} D(j)_m^l \quad [4]$$

where $D(j)_m^l$ and $A(j)_{l,m}$ are, respectively, the l-pole operator and the expansion coefficients for the jth atom centered at R_j in the vicinity of the terbium ion. The $A(j)_{l,m}$ coefficients then appear in the usual way in the first-order perturbative correction to the f-level wavefunction.

A number of approximations are needed to simplify the calculation; for technical details the reader is referred to Judd (29) or Ofelt (30). Reasonable assumptions include the following: we assume that the effective dielectric constant caused by all but the nearest neighbors is constant in time, and we neglect possible closed-shell excitations and splittings within the excited multiplet (which are small compared to the energy differences between the ground and excited states). A more subtle point is the assumption that the coefficients in the expansion of the potential are *time-independent*; that is, we neglect vibrational mode couplings, which can give rise to time dependence in the oscillator strength. At very high temperatures we assume, as stated earlier, that the protein structure averages rapidly over the different conformational states, whereas at low temperatures (in the glassy state) we take the coefficients to be time-independent. The intermediate region poses an interesting theoretical problem.

Within our present assumptions the oscillator strength can be shown to scale as

$$F_{if} \simeq B_{if} \sum_{j,l} R_j^{-(l-1)}, \quad [5]$$

where the sum is over the configuration of multipoles labeled by the subscript j at a distance R_j from the rare earth ion and with multipole moments of order l. Since we must connect 4f (angular momentum $l = 3$) states in the initial and final configurations the only nonvanishing contributions to F_{if} in the initial and final states are given by $l = 1, 3, 5, 7$ [this results from the properties of the $3 - j$ symbols used in the full wavefunction expansion (29)]. Furthermore, the $l = 1$ term corresponds to a finite monopole field at the ion position, which would imply that the terbium is not in an equilibrium position. Thus, the $l = 1$ term must be discarded. If we make the further simplifying assumption of a single multipole at a distance R from the terbium ion, then the decay rate Γ_{if} will scale as:

$$\Gamma_{if}(R) = \frac{A}{R^8} + \frac{B}{R^{12}} + \frac{C}{R^{16}}, \quad [6]$$

*Gol'danskii, V. I., Proceedings of the Eighth International Biophysical Congress, July 29–August 4, 1984, Bristol, U.K.

where A, B, and C are constants containing the detailed atomic parameters of the problem. For simplicity, we retain only the lowest order (in R) term; retaining higher order terms in the computer fits leads to no significant improvements.

If the environment rapidly averages over all possible conformational substates, then we would expect to observe a simple exponential decay for excited terbium ions. However, as mentioned in the Introduction, a protein not only can exist in a large distribution of conformational substates but also is probably "frozen" into a glassy positional distribution at low temperatures. A given conformational substate is now interpreted as that substate which has the value R of the mean symmetry position of the surrounding multipole. Further, we take the distribution in R to be gaussian:

$$P(R) = \frac{\exp[-(R - R_0)^2/2\sigma^2]}{\sigma(2\pi)^{1/2}}, \qquad [7]$$

where $P(R)$ is the probability for finding a multipole at position R, the mean position is R_0, and the width of the distribution is σ. The choice of a gaussian distribution is not mandatory, and we found that equally good fits could also be obtained with a "square" distribution centered at R_0 with half-width σ, which are really the only crucial parameters in the theory. Since calmodulin has been crystallized (11) and its detailed structure determined to within 3 Å, it is possible to make a reasonable guess that R_0 is approximately 2 Å. The distribution σ in the distances is obviously dependent on the state of the protein (liquid environment, crystal, frozen glass), its thermal history (quickly cooled or slowly cooled), and the protein itself. We will thus treat σ as a variable, although we point out that according to the work of a number of others (8, 23, 36), the radial static disorder in protein crystals can range from 0.1 to 0.3 Å. One test of our simplified theory, therefore, is that comparison to experiment should yield σ within this range. At high temperatures the protein is fluctuating among substates on a time scale that is fast compared to the natural decay rate $1/\Gamma_0$, so that each terbium ion sees an effective field due to the ions at an average effective distance R_0. At temperatures below 210 K, at least in our particular solvents, the protein is apparently frozen into a distribution of conformational states given by Eq. 7.

A one-parameter fit to Γ with R_0 fixed at 2.0 Å and σ fixed at 10^{-3} Å (that is, an exponential time process) was done for the 290 K terbium–calmodulin data in order to establish the size of the effective temperature-independent matrix elements. The value of A in Eq. 6 so derived, 4.0 $Å^8 \cdot sec^{-1}$, was then held fixed for all low-temperature fits. We then fit the different decay curves as temperature was lowered by allowing R_0 and σ to be variables. In principle, A could also be a variable in the fits, but no significant improvement in χ^2 was obtained. This is important, because A depends on microscopic details independent of temperature.

CONCLUSIONS

Table 1 presents the results of the low-temperature fits. Notice that the derived values for the width of the conformational distribution σ at temperatures well below what appears to be a rather sharp transition are in good agreement with the x-ray crystallography data of static disorder in proteins. Also, the quickly cooled sample has a significantly wider distribution for the disorder width than the slowly cooled sample, indicating that some amount of annealing is possible in the structure of the protein as it is cooled.

There is further information that can be extracted from these data—namely, the temperature dependence of the conformational relaxation rate. If the ions surrounding the terbium move at a rate much slower than the mean decay rate of the terbium, then the emission decay will be nonexponential. If, on the other hand, the rate of conformational motion is much faster than the mean terbium decay rate, then only the mean decay rate will be observed. These same ideas have been discussed in another context by Austin *et al.* (2), and in fact the similarity between the results is striking despite the dissimilarity between the two techniques. At high temperatures we therefore expect that the decay should be fit by a narrow σ, but the same values of R_0 and A should be common to fits at all temperatures. The data in Table 1 and Fig. 3 confirm this expectation.

It is intriguing that the distribution measured by σ changes rather suddenly over a narrow temperature range. Several important possibilities need to be considered in interpreting this phenomenon. First, the solvent used in this experiment, glycerol/water, has a liquid→glass phase transition at 190 K. Clearly, it is entirely possible that the glass transition of the solvent drives the glass transition of the protein, if the two systems are strongly coupled. In fact, two groups of researchers (6, 32) have argued that the adsorbed water on the protein surface is the driving element in dynamical transitions in proteins. The reason that one must restrict the argument to adsorbed water is that the dynamical transitions for proteins in pure ice seem to occur at the same temperature as the glycerol/water transitions, so that the macroscopic solvent alone cannot be the sole controlling factor.

Independent of the source of the glass transition, including the possibility that the transition may be internal and local, the similarity of our solvent system with the solvent used by Frauenfelder and coworkers (2, 3) allows us to compare the relaxation rates of calmodulin with myoglobin. As we expect for any glassy system, the temperature at which relaxation is seen to occur is a function of both the characteristic time of the measurement and the characteristic size of the molecular group that the technique actually measures. In our case, individual atomic movements near the terbium ion are responsible for relaxation, as is also true for carbon monoxide recombination by movement in the vicinity of the heme group in hemoglobin (33).

Our characteristic time is the lifetime of the terbium state in the protein pocket, 1.3 msec. Chemical recombination processes, especially at low temperatures, have a rather ill-defined characteristic time window. We can choose, on the basis of the present interpretations of the low-temperature recombination events, the time at which the geminate kinetics turn over from a power law to an exponential as the time

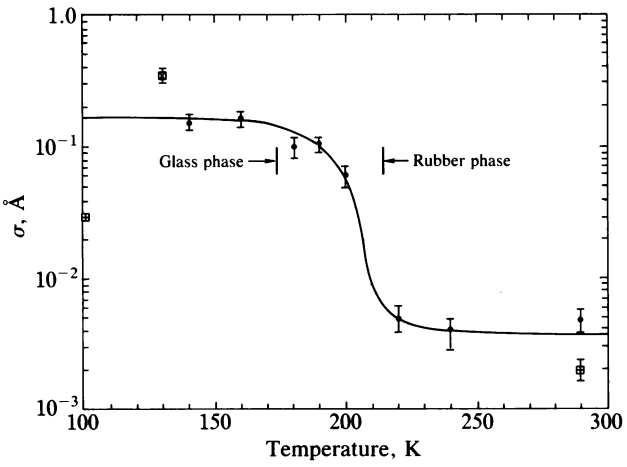

FIG. 3. Semilogarithmic plot of the disorder width σ (assuming a gaussian distribution) vs. temperature for a slowly cooled terbium/calmodulin sample (●), terbium in glycerol (⊞), and a rapidly cooled terbium/calmodulin sample (⊠). The solid curve is present to guide the eye and does not have a theoretical basis. The glass-like region of the protein is labeled on the low-temperature side, and the rubber phase is labeled on the high-temperature side.

at which structural microfluctuations occur. Visual inspection of the data in the paper by Austin *et al.* (2) indicates that crossover from geminate power law to geminate exponential times comparable to the terbium lifetime of 1 msec occurs at a temperature of approximately 215 K in myoglobin, very close to our observed temperature of 210 K for change from nonexponential to exponential luminescence decay. Thus, although the proteins and the techniques are quite different in the two experiments, the energy-barrier distribution between conformational substates may be similar.

Finally, as we will discuss at length in a future paper, we would like to introduce what we believe is a new concept to globular proteins, but an old idea in polymer chemistry. We believe that the phase transition seen at approximately 210 K is analogous to a glass→rubber phase transition seen in crosslinked polymers (34, 35). A glass is a frozen disordered solid on experimental timescales, whereas a rubber is a crosslinked polymer in which rotational motions of the polymer backbone, but not translational motion, are allowed. It should also be noted that since a typical globular protein contains a substantial amount of water, the protein may perhaps be better characterized as a gel rather than a rubber-like material. "Rubber" may be an inelegant expression, but it might well describe the physics of the macromolecule. It is also possible that the glass→rubber phase transition in biomolecules, if it exists, is actually relatively independent of the solvent and not driven by the bound water. An obvious extrapolation of our experiment is to measure the terbium glass→rubber phase-transition temperatures in a variety of solvents to establish the degree of solvent independence.

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