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Of spherical cows, cloudy crystal balls, and proteins

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Abstract

Although proteins perform a vast multitude of tasks in living organisms, perhaps the most fascinating and least well understood is the nanoengine aspect of protein action, where chemical energy is turned into mechanical motion. In order for this to happen a protein must change chemical bond energy into physical displacement via some sort of a conformational change of the protein. The critical first step of this process must be the transient storage (self-trapping) of chemical energy into some metastable strained conformation of the protein. We discuss how the early work of Irwin C. "Gunny" Gunsalus with Hans Frauenfelder and Peter Debrunner led to insights into the picosecond dynamics of proteins, the initial functionally important motions.

It is a great treat to write a paper in honor of Irwin C. "Gunny" Gunsalus. We first meet Gunny when we started out as graduate students in Hans Frauenfelder's and Peter Debrunner's budding groups in biological physics at the University of Illinois. Outside of Hans and Peter (who were just a bit ahead of us), we graduate students knew of course nothing about biology or biochemistry, so Gunny was our one friendly source of guidance into a new world. Of course, we use the word "friendly" with some care, because Gunny was also known to not suffer fools gladly, make a stupid statement, or be lazy on a Saturday morning and you could easily find your head rolling down the hallway. We think we avoided that fate with Gunny, but not RHA with Hans. RHA's head is still rolling, and rightly so.

Gunny decided that he wanted to work with physicists, or he was drafted into working with us. We are not sure about the details. The problem with physicists is that they like to boil all of the phenomenology of Nature into a few basic "laws" from which everything else can be derived. One of the great pleasures in freshman physics mechanics is to start any problem by saying, "Well, $\vec{F} = d\vec{p}/dt$," and proceed (hopefully) to use this actually rather sophisticated vector equation (it isn't as trivial as you think!) to solve any freshman mechanics problem. This is the origin of the really old joke which we unfortunately will repeat here: A farmer from Gibson City comes to Urbana to find out why his cows aren't giving milk. He goes to Loomis Lab because he hears that physicists are smarter than biologists and asks a theorist there, maybe David Pines, if he can help the problem of his cows not giving milk. The theorist thinks for awhile, runs to the blackboard, and sketches a circle and says: "Consider a spherical cow." Like any joke there is always an element of truth in the humor. It *is* true that we like to consider spherical cows, in the sense that we like to find overriding general principles. Of course we are sure physicists are no different than other scientists in that regard; all scientists want to find general principles of importance. What makes a physicist a little different from a biologist is that there is (usually) a mathematical formalism in which the laws are couched that allows us, *without experiments*, to make quantitative predictions about what future experiments should reveal. If the future experiments fail to confirm the predictions, the theory has been falsified. So while we posit spherical cows, our cows can be popped and exploded.

When we started working with Gunny, we were of course encouraged to work on Gunny's favorite protein, cytochrome P450 [1–3]. Physicists like heme proteins because they are colored and you can see them, even better if they are not fully coordinated and can bind carbon monoxide, a ligand which can usually be easily dissociated with a photon. This had the huge advantage

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for us that it was possible to start a reaction in femptoseconds and follow the progress versus time, hard to do normally in biochemistry. We started doing some low-temperature photolysis experiments to complement the Mössbauer work being done at the time by Hans and Peter, figuring that we would knock off the ligand at low temperatures, it would stay off, and we would warm things up until the recombination began. To our great surprise this is *not* what happened; instead the recombination continued at cryogenic temperatures and in a very nonexponential way. *I* remember Hans saying that either we had found a dead cat or something really wonderful had been discovered. It turned out to be the latter. The whole picture of energy landscapes of proteins emerged from that work, and Gunny's name is attached to those early and important experiments [4–8].

So, having grandly flourished $\vec{F} = d\vec{p}/dt$, we want to talk about another general "law" of condensed matter physics which is intimately connected with Gunny's original work with Hans Frauenfelder and Peter Debrunner on the Mössbauer effect in cytochrome P450 proteins [9], and connects up with some high-tech picosecond pump-probe mid-infrared experiments RHA has been doing lately with Professor Aihua Xie (also a Frauenfelder alumnus, who unfortunately didn't get a chance to overlap with Gunny) and Dr. Lex van der Meer. We intend here to fully be in the spherical cow mode by the way, in the hopes of finding something general about the work Gunny did with The Physicists on how enzymes can make structural transitions from inactive to active conformations. Hans has coined the words "functionally important motions" to describe the large-scale conformation changes in a protein which are critical for enzyme action.

The Debye–Waller factor as a unifying concept in Mössbauer Effect, X-ray scattering and infrared spectroscopy

The Mössbauer effect is the emission of a *monenergetic* gamma ray from the excited state of a nucleus. Ordinarily when a gamma ray is emitted from the nucleus of an atom covalently bonded in a protein, such as Fe^{57}, there is a wide spread in the energy of the emitted gamma ray because the recoiling nucleus can excite many different collective modes in the lattice that the Fe nucleus is embedded in. This doesn't happen in the Mössbauer effect. A certain fraction of the gamma rays come out with an incredibly small (on the order of 10^{-22}) energy spread of the mean energy E_o. The collective modes which normally carry away the recoil energy and linear momentum of the nucleus are called "phonons" in condensed matter lingo [11]. Gunny, Hans, and Peter were pioneers in the exploitation of the amazingly high spectral resolution of the Mössbauer effect to probe the local covalent environment of the iron nucleus in cytochrome P450, an extremely important enzyme responsible for drug detoxification in the liver [8]. But we don't want to talk about this important work today; the subject is spherical cows, cloudy crystal balls, and large-scale dynamics in proteins.

The word "lattice" is used here carelessly. Normally, in the solids that condensed matter physicists talk about a lattice is a highly regular repeating 3-D array of atoms which stretch off to infinity in elegant repetition, that is, a crystal [11]. But, Gunny, Hans, and Peter were working on proteins and they *don't* look like a highly repeating array of atoms. A protein macromolecule forms a connected lattice of quite definite structure, if a highly complex lattice, and these complex lattices surely have collective modes. We implied in the title to this paper that a protein macromolecule looks like a cloudy crystal ball, the cloudy implying that the crystal of the condensed matter physicist is actually full of what they call defects but what a biologist would call secondary and tertiary structures. The spherical cow approach to handling the problem of all these interacting atoms in a protein is to reduce the problem to a crystalline, harmonic lattice with the atoms connected to one another by harmonic springs...a crystal of sodium chloride springs to mind, with two masses M_{Na} and M_{Cl}. When you do the rather subtle calculation of the allowed vibrational modes of such a "protein," you find that the vibrations separate into two distinct bands: the optical phonon branch in which the adjacent atoms beat against each other and the acoustic branch where the adjacent atoms move with each other. The names "acoustic" and "optical" arise from the fact that when the atoms beat against each other the transition moment for optical (infrared actually) absorption is large, while the motion when atoms move with each other is like a sound wave. The wavevector $\vec{\mathbf{K}}$ is a critical concept here and represents 1/length over which the displacements of the atoms $\vec{\mathbf{u}}$ from their equilibrium repeat themselves in the direction $\vec{\mathbf{n}}$: $\vec{\mathbf{K}} = \frac{2\pi}{\lambda}\vec{\mathbf{n}}$. Note that at $\vec{\mathbf{K}} = 0$ the wavelengths of the phonons are infinite; this is the special zero-phonon which couples all of the atoms coherently together as one mass motion. Note also that the low-energy acoustic modes which represent atoms moving collectively in the same direction as their neighbors are split from the optical modes. The optical modes represent atoms moving in the direction opposite of their nearest neighbors, which results in the bizarre fact that at $\vec{\mathbf{K}}_{\mathrm{optical}} = 0$ the energy of the optical phonon is at a maximum while the energy of the acoustic phonon is at a minimum! Very strange.

There is an important number which connects up these phonons with the rigidity of the lattice, and it connects as we will see in a little bit to how rigid a protein is *in its core*. As the acoustic phonons increase in energy, at some point the wavelength λ_{D} becomes less

than the spacing between the atoms; the wave no longer can propagate through the lattice but instead exponentially dies. This cutoff in modes can be expressed as an energy by multiplying the angular frequency of the highest possible phonon ω_D by $\hbar$, and turned into a temperature called the Debye temperature T_D by dividing by Boltzmann's constant k:

$$T_D = \frac{\hbar\omega_D}{k} = \frac{\hbar v}{k}[6\pi^2\rho]^{1/3}, \tag{1}$$

where v is the speed of sound in the protein and ρ is the density of atoms in the protein. Before you reach this critical cutoff, the density of the phonon acoustic modes $D(\omega)$ goes simply for a spherical cow or a spherical protein as $\sim \frac{\rho\omega^2}{2\pi^2 v^3}$. We'll need to know that the average number of phonons $\langle n(\omega)\rangle$ of frequency ω at temperature T is given by the Planck distribution:

$$\langle n(\omega)\rangle = \frac{1}{\exp[\hbar\omega/k_B T] - 1}. \tag{2}$$

The connection of these phonon collective modes with the Mössbauer effect, with which Gunny first started to work with Hans and Peter, comes from the fact that the Mössbauer effect can be observed only if the gamma ray emitting atom recoils as if the recoiling mass is not that of the Fe^{57} nucleus but rather the *entire* mass of the lattice. In the words of condensed matter physics, the wavelength of the excited phonon which carries away momentum is infinite, or the wave vector $K = \frac{2\pi}{\lambda} = 0$. This is known as a zero-phonon transition, or within the Mössbauer community a recoil-less transition, since although the momentum of the recoil must always be equal and opposite to the gamma ray momentum, the energy carried away scales inversely with the mass of the object; hence a zero-phonon carries away zero energy, leaving all the energy in the monochromatic gamma ray. The mere fact that you can even see the Mössbauer effect in a protein tells you that zero-energy phonons exist in a protein and that large-scale collective modes exist. Of course, it isn't very surprising or magical that you can observe the Mössbauer effect in a protein, since the Mössbauer effect can be observed in any solid, crystalline, or glass or protein [10].

There is also a connection between the recoil-less fraction in a Mössbauer transition and the intensity of X-ray spots in an X-ray diffraction pattern. Of course, X-ray crystallography remains the premier way to determine at the angstrom level of resolution protein structures. When an X-ray (a photon) scatters from an atom, the change in momentum of the scattered photon must be carried away by phonons in the lattice. If a zero-phonon is excited, no energy is lost and the X-ray elastically scatters off the lattice. If finite wavelength phonons are excited, then the X-ray loses energy and does not coherently scatter off the lattice, resulting in a decrease in the intensity of the diffraction spot on the detector. The amount by which the intensity of the diffracting spot is lost is called the Debye–Waller factor **W** [12]:

$$I(\mathbf{W_D}) = I(T=0)\exp[-2\mathbf{W_D}] = \langle \exp[i\vec{G}\cdot\vec{u}_j]\rangle^2, \tag{3}$$

where $I(T=0)$ represents the scattering intensity at temperature $T=0$, $\vec{G}$ represents the change in wave-vector of the scattered X-ray, $\vec{u}_j$ is the sum over change in the atomic coordinates in the lattice with thermal excitation, and the angle brackets $\langle\cdots\rangle$ represent a sum over the thermally excited phonons. In the case of a zero-phonon scattering event, $\overrightarrow{u_j} = 0$ since the entire lattice recoils and the displacement of the atoms is effectively zero. Such an elastically scattered photon is directly connected to the Mössbauer effect, since you only see a recoil-less event when an acoustic phonon of infinite wavelength representing no local atomic displacement is excited. In the case of the Mössbauer effect, the recoil-less fraction f is given by [13]

$$f = |\langle\psi_o|\exp[-i\kappa\cdot\vec{r}]|\psi_o\rangle|_T^2, \tag{4}$$

where ψ_o is the initial wavefunction of the solid, κ is the wave vector of the emitted gamma ray, and $\vec{r}$ is the position of the nucleus and the average is over all thermally accessible states at temperature T. Eqs. (4) and (3) should look awfully similar. In fact, "it can be shown" that [14] Eq. (4) can be written as

$$f \sim \exp[-\kappa^2\langle x_{vib}^2\rangle], \tag{5}$$

where $\langle x_{vib}^2\rangle$ is the thermal average over all the vibrational excursions of the nucleus. X-ray looks at all the vibrational excursions, Mössbauer just at the excited nucleus. Doing the thermal average over all the allowed modes in either case (this can be approximated using the expression for the Debye acoustic modes we gave in Eq. (2)) is actually a nasty business since the resulting integrals can't be done analytically, even for the spherical cows we are considering. See below.

Finally, to tie things up for the next section where we actually discuss experiments we need to return to the optical absorption of phonons in the infrared where the acoustic and optical bands we first mentioned. To make the connection between IR modes and the Debye–Waller factor we suggest reading the book *Physics of Color Centers* [15], which has some nice articles by some great Urbana physicists: Miles Klein and Charles Slichter. A color center is a defect in a lattice, which interacts with the phonons of the lattice, which perturbs the energy levels of the defect. Normally the defect is deep enough that its excitation levels are in the visible, hence the name color center. But we can equally view the defect as an amino acid in a protein macromolecule, in which case the excitation levels are in the infrared and the effect of the phonons of the "lattice"...the protein itself...is to perturb the IR spectroscopy of the level. The spectroscopic effect we are most interested in again

is the zero-phonon band, the same band that gives rise to the Mössbauer effect and the same band that gives intensity to X-ray diffraction in protein crystallography, all things that Gunny got connected up with when he started working with The Physicists.

Because a color center interacts with a whole spectrum of phonon modes, the effect of the interaction is to broaden the linewidth of the color center transition. However, there is something special that happens at low temperatures; we will define what we mean by "low" in a second. If the lattice is very cold, then the phonons cannot be excited and the spectral line narrows due to loss of higher order phonon excitations, except for one special line, the zero-phonon line. Since it represents a recoil-less transition that costs no energy, it is the sole remaining phonon line that survives at $T = 0$. As the temperature changes, other phonons are activated and the area of the zero-phonon line decreases with increasing temperature. If we use the Debye approximation for the density of the phonons $D(\omega)$ as a function of the frequency of the phonon ω, namely $D(\omega) \sim \omega^2/2\pi^2 v^3$, where v is the speed of sound in the lattice as above, then we can calculate that the integrated intensity of the zero-phonon line, *which once again is the same Debye–Waller factor that we saw in X-ray diffraction and the Mössbauer effect*. The calculation of the intensity of the zero-phonon line intensity is **the same** as the calculation of the recoil-less fraction and is **the same** as the temperature-dependence of a spot in an X-ray diffraction image. In order to calculate any of these temperature-dependent effects, in our spherical cow approximation of isotropic Debye modes, the nasty integral over the Debye continuum of modes using the Planck distribution function has to be done. Actually the integrals cannot be done analytically, but the integral should scale for $T < T_D$ as [15]

$$W_D \sim W_o \exp\left[-\frac{T}{T_D}^2\right]. \quad (6)$$

At temperatures greater than the Debye temperature T_D the Debye–Waller factor simply scales with T since in this "classic" regime we simply have that $\langle x^2 \rangle \sim k_B T/\gamma$, where γ is the effective spring constant of the elastic forces holding the atoms in place. However, if $T_D > T$, this classical approximation is very much wrong. We will see below that probably T_D is greater than 300 for the internal core of a protein.

The hard work is done now and we can look at some results from our lab that probe this unifying view of protein dynamics, and we hope tell us a bit about how proteins function dynamically. Fig. 1 shows what we have right now: a protein as a spherical crystal ball. We tried to make things a little more realistic by shading the shell of the protein to indicate that the part of the protein in contact with the solvent is probably floppy to some extent, but the interior we view as a rigid matrix.

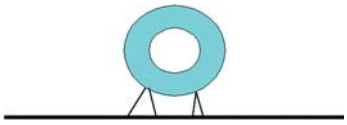

Fig. 1. A spherical cow grazing outside the city limits of Urbana–Champaign, Illinois. The landscape in the background is basically correct to scale.

We drew this cow this way based on the fundamental analysis of the X-ray crystallographic Debye–Waller factors determined by Frauenfelder et al. [16]. Note how the Debye–Waller factors are smallest within the protein core and large at the surface.

The zero-phonon line in the amide I spectrum of myoglobin

It would be nice if proteins were spherical crystal balls, as the analysis so far has assumed. They are **not** crystal balls; if they were, they would be very boring. In fact, many proteins are capable of making dramatic conformational changes from one basic conformation to another one, and these functionally important motions are critical to protein motion. We won't go very far in this brief note in trying to address the physics of these large-scale motions. The first step we will make here is to make the crystal balls cloudy: we'll talk about how the infrared absorption spectrum of a protein can be viewed as something like a color center in a crystal, that is, a chromophore which interacts with the phonon field of the Debye modes. How strongly it interacts remains beyond the scope of this note, but interact it does, as it must.

The fundamental papers on the physics of myoglobin to which Gunny's name is permanently attached [4–8] serve as a reminder of the importance of having a reasonably well-understood or at least well-measured protein into which you try to squeeze your spherical forms in an attempt to understand things. A protein such has myoglobin (Mb) is over 85% α-helix. The α-helix is a very simple and beautiful secondary structural motive in protein crystallography, and for physicists it is extremely interesting because it is a quasi-one-dimensional structure which is held together by the very polar hydrogen bond. This means that the α-helix resembles a solid state object which has some resemblance to the Debye solid we described in the earlier section. As we noted in Fig. 2, the Debye–Waller factors for Mb are relatively small in the center of the protein and along the α-helix stretches, indicating that it is within the core of a protein that our solid-state picture of the vibrational modes of a protein may be valid. At the surface, the validity of the harmonic approximation is gravely doubtful.

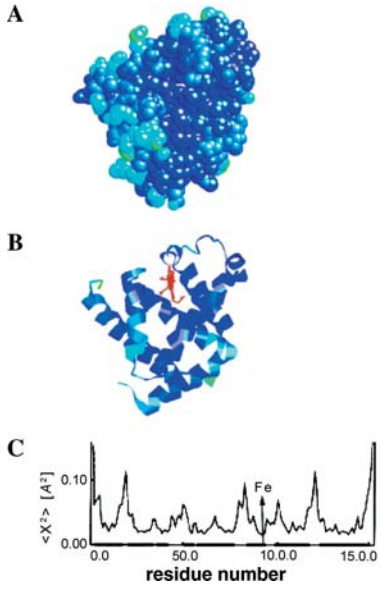

Fig. 2. The thermally averaged vibrational factors $\langle r^2 \rangle$ as determined by the Debye–Waller factors from X-ray crystallography. (A) Color-coded all-atom view of $\langle r^2 \rangle$ Mb; the deeper the blue, the smaller the $\langle r^2 \rangle$. (B) A ribbon picture of MB with color-coded $\langle r^2 \rangle$; the heme is shown in red. (C) A linear view of the $\langle r^2 \rangle$ as a function of residue number. Taken from [16].

We now will concentrate on the infrared absorbance spectrum of Mb, and in particular the so-called amide I band at about 6 μm which is due to a highly delocalized C═O stretching mode of the carboxylic acid radical of the amino acid group. We don't have space to go into why we know that this transition is highly delocalized, which comes out of picosecond nonlinear spectroscopy [17], but the fact that it is gives further credence to the view we are pushing here that we can view the interior of the protein as some sort of a solid. A further test of this idea would be if the amide I band at 6.0 μm would show as a function of temperature the same sort of recoil-less fraction zero-phonon mode that is seen by Mössbauer spectroscopy as we stated above and in the X-ray crystallography.

It is critical in such IR experiments to dissolve the protein in a deuterated solvent because the water H_2–O scissors mode is also at that wavelength and obscures the amide I band at 6.0 μm. It is also interesting to note that when a protein like Mb is placed in a deuterated solvent, not all the hydrogens exchange to deuterons: the inner core of the protein does not exchange its deuterium atoms. This is why there are two amide II bands (the amide II band is due to a "scis-scissoring" N–X motion) in our sample: a band at 6.50 μm due to the N–H mode in the inner core of the protein, and a band at 6.90 μm which is the deuterated N–D modes at the surface of the protein where hydrogen exchange has occurred. The amide I band, which is a C═O stretch, is only weakly coupled to the hydrogens and is relatively sensitive to deuteration. With this warning, we present Fig. 3, which shows the temperature dependence of the IR absorbance spectrum of sperm whale myoglobin as a function of temperature from 5 to 280 K. The bands between 2.5 and 5.5 μm which are due to nonexchanged N–H stretching motions are seen to shift with temperature to shorter wavelengths, as can be easily seen in Fig. 4, which shows the difference spectra of the Mb absorbance as a function of temperature. However, the bands associated with the protein between 6 and 8 μm which are components of the amide I and II bands show not a shifting of the bands with temperature but instead either increased oscillator strength for the amide II bands or the appearance of a new red-shifted band at lower temperatures for the amide I band.

We can then integrate the area of the zero-phonon band between 6.06 and 6.24 μm and try to fit the

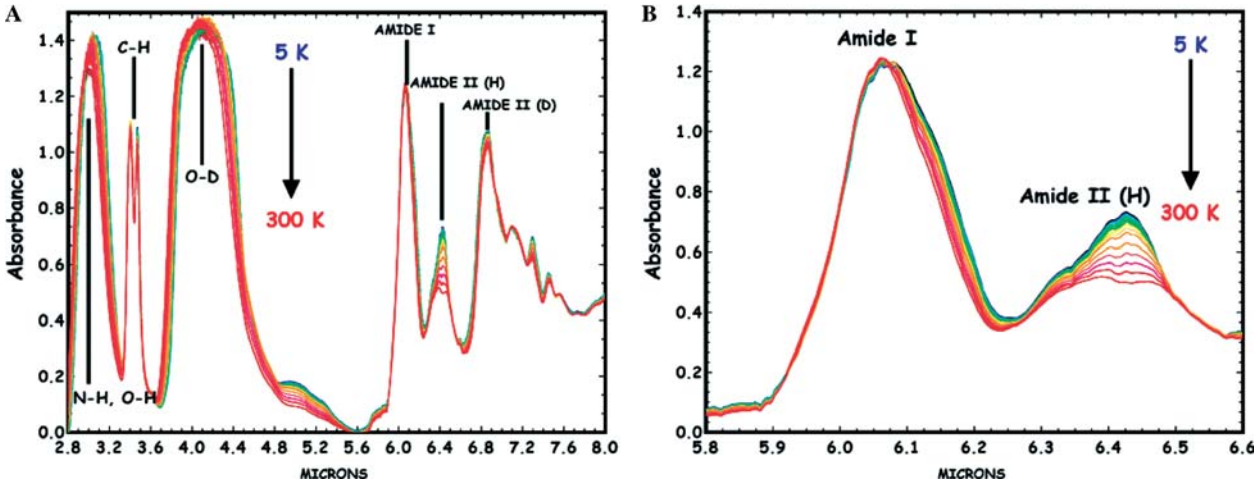

Fig. 3. (A) The infrared absorption spectrum of sperm whale myoglobin as a function of temperature from 5 to 300 K, from 2.8 to 8.4 μm. (B). Enlarged view of the amide I and amide II regions of Mb as a function of temperature.

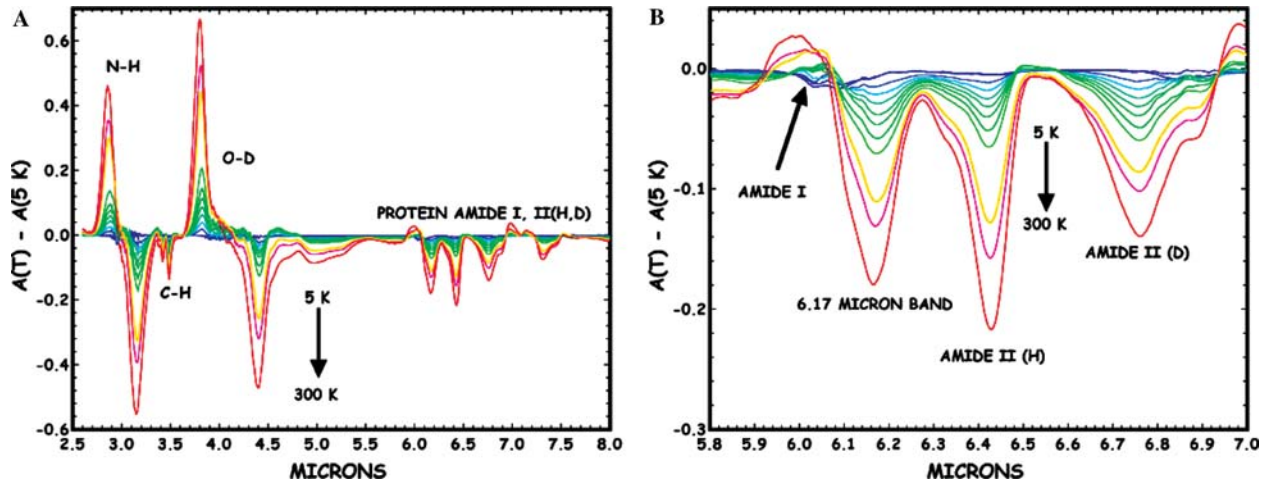

Fig. 4. (A) The infrared difference absorption spectrum of sperm whale myoglobin as a function of temperature from 5 to 300 K, from 2.8 to 8.4 μm. These curves came from Fig. 3 by subtracting the absorbance at temperature T from $T = 5$ K. (B). Enlarged view of the difference of the amide I and amide II regions of Mb as a function of temperature.

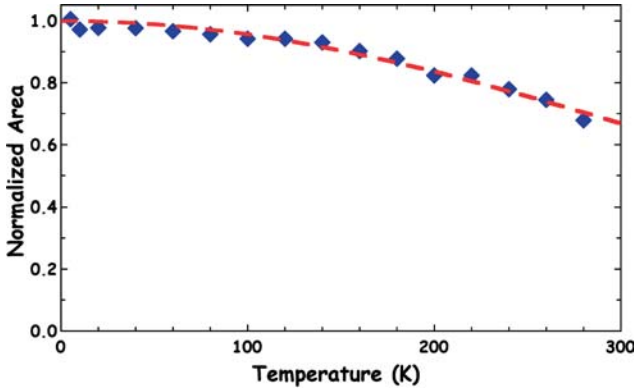

Fig. 5. The integrated absorbance of the zero-phonon band in Mb plotted vs. temperature (solid diamonds). The fit of Eq. (6) to this area is shown as the dashed line; the fit shown has a Debye temperature T_D pf 470 K.

temperature dependence of this area to Eq. (6) to extract the effective Debye–Waller factor of the α-helix core of Mb. Fig. 5 shows the results of this attempt. The best fit of Eq. (6) yields a value for the Debye temperature T_D for the core of the protein, or at least the α-helix section, of 470 K, well above room temperature. This result only gives emphasis to the belief that the interior of a protein is truly rigid at room temperature.

This result should not, ever, give rise to the mistaken belief that because the interior of the protein is rigid it cannot make large conformational changes; there is ample evidence for that. What it does show is that the conformational motions are not the result of some fluid flow, but are due to substantial inputs of free energy and that parts of the protein move as rigid parts...like a nanomachine if you will. Only future experiments, which we hope to do, can elucidate the nanomachine dynamics of these truly fascinating molecules.

Conclusion

We have tried to show how the recoil-less fraction in the Mössbauer effect, a technique that Gunny started with Hans and Peter more than 30 years ago, still has some legs. It is of importance in X-ray crystallography of proteins and in IR spectroscopy. But, much more importantly, it also tells us about the rigidity of the inner core of a protein and gives us some idea about how rapid conformational changes are transmitted to other parts of the protein, conformational changes that are critical for protein function and catalysis as in his favorite, cytochrome P450-camphor. We thank him for being such an inspiration to us all. Actually what is most inspiring is to realize that Gunny was at roughly the age of RHA when he *began* to collaborate with Hans and Peter! There perhaps is still time to do something really great after all, with some luck and a whole lot of hard work.

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