Low-Frequency Protein Motions Coupled to Catalytic Sites

Christopher M. Cheatum

Department of Chemistry, University of Iowa, Iowa City, Iowa 52242

email: christopher-cheatum@uiowa.edu

ORCID: 0000-0003-3881-3667

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Abstract

This review examines low-frequency vibrational modes of proteins and their coupling to enzyme catalytic sites. That protein motions are critical to enzyme function is clear, but the kinds of motions present in proteins and how they are involved in function remain unclear. Several models of enzyme-catalyzed reaction suggest that protein dynamics may be involved in the chemical step of the catalyzed reaction, but the evidence in support of such models is indirect. Spectroscopic studies of low-frequency protein vibrations consistently show that there are underdamped modes of the protein with frequencies in the tens of wavenumbers where overdamped behavior would be expected. Recent studies even show that such underdamped vibrations modulate enzyme active sites. These observations suggest that increasingly sophisticated spectroscopic methods will be able to unravel the link between low-frequency protein vibrations and enzyme function.

Introduction

Enzymes exhibit tremendous catalytic power, and the molecular mechanisms by which they regulate their function and achieve such catalytic power remain a subject of ongoing study. That structure is essential for function has long been the paradigm of biochemistry.(1) It is also clear, however, that the relationship between structure and function is mediated by protein dynamics.(2-14) Motions of proteins across a wide range of time scales can regulate events such as ligand binding, conformational transitions, and even chemical reactivity. Understanding how protein dynamics influence enzyme function remains an unresolved frontier in molecular enzymology with the potential to enable novel approaches to directed evolution, drug discovery, and enzyme engineering.

Proteins exhibit motions at a wide range of time and length scales, from stretching and bending vibrations involving one or two bonds with vibrational periods of 10's to 100's of femtoseconds to the relative motions of whole protein domains with respect to one another at the microsecond to millisecond time scale. As with the overall protein structure, the dynamics are hierarchical, so that the motions at the shortest time and length scales are determined by the organizing structure at longer time and length scales. In addition, because of the many orders of magnitude spanned in the dynamics of a protein, these motions move from being underdamped vibrations for the highest frequency modes to overdamped diffusive motions for the lowest frequency modes (See Sidebar for a discussion of underdamped and overdamped oscillators). Because of the large molecular size and the structural complexity, proteins exhibit many vibrational degrees of freedom, and these modes are dominated by inter-residue interactions forming a rich, coupled network of motions.

It is widely accepted that the three-dimensional folded protein structure of an enzyme is an evolved property that is intimately tied to function. Clearly, that folded structure also determines the interactions between residues that ultimately govern the protein dynamics as well. Thus, the protein motions must also be an evolved property of the protein encoded in the primary sequence. There are many models that suggest a role for protein motions in enzyme catalysis and its regulation. While there is considerable evidence to support these models, much of that evidence is indirect in nature. Spectroscopic methods of many kinds have been used to probe the motions of proteins. Nevertheless, unambiguous experimental evidence for the coupling of low-frequency protein vibrations to the catalytic site remains a frontier problem in enzymology. This review will begin by considering models for the influence that protein motions may have on catalysis along with some of the indirect evidence in support of such models. Next it will consider methods for probing low-frequency protein motions directly and what is known about protein dynamics from such studies. It will then turn to the evidence for coupling of such low-frequency modes to catalytic sites. Finally, we will consider the unresolved questions and future prospects for this research area.

MODELS FOR HOW PROTEIN MOTIONS COULD AFFECT CATALYTIC SITES

That proteins must have dynamic rather than static structures in order to function is self-evident. Clearly, protein motions are involved in ligand binding and release as active-site pockets must open and close to facilitate binding and release. Many proteins undergo substantial structural rearrangements to adopt the reactive conformation, consistent with ideas like the induced-fit model of enzymes. All of these processes involve primarily motions on the microsecond to millisecond time scale or longer. What role, if any, that protein motions at shorter time scales play in facilitating the catalyzed reactions in enzymes, however, remains a subject of considerable debate. It has long been thought that the turnover rates of enzymes being as slow as they are means that the shorter time scale protein motions are fully averaged over on the time scale of the reaction and that averaging over such motions makes them irrelevant. Nevertheless, this averaging is also the process of sampling the distribution of local structures at or near the transition state and is, therefore, essential for reactivity. There is also the possibility that the protein itself can be an explicit part of the reaction coordinate meaning that the low-frequency modes of the protein could become directly coupled to barrier crossing. These ideas are at the heart of the potential role of low-frequency protein motions in catalysis.

Activated Tunneling

The first set of models that we will consider are models for hydrogen transfer reactions, which are among the most common reactions in enzymes and have been widely studied. Though at first controversial, it has been known for many years that the kinetics of hydrogen transfer reactions, whether of proton, hydrogen atom, or hydride, all depend significantly on quantum tunneling of the hydrogen nucleus.(15-18) These reactions are among the most studied, in part, because they are so readily accessible by primary kinetic isotope effects (KIEs) because substitution of the transferring hydrogen with deuterium or tritium causes a large change in mass that leads to a substantial isotope effect, even in the semi-classical limit without a contribution from tunneling. Although it was long expected that tunneling contributions should lead to further inflation of the KIE, it is now understood that many enzymes are in a deep tunneling regime where both deuterium and tritium can also tunnel efficiently meaning that the KIE can be less than would be anticipated based on semi-classical transition-state theory. The conventional transition-state theory model for the reaction that identifies the motion of the hydrogen nucleus between hydrogen donor and hydrogen acceptor as the reaction coordinate proves to be an incomplete model for the reaction that does not account for the most important degrees of freedom. Thus, a variety of models have been developed to describe such reactions in enzymes and go by a variety of names including Marcus-like models, multidimensional tunneling models, full tunneling models, environmentally coupled tunneling models, and activated tunneling models.(19-29) These models, which we will refer to as activated tunneling models, describe essentially the same phenomenology and have proven to be a powerful framework for interpreting temperature dependent KIE experiments in enzymes.

The basic premise of the activated tunneling model for enzyme-catalyzed hydrogen transfer reactions is that the hydrogen motion, itself, is not the reaction coordinate. Rather, the hydrogen motion is characterized by a double-well potential where one well corresponds to the hydrogen bound to the donor, and the other well to the hydrogen bound to the hydrogen acceptor, as shown in the lower panel of Figure 1. The reactant state is one in which the hydrogen-donor well is lower in energy compared to the hydrogen-acceptor well. The converse is true of the product state. The reaction coordinate involves motion of the heavy atoms in the environment, in this case the enzyme, that destabilizes the hydrogen-donor well and stabilizes the hydrogen-acceptor well. This model is phenomenologically similar to Marcus electron transfer theory.(30) Plotting the zero-point energy levels of the double-well potential as a function of motion in the heavy-atom "solvation" coordinate, in this case motion of the enzyme, gives rise to two diabatic potential surfaces. The point at which these surfaces cross is the point at which the two wells in the double-well hydrogen atom potential are degenerate. This crossing point, known as the tunneling ready state, is the effective transition state for the reaction. Mixing of the two diabatic curves gives rise to a single adiabatic minimum free energy curve as a function of the heavy atom coordinate. Using conventional transition state theory as a function of this reaction coordinate, the rate constant for the reaction is,

$$k(T) = \frac{|V|^2}{\hbar}\sqrt{\frac{\pi}{\lambda k_B T}}\, e^{-(\Delta G^\circ + \lambda^2)/4k_B T\lambda} \int_0^\infty P_{Tunn}(m, DAD)\, e^{-E(DAD)/k_B T}\, dDAD$$

where V is the coupling between the two diabatic states, ΔG° is the driving force of the reaction, λ is the reorganization energy in the heavy atom coordinate, $P_{Tunn}(m, DAD)$ is the tunneling probability for a particle of mass m at donor-acceptor distance DAD, and $e^{-E(DAD)/k_B T}$ is the Boltzmann probability distribution of donor-acceptor distances (DADs). This expression has two terms, one that includes everything up to the integral and is essentially the rate of reaching the tunneling ready state, which follows the usual transition-state theory expression for approaching the barrier along the potential in the heavy atom coordinate. The temperature dependence of the rate constant is dominated by this term and is the same independent of the transferring isotope because it involves only motion of the heavy atoms. Unlike in Marcus electron transfer theory, however, the probability for the hydrogen nucleus to tunnel between the wells is not necessarily unity. Instead, it depends on the distance between the hydrogen donor and acceptor, or donor-acceptor distance (DAD), and the height of the barrier between the wells. The thermally averaged tunneling probability is given by the integral of the tunneling probability over the Boltzmann distribution of donor-acceptor distances.

The thermally averaged tunneling probability is the critical feature for the temperature dependence of the KIE. If the DAD distribution is narrow and peaked at short distances, then the tunneling probability for all of the hydrogen isotopes will be comparatively high leading to smaller values for the KIE. In addition, because the DAD distribution is only weakly dependent on temperature as a result of the narrow distribution, then the isotope effect itself is, essentially, temperature independent. In contrast, if there is a broader distribution of DADs, then thermal sampling of that distribution and the corresponding variation in the tunneling probability of the hydrogen isotopes as a function of the DAD gives rise to temperature dependent KIEs. In this way, the temperature dependence of the KIE is, within this framework, indicative of the thermal sampling of the DAD distribution at the tunneling ready state. To the extent that the enzyme has evolved to generate a narrow DAD peaked at short distances, then the KIE will be temperature independent, as is often seen in wild-type enzymes. Mutations of residues that alter the conformational ensemble present at the tunneling ready state resulting in a broader distribution of DADs peaked at longer distances will give rise to temperature dependent KIEs.

There are, by now, many studies that have used the temperature dependence of the KIE as just described to probe the DAD sampling in a number of different enzymes.(9; 12; 31-47) As these studies have been thoroughly reviewed elsewhere, we will focus our discussion on the major themes of this work. First, as already noted, most well-evolved, wild-type enzymes exhibit temperature independent KIEs suggesting that there must be some evolutionary pressure for narrow DAD distributions. Second, active-site mutations of residues that serve to narrow the DAD distribution, typically bulky hydrophobic residues that sterically restrict the relative positioning of the donor and acceptor, lead to temperature dependent KIEs in proportion to the magnitude of the perturbation. Third, mutations of distal residues that participate in coupled networks of motion involved in approaching the transition state and that contribute to determining the conformational ensemble at the tunneling ready state also give rise to temperature dependent KIEs, even though these residues are, in some cases, more than 20 Å away from the active site. Furthermore, making two mutations within such networks results in more than additive effects on the temperature dependence of the KIE suggesting that the effects on the distribution of DADs of the residues are synergistic. Even though these coupled network residues may involve motions at the microsecond to millisecond time scale,(48) they are important in constraining the conformational ensemble of DADs at the tunneling ready state. Thus the thermal motions sampled at the transition state are determined by the properties of these coupled networks that act on longer time scales. Finally, there is growing evidence for the idea that the properties of the tunneling-ready state are an evolved feature of enzymes that catalyze hydride and hydrogen atom transfer reactions, particularly in NAD(P)-dependent enzymes.(36; 49) These observations are particularly intriguing as the chemical step is rarely rate limiting, so it is surprising that there would be significant evolutionary pressure on this step in the catalytic cycle.

In spite of the compelling nature of the KIE results and their support for the activated tunneling model, the main weakness of this work is the fact that the interpretation of the KIE measurements though consistent with the model, is indirect. Though the temperature dependence of the KIEs are interpreted as reflecting the thermal sampling of the DAD distribution, they are not a direct measure of such motions. Furthermore, The KIE temperature dependence reflects the ratio of integrals of the tunneling probabilities for the transfer of different hydrogen isotopes each integrated over the thermal distribution of DADs. Though the integrals are certainly dependent on the distribution of DADs, many different models for the DAD distribution can give rise to similar temperature dependencies for the KIE.(27) Thus, there is no unique way to extract any details of the DAD distribution from the temperature dependence of the KIE itself. Finally, the KIE data could also be consistent with other models of the reaction kinetics. That the KIE results are consistent with and can be interpreted in terms of the phenomenology of the activated tunneling model does not, in itself, show that this model is correct. Clearly, a full characterization of the chemical reaction dynamics for hydrogen transfer reactions in enzymes will require further information about the structure and structural dynamics of conformational sampling at or near the tunneling ready state of the enzyme catalyzed reaction.

Rate Promoting Vibrations

A closely related idea that has been proposed to understand the role of protein dynamics in enzyme-catalyzed reactions is the rate-promoting vibration proposed by Schwartz and coworkers. Originally, this idea arose from studies of the spectral density of enzymes in conformations that sit just on either side of the reaction barrier.(50-52) These spectral densities showed evidence of protein modes with frequencies on the order of 100 cm_{-1} or so that were coupled to the reaction coordinate. This proposal engendered a great deal of controversy as the idea of underdamped oscillations of the protein at such time scales was itself surprising but that such modes should be coupled to the reaction coordinate for the enzyme catalyzed reaction raised questions about the equilibrium nature of the transition state ensemble and the effect that such fast dynamics could have on the free energy barrier for the reaction.

Building on their earlier work, Schwartz and coworkers showed that they could identify similar rate-promotion motions of the protein as part of the reaction coordinate using transition path sampling methods.(4; 5; 53; 54) Transition path sampling is an approach for Monte Carlo sampling of the rare event trajectories that connect reactant and product basins in a complex multidimensional configuration space.(55-57) A critical feature of this approach is that is capable of identifying, in an unbiased way, the locus of points in figuration space such that there is a 50-50 probability of proceeding to ether reactants or products when trajectories start with random velocities. This locus of points is called the separatrix and functions as the multidimensional statistical analog of the transition state. Importantly, by identifying the degrees of freedom both along and orthogonal to the separatrix, it is possible to characterize both the nature of the transition state ensemble and the reaction coordinate for the reaction. Using this approach, they have been able to identify modes of the protein that participate directly in the reaction coordinate. Most significantly, they have made the case that these motions do not just accompany the reaction but, in fact, cause the reaction as a necessary component of the passage from reactants to products.(53)

The studies identifying rate promoting vibrations in enzymes have focused on three main systems, human dihydrofolate reductase, purine nucleoside phosphorylase, and lactate dehydrogenase. Some of the most interesting results of those studies involve efforts to validate the existence and significance of the rate promoting vibrations. One computational study tested the significance of the rate promoting vibration identified in lactate dehydrogenase.(58) They deposited energy into the active site by coupling the nicotinamide ring of the cofactor to a 1000 K Nose-Hoover thermostat. This thermostat continuously injects energy into the active site. They then propagated the dynamics forward in time for 20 ps while they monitored the temperature of all of the residues of the protein as a function of time. Not surprisingly, the residues closest to the active site experienced the largest temperature increase, and the temperature increase fell off monotonically with distance, on average. Interestingly, though, the residues that had previously been identified as participating in the rate promoting vibration consistently exhibited higher temperatures than the other residues at the same distance from the active site. The left panel of Figure 2 shows the protein structure color coded in concentric spherical shells of varying distance from the active site and identifies the specific residues involved in the rate promoting vibration. The right panel of Figure 2 shows the temperature as a function of time for residues that participate in the rate promoting vibration as well as the solvent and the overall protein. While the temperature change is larger for residues closer to the active site, the change is the largest for the residues in the rate promoting vibration. The authors concluded from this work that the energy relaxation is anisotropic and that the rate promoting vibration serves as the preferred relaxation pathway for energy to flow out of the active site and into the bath. They suggest that this result reinforces the centrality of this mode to enzyme function.

In another series of studies Schwartz and his collaborator Vern Schramm have developed the idea of isotope labelling the protein itself as a strategy to shift the timescale of the protein motions somewhat due to the mass modulation.(4; 59-62) They assume that this perturbation affects only the protein mass and not the inter-residue interactions. Although it is possible that the isotopic substitutions could change the strength of the intermolecular forces and perturb the overall electrostatics of the protein, they take care to assess these effects to the extent that it is possible to do so and have found that, for some systems at least, the perturbations to the potential are modest or even negligible and the effect is predominantly a mass effect. They term such heavy enzymes "Born-Oppenheimer" enzymes, and the approach has now been applied to a number of systems.(4; 46; 60; 63; 64) In purine nucleoside phosphorylase, which they studied extensively both experimentally and computationally, they found that the protein mass modulation causes miss timing of the rate promoting vibration leading to a decrease in the probability of finding the transition state. They observe this decrease on the rate of the chemical step even though the observed steady-state kinetic parameters for the enzyme turnover remain unchanged suggesting that, while the chemical step is altered, the slow protein motions that control substrate binding and product release are unchanged. More significantly still, using the transition path sampling methods, Schwartz and co-workers were able to predict a mutation that would recover the function of the heavy enzyme and showed that mutating Phe 159 to Tyr resulted in an enzyme where the heavy exhibited a faster rate constant for the chemical step than did the light enzyme. The experimental corroboration of this prediction offers significant support for the interpretation of the rate promoting vibration.

Much as with the activated tunneling models, however, the main weakness of the work suggesting that a rate promoting vibration participates in the reaction coordinate for the catalyzed chemical reaction in some enzymes is that the experimental evidence in support of this model is indirect. There are, to date, no direct experimental measurements of such vibrational modes in the active site of an enzyme. That being said, the extensive computational studies have resulted in predictions for the behavior of such systems that may be tested experimentally. In some cases, such as the heavy enzyme work, those predictions have been tested by enzyme kinetic studies and proven to be supportive of the predicted behavior. In other cases, particularly the prediction of anisotropic energy flow along the rate promoting mode, there are ready opportunities to test the computational predictions spectroscopically and such experiments would go a long way towards providing more direct evidence for this model.

EXPERIMENTAL APPROACHES TO OBSERVING LOW-FREQUENCY MOTIONS IN PROTEINS

The first step in understanding the role of low-frequency protein motions in the catalytic function of enzymes is to characterize the motions themselves. Already this task is a significant experimental challenge as there are many low-frequency modes of the protein, and they are often significantly overdamped, which will make spectra appear broad and featureless in most cases. Nevertheless, the significance of these motions to enzyme function is such that many efforts have been made to develop tools to probe low-frequency protein dynamics. To be precise, we will focus our discussion on motions with frequencies in the range from 1-100 cm^{-1} or 0.03-3 THz. These frequencies correspond to underdamped vibrational periods of >30 ps down to 300 fs. Motions at these time scales fall into an interesting range where the damping crosses over from being fully overdamped at the lowest frequencies to being fully underdamped at the highest of these frequencies.

NMR spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is among the most powerful and widely used methods for probing protein dynamics at picosecond to nanosecond time scales.(7; 65-69) Thus it is necessary to discuss this method, though it will not be a major focus NMR does not directly probe the low-frequency vibrational motions of proteins. Rather than probing the motions of the protein and their timescales directly, NMR uses the time scales for reorientational relaxation of individual nuclei with respect to the external magnetic field to characterize the local fluctuations of the bond vector. Of course, overall tumbling of the protein molecule also makes a substantial contribution to the relaxation, limiting the molecular size of the proteins to which these methods are applicable. To separate tumbling from reorientation due to protein dynamics, Lipari and Szabo proposed an approach known as the "model-free" formalism to extract an orientational order parameter, S^2, and a characteristic reorientation time, τ_e, for the bond vector of each NMR-active nucleus.(70; 71) The order parameter is bounded between 0 and 1 and reflects the spatial restriction of the motion. An order parameter of 1 means that the bond vector undergoes no orientational relaxation, and an order parameter of 0 means that the bond vector is undergoes complete orientational diffusion. The time constant, τ_e, is the time constant for the reorientational diffusion and typically takes a value between 0 and 100 ps though it can extend to a few nanoseconds.

The real power of NMR spectroscopy lies in the fact that this approach yields residue-specific information about the dynamics. So, for example, the most common experiment involves ^{15}N labeling of the amide backbone, which then reports on the ^{15}N-H bond vector reorientation. Another powerful tool is to label side-chain methyl groups with ^{13}C and one or two ^{2}H atoms, then the ^{2}H relaxation is encoded in the ^{13}C-^{1}H signal and reports on the dynamics of the side chains. Not surprisingly, the order parameters of the backbone amides tend to be relatively high, i.e. >0.8, since secondary structures of the protein make the backbone relatively rigid. Flexible regions of the backbone often correspond to unstructured loop regions where the order parameter will tend to be in the range 0.4-0.6 though the range of accessible values for the backbone order parameters depend very much on the protein and its structural properties. In contrast, the side-chain order parameters tend to be much more heterogeneous, spanning the range of values from just a little more than 0 to just a little less than 1 with no relationship to the protein secondary structure. As for the correlation time, τ_e, it might be anticipated that this quantity would be the most interesting value as it would report on the time scale of the motion, and, to some extent, that is true. Importantly, however, τ_e is an effective correlation time in the model-free formalism that is related, along with the generalized order parameter S^2, to the integral of the orientational correlation function. As such, it does not reflect the details of the functional form of the correlation function. That is to say that the correlation function could involve multiexponential decays corresponding to a set of overdamped low-frequency modes or even underdamped oscillatory motions, and the integral of the correlation function would, nevertheless, give a single value for the correlation time that does not offer insight into the detailed nature of the molecular motion. Consequently, it is impossible to tell of the reported dynamics involve underdamped or overdamped motions or exactly what frequencies and relaxation time scales are actually present.

NMR methods suffer several other weaknesses in addition to the limitation of understanding the nature of the dynamics. First, NMR relaxation experiments report dynamics through the lens of orientational relaxation, which is sensitive to angular fluctuations of the bond vectors but not to translational motions. Consequently, NMR reports on a particular subset of the protein motions. Second, NMR spectroscopy requires isotopic labeling of the protein, which is assumed to be nonperturbative. It is possible, however, that both the structure and dynamics of the protein could be perturbed by isotopic substitutions as both the masses and, potentially, the hydrophobic and hydrogen bonding interactions between residues could be affected by the change in isotopic composition.(60-62) Finally, the application of NMR spectroscopy is limited by the size and solubility of the protein. Although the systems that are accessible by NMR methods continue to grow, it remains a tool that is most applicable to modestly sized enzymes.

Low-Frequency Protein Spectroscopy

The most obvious way to directly characterize the low-frequency vibrational motions of proteins is to directly measure the low-frequency vibrational spectrum. Much work has been done in this area, but the measurements are difficult. The easiest approach in some ways is Raman spectroscopy, which does not require exotic sources or detectors, but the signal is weak and the low-frequency spectral region is challenging due to the proximity of the intense Rayleigh scattering. Absorption spectroscopy in this region has long been troubled by the difficulty of generating and detecting light of the appropriate wavelength and the challenges posed by the strong water background absorbance. In spite of these difficulties, much work has been done to probe the low-frequency vibrational spectra of proteins with considerable success.

Raman spectroscopy measures molecular vibrations and is capable of measuring even the low frequency vibrational modes of proteins in aqueous solution. Raman works based on inelastic scattering of photons. A portion of the photon energy goes into exciting a vibrational mode of the molecule and the photon energy is shifted by an amount corresponding to the energy of the molecular vibration that is excited. Thus, the Raman spectrum is plotted as intensity versus Raman shift to reflect that the energy shift in the photon energy reflects the energy of the molecular vibration that is excited. The intensity of Raman scattering tends to be extremely weak. This fact, combined with the small frequency shift of low-frequency modes, poses a particular challenge in measuring Raman spectra for low-frequency vibrations as they can be quite difficult to effectively separate from the (unshifted) Rayleigh scattering of the excitation source. Nevertheless, Raman was among the earliest methods used for measuring low-frequency vibrational spectra of proteins both because of the difficulties of generating far-infrared light for direct absorption experiments and because of the ability to discriminate against the water background. The water background is, in fact, among the most significant advantages of Raman spectroscopy of biological samples. Because water exhibits an extremely small Raman scattering cross section, the background signal from the aqueous solvent is generally negligible, in stark contrast to the situation in infrared spectroscopy.

The earliest reports of low-frequency Raman spectra of proteins go back to the early 1970's.(72-78) Brown et al. showed that there is a distinct peak at 29 cm^{-1} in the Raman spectrum of lyophilized powders of α-chymotrypsin that is not present in the denatured protein.(75) Subsequent studies have identified features in the same frequency region in the Raman spectra of a range of other proteins. Table 1 lists the frequencies of the low-frequency Raman transitions observed in several different proteins. It is remarkable that these proteins span a range of secondary structures and molecular weights and include both monomeric and dimeric proteins, yet most of them exhibit a low-frequency transition in the same general region between 10 cm^{-1} and 30 cm^{-1}. These transitions have been attributed to collective vibrations of the folded protein that are characteristic of the protein and its folded structure, but the transition frequency does not appear to depend sensitively on the precise secondary structural composition or the size of the protein. Indeed, the precise vibrational character of these low-frequency modes has been the subject of considerable debate, but it is clear that these are highly collective modes and that they are present in the folded protein in solution, in crystals, in hydrated films, and in lyophilized powders suggesting that they represent a characteristic motion of the globular folded protein itself and do not depend on the environment of the protein.(73-75; 79; 80)

With the development of effective sources and detectors, direct absorption spectroscopy in the far-infrared or THz region of the electromagnetic spectrum has become available as a way to measure the low-frequency vibrational spectra of biomolecules.(81-85) Much as in conventional FTIR absorption spectroscopy, the spectra are collected in the time domain. Often, pulsed THz sources and detectors use optical rectification of femtosecond laser pulses in an antenna. For aqueous proteins, the absorption spectra tend to be very broad and featureless much like that of the water background. Though the background absorbance due to water poses an obstacle, it is sometimes possible to isolate the spectral response of the protein, but the broad featureless nature of the resulting absorption spectra makes it difficult to identify collective modes of the protein that could have biological significance. Nevertheless, measurements of lysozyme crystals do exhibit underdamped features in the absorption spectrum that vary with crystal orientation demonstrating that the observed transitions are modes of the protein and not the solvent.(86) The strongest feature is at 45 cm^{-1} and is present in both hydrated and dry crystals. That frequency was also observed as a shoulder in the Raman spectra of lysozyme crystals.(87) There are also significant features at 68 and 80 cm^{-1}.

Optical Kerr effect (OKE) spectroscopy is another approach to directly measure low-frequency spectra in proteins.(88-90) OKE is, in principle, equivalent to spontaneous Raman spectroscopy but is, in practice, more effective than conventional Raman spectroscopy for measuring low-frequency modes because it is not perturbed by the strong Rayleigh line that poses such a problem in Raman spectroscopy. One particularly powerful demonstration of this approach involves OKE experiments on aqueous lysozyme solutions in the presence and absence of an inhibitor.(91) The OKE measurements show a large and distinctive underdamped mode at 80 cm^{-1} that shifts to higher frequency upon inhibitor binding.

Impulsive Spectroscopy

With the development of femtosecond spectroscopies came a new approach to measuring the vibrational properties of proteins.(92-96) Impulsively exciting a protein introduces a sudden perturbation that causes the protein to oscillate at the natural frequencies of the system, much like a hammer striking a bell. Such impulsive excitations typically involve electronically exciting a chromophore, which will induce both excited-state and ground-state dynamics. In most cases, the excited-state dynamics will dominate the observed response and the resulting vibrational coherences will reflect the excited-state motions involved with carrying the system to equilibrium from the Franck-Condon region on the excited state, where the system lands upon photoexcitation. There are many examples of biologically relevant excited-state dynamics such as in photosynthetic systems, light-harvesting proteins, and proteins involved in the primary steps of vision.(97-100) In some systems, excitation of a chromophore, often a metal center, that either dissociates a ligand or rapidly non-radiatively decays to a highly vibrationally excited ground electronic state can induce low-frequency oscillations of the chromophore and surrounding protein that are characteristic of the intrinsic ground-state vibrational modes of the system.(93-96; 101-115) This approach, which is sometimes referred to as vibrational coherence spectroscopy, offers a unique strategy for probing the low-frequency vibrational modes coupled to the active sites of certain proteins.

The most common way of implementing this method is conventional electronic transient absorption spectroscopy. A visible or UV pump pulse excites a chromophore in the protein inducing the perturbation that initiates the nonequilibrium dynamics. A second visible or UV probe pulse then probes the electronic absorption spectrum as a function of the time delay between pump and probe. Because the vibrational motions modulate the intensity of the electronic absorbance, the pump-probe transients exhibit oscillatory time dependence. The Fourier transformation of the pump-probe response yields the spectrum of low-frequency vibrations of the protein. Though the earliest studies of the ground-state vibrational dynamics of proteins using this method focused primarily on myoglobin,(92; 94-96; 112; 114) the approach has since been extended to a range of different proteins including, cytochrome c,(93; 102; 104; 109; 110) cytochrome P450,(106; 110) cystathionine β-synthase,(108) blue-copper proteins,(111; 113) and MoFe nitrogenase.(115) In most of the heme systems, the spectra of low-frequency vibrations are dominated by modes of the heme itself, which is not surprising since the method is sensitive only to those vibrations that perturb the electronic absorption being probed. In other cases, however, it is clear that the low-frequency vibrations are underdamped modes that belong to the protein, and there is considerable speculation about the potential functional relevance of such motions. One example is the blue-copper protein, rusticyanin, which exhibits oscillatory transients in the visible upon excitation of the visible absorption band of the copper center.(111) The exact oscillatory dynamics are dependent on the probe wavelength, and the Fourier power spectrum of the oscillations at two wavelengths are shown in Figure 3. Note, in particular, the 38 cm_{-1} mode that is prominent in the data probed at 625 nm. This feature is identified as a ground-electronic state motion assigned to a delocalized mode of the protein, but the functional significance of this motions remain a matter of speculation.

Another closely related approach that has been developed recently involves probing not by spectroscopy but by X-ray scattering.(101; 116-118) As with the previous pump-probe studies, a short laser pulse excites an electronic transition initiating nonequilibrium protein dynamics, but now the protein structural response can be determined directly using femtosecond X-ray pulses from a free electron source using both small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS). These methods allow for measurement of not just the motions at or near the spectroscopic probe, such as the heme, but also the overall motions of the protein itself. The initial studies have focused on dynamics in myoglobin and hemoglobin following ligand dissociation and photoactive yellow protein excited-state motions, and there remain few examples of such measurements. Interestingly, the results of studies of myoglobin dynamics following dissociation of CO show that the whole protein is involved in the response in what is termed a "protein quake." This approach, which is in the early stages of development, holds significant promise for time resolving the protein motions following a photo-triggered event, for which there are many potential applications.

Two-Dimensional Infrared spectroscopy

Two-dimensional infrared (2D IR) spectroscopy is a powerful tool for probing the solvation dynamics in the environment of a vibrational probe molecule.(119) By locating such a vibrational probe at a selected location in a protein, such as the active site of an enzyme, it becomes possible to measure the enzyme dynamics with the added specificity that the observed dynamics will be unique to the particular region of the protein where the probe molecules is localized.(120) Thus, 2D IR requires a high frequency oscillator to be bound in a desired location in the protein. Several different probes have been used. The most common is a carbon monoxide ligand bound to a heme center.(121-132) Some other alternatives have included nitriles, which can be introduced either as part of an unnatural amino acid(133-135) or in an inhibitor,(136; 137) and azide anion,(138) which binds as a competitive inhibitor to a number of enzymes. In addition, several recent studies have used azido derivatized unnatural amino acids as a way of introducing a suitable vibrational chromophore.(121; 139-141)

Regardless of the particular chromophore that is used, the approach to measuring the low-frequency motions is the same. Similar to a pump-probe experiment, a 2D IR spectrum measures the change in absorbance of a chromophore due to direct excitation in the infrared. A 2D IR spectrum plots the intensity of the absorption change as a function of the excitation (pump) and detection (Probe) frequencies for a fixed time delay between excitation and detection. In a complex environment like a protein, the ensemble of oscillators in the sample have a distribution of frequencies reflecting the heterogeneous distribution of structural environments. For short time delays between excitation and detection, the environments do not have time to change and the frequency at which the oscillators are excited and detected are highly correlated leading to a 2D IR lineshape that is elongated along the diagonal, $\omega_{\text{pump}} = \omega_{\text{probe}}$. For longer delays between excitation and detection, however, a molecule that starts in one environment will have time to experience structural fluctuations leading to a new structure and, correspondingly, a new transition frequency. Thus as the pump-probe time delay increases the 2D IR lineshape will evolve from being elongated along the diagonal to being increasingly rounded as the transition frequencies, which are initially highly correlated, become increasingly less correlated as a result of the fluctuations in the local environment. That is to say, that the time evolution of the 2D IR lineshape of a protein as a function of the waiting time, T, between excitation and detection is a direct measure of the protein dynamics.

More formally, the waiting time dependence of the 2D IR lineshape is a direct measure of the frequency fluctuation time correlation function (FFCF) of the protein-bound oscillator. There are a number of experimental approaches that have been developed to extract the correlation function from the 2D IR data, but they all essentially give the same result with slight variations and sensitivities to experimental distortions.(142) A full discussion of these is beyond the scope of this review, and it is sufficient to know that the centerline slope (CLS) analysis is the most common method and involves creating a centerline by locating, at each pump frequency, that probe frequency at which the 2D IR spectrum goes through a minimum corresponding to the loss of absorption or ground-state-bleach and stimulated emission signal on the diagonal in the 2D IR spectrum.(120) The slope of the centerline reflects the amount of correlation between excitation and detection frequencies for the particular waiting time of that spectrum. A plot of the CLS versus the waiting time is directly proportional to the full FFCF. The FFCF is a direct measure of the protein dynamics with the caveat that it only measures that subset of the protein motions that affect the vibrational frequency of the chromophore. This feature offers a unique advantage in that, rather than probing all of the low-frequency modes of the protein, 2D IR is selective for only those modes that interact with the chromophore. Placing the chromophore at an important location in the protein, like an enzyme active site, allows for selective observation of modes of the active site.

For most of the systems that have been studied to date, the FFCF shows similar behavior. There is typically a multiexponential decay with time constants ranging from 1 to a few tens of picoseconds along with a long-term apparently static offset indicative of those dynamics of the protein that occur on timescales that are much slower than the vibrational relaxation of the chromophore used for the 2D IR experiments. This multiexponential picosecond decay is the result of many overdamped protein motions that arise from the complicated and heterogeneous ensemble of protein environments that interconvert diffusively on the picosecond time scale. A number of enzymes exhibiting this same basic behavior have been studied previously including HIV-1 reverse transcriptase,(136; 137) horseradish peroxidase,(143; 144) cytochrome P450cam,(123; 124; 132; 145; 146) and complexes of plastocyanine with cytochrome f.(147) As an example of the kinds of information that 2D IR measurements of the overdamped protein dynamics can afford, in cytochrome P450cam the Thielges group has shown that the time scales for decay of the FFCF correlate with the degree of regioselectivity that is observed for different substrates.(132; 145) Based on these observations, they have proposed a model in which the substrates can adopt distinct conformational states that are stabilized differently by different substrates. The key to the regioselectivity, then, is the flexibility of the two conformations, which is revealed clearly in the 2D IR spectroscopy.

The notable exception to the typical overdamped behavior of the FFCF decay comes from recent work probing the dynamics of the enzyme formate dehydrogenase using the azide anion, which is a tight-binding inhibitor that, along with the cofactor NAD^+ (Nicotinamide Adenine

Dinucleotide), forms a complex that is a mimic of the transition state structure of the enzyme.(32; 138) The azide anion in this complex also serves as the 2D IR chromophore. Shown in Figure 4 is the CLS decay for the azide anion, and, unlike the previous enzymes that have been studied, the decay is not multiexponential but oscillates. In fact, there are at least two oscillatory frequencies, one oscillation at 9 cm^{-1} and another with a frequency of 24 cm^{-1}. There may be additional higher frequency oscillations that contribute as well, though they make much smaller contributions. Although the oscillations in the FFCF decay determined from the CLS values for the 2D IR spectra are quite distinct, there are no corresponding oscillations in the peak amplitude or volume, meaning that the oscillations cannot arise from coherent excitation of low-frequency modes of the protein that are anharmonically coupled to the azide vibration. Instead, these oscillations are the statistically averaged behavior of the protein ensemble at equilibrium. The observation of such underdamped oscillatory motions is unprecedented in 2D IR experiments. Of course, the other measurements we have already discussed make it clear that there are such underdamped low-frequency vibrational modes in proteins, but the 2D IR approach selects out only those modes of the system that perturb the vibrational mode that is being probed. That means, in this case, that the oscillatory dynamics are necessarily acting at the active site where the azide is bound, which is an important step towards linking the motions to the catalytic properties of the enzyme.

EMERGING CONSENSUS AND FUTURE PROSPECTS

It is interesting to note that each new spectroscopic method that is used to study the low-frequency motions of proteins finds evidence for underdamped vibrational modes in the 10-100 cm^{-1} region, and, in each case, this result is reported as surprising. Indeed, spectroscopically, the existence of underdamped vibrations of proteins at such low frequencies is surprising since the vibrational density of states at these frequencies should be high, solvent friction for large amplitude motions at these low frequencies should be significant, and thermal population of these states at room temperature along with solvent friction would be expected to result in overdamped behavior. Nevertheless, given the number of observations of underdamped low-frequency vibrations in many different proteins and using a wide range of different spectroscopic methods, it is now clear that such vibrations are probably common in proteins and should be expected to be observed spectroscopically. The critical question is, are these underdamped low-frequency vibrations present in enzymes as a merely incidental consequence of the overall protein structure or are such modes of vibration functionally important. Similarly, one might ask whether such vibrations are an evolved property of the enzyme only in the trivial sense that the enzyme itself is an evolved structure and it happens to have these characteristic vibrations or if the vibrational modes themselves have some functional relevance and arise in a causal way from evolutionary pressure on the organism. These related questions are at the heart of the ongoing effort to elucidate the relationship between enzyme structure, dynamics, and function.

Spectroscopic studies that probe low-frequency vibrations of proteins in the catalytic site are at the frontier of the field. One of the most robust ways of assessing functional properties in enzymes is to perform site-directed mutagenesis experiments and determine the impact of selected mutations of key residues on catalytic function. In this context such experiments could also involve spectroscopic studies of the protein vibrations to determine if the changes in function correlate with changes in the vibrational dynamics of the protein. Another approach would be to study the vibrational energy transfer within the protein using an experiment much like that proposed by Schwartz and coworkers to track the vibrational energy transfer pathways following injection of thermal energy at a specific protein site. A recent report of the development of an azulene derivatized unnatural amino acid provides a way of depositing large amounts of vibrational energy on a sub-picosecond time scale, which is critical for such studies.(148) The report also shows that an azido derivatized substrate can detect the thermal energy following photoexcitation of the azulene holding he promise that experiments like these could be used to map energy flow and thereby understand communication pathways in proteins. Finally, the 2D IR experiment on formate dehydrogenase showing underdamped vibrations in the enzyme active site offers the exciting possibility of studying active-site and remote mutations to test for key catalytic residues and communication pathways. Further experiments on isotopically substituted (heavy) enzymes can also test the effects of these isotope substitutions that have been proposed to alter the timing of rate promoting vibrations. Thus, the future prospects of being able to determine the functional relevance of underdamped, low-frequency protein vibrations for enzyme catalysis are promising.

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Table 1: Raman frequencies for low-frequency modes of several proteins. Adapted from Ref. (72)

Protein	Molecular Weight (kDa)	Observed Raman Transition Energy (cm^{-1})
Insulin	11.6 (dimer)	22
Lysozyme	14	25
α-Chymotrypsin	22.6	29
Pepsin	35	20
β-Lactoglobulin	36 (dimer)	25
Ovalbumin	44	22
Concanavalin A	55	20
Bovine Serum Albumin	67	14
Bovine Immunoglobulin G	150	28
Adolase	158	32
Thyroglobulin	669	17

Sidebar – Damped Harmonic Oscillator

Molecular vibrations can be modelled as damped harmonic oscillators. The general behavior is described by the differential equation,

$$\ddot{x} + \gamma \dot{x} + \omega_0^2 x = 0$$

Where ω_0 is the frequency of the undamped oscillator and γ is the damping coefficient.

The characteristic equation has roots,

$$\frac{-\gamma \pm \sqrt{\gamma^2 - 4\omega_0^2}}{2}$$

Underdamped Oscillator

If $\gamma^2 < 4\omega_0^2$, then the argument under the square root is negative and the root is complex valued giving a complex exponential solution, which corresponds to damped sinusoidal motion with a frequency,

$$\omega_{obs} = \frac{\sqrt{\gamma^2 - 4\omega_0^2}}{2}$$

The general solution is of the form,

$$x(t) = Ae^{-\gamma t/2} \cos(\omega_{obs} t + \phi)$$

Thus, if the friction is low and the oscillator frequency is high, then the oscillator will exhibit slowly decaying oscillatory motion.

Overdamped Oscillator

If $\gamma^2 > 4\omega_0^2$, then the argument under the square root is positive, and both roots are negative. Thus the solution takes the form of a biexponential decay,

$$x(t) = c_1 e^{\left(-\gamma + \sqrt{\gamma^2 - 4\omega_0^2}\right)t/2} + c_2 e^{\left(-\gamma - \sqrt{\gamma^2 - 4\omega_0^2}\right)t/2}$$

Thus, if the damping is large and the oscillatory frequency is low, then the system cannot oscillate. It relaxes asymptotically back to it equilibrium.

Critical Damping

If $\gamma^2 = 4\omega_0^2$, then the argument under the square root is exactly 0. The characteristic equation has repeated real roots of $-\gamma/2$, and the general solution takes the form,

$$x(t) = e^{-\gamma t/2}(c_1 + c_2 t)$$

As in the overdamped case, this solution does not oscillate.

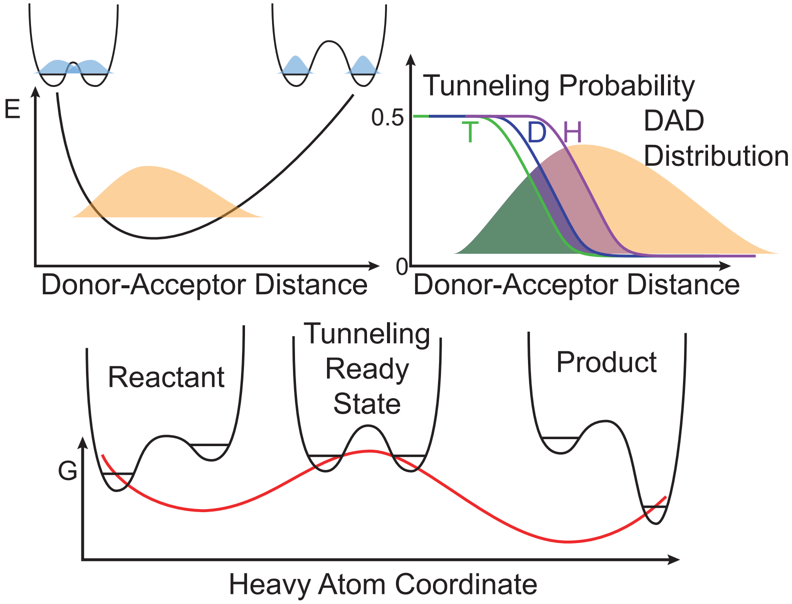

Figure 1: The activated tunneling model. The hydrogen nucleus occupies a double well potential. Plotting the zero-point energy of each well in the hydrogen potential creates two diabatic curves that, when coupled, produce the adiabitc potential shown in red that is the effective reaction coordinate. At the barrier in this coordinate, known as the tunneling ready state, the hydrogen nucleus can tunnel between the two wells. The upper left panel shows the thermal distribution of donor-acceptor distances (DADs) determined by the DAD potential. The upper right panel shows the overlap between the tunneling probabilities for the three hydrogen isotopes, H, D, and T, and the DAD distribution.

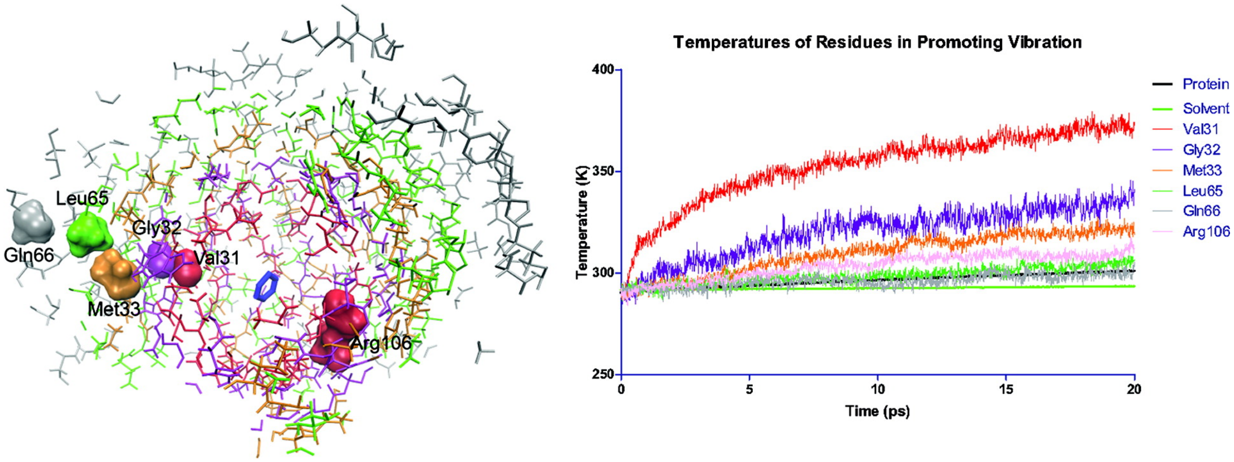

Figure 2: (Left) Different spherical shells containing members of the promoting vibration. Highlighted residues represent the different members of the promoting vibration. (Right) Temperatures of each the residues. Promoting vibration residues closer to the active site maintain higher temperatures throughout the course of the simulation. Reproduced with permission from Reference 58.

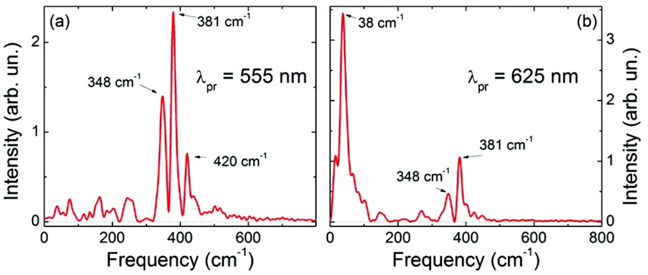

Figure 3: Fourier transform power spectra of the oscillatory components of pump-probe signals at two different probe wavelengths in rusticyanin. Reproduced with permission from Reference 111.

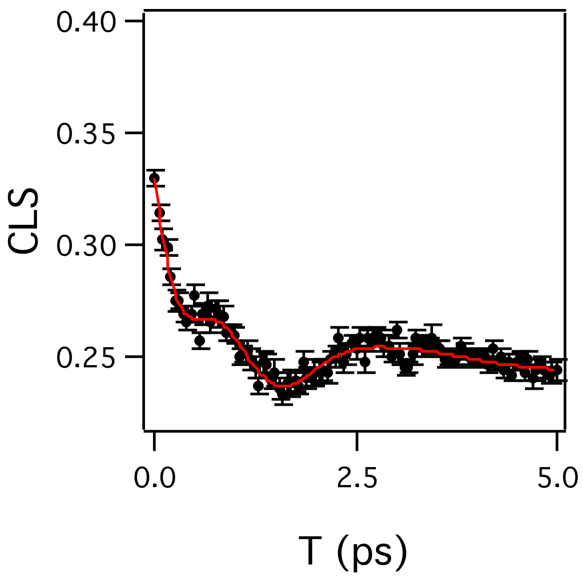

Figure 4: CLS decay data of azide bound to formate drhydrogenase withNAD+. The circles are the data, and the solid line is the result of a fit to a sum of exponentials with two oscillatopry terms with frequencies of 9 and 24 cm^{-1}. Reproduced with permission from Reference 138.