Diffractive Optics Based Nonlinear Optical Studies of Protein Motions: From Inertial Collective Modes to “Protein Quakes”

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Recent advances in nonlinear spectroscopy are discussed as a means to probe in real time the highly correlated structural changes involved in functionally significant protein motions - over nine decades in dynamic range. The dynamics and energetics of these processes can be followed with unprecedented sensitivity by using diffractive optics to generate the proper beam geometries, along with a reference for true phase sensitive detection of the signal field. Conventional descriptions in terms of glass dynamics are found not to hold over the complete dynamic relaxation pathway. A paradigm based on the coupling of reaction coordinates to collective modes is invoked to explain the biomechanics and energy transduction processes involved in deterministic protein motions.

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Biological processes in nature are driven by the formation and breaking of chemical bonds. The energy associated with these bonds is ultimately responsible for driving various biological functions. Exactly how the energy in a chemical bond becomes associated with dice”. In order to understand these processes, information about the strain-inducing structural dynamics and energetics are required. This information typically involves very low energy relaxations that correspond to small (<0.1 Å) amplitude rms motions of the protein residues. An extremely sensitive method is required for this; four-wave mixing spectroscopies provide interferometric sensitivity and can be constructed to access the desired information.

The challenge is to further increase the temporal range and sensitivity of four-wave mixing to cover the enormous dynamic range of the relevant relaxation processes involved in biological systems. Ideally, one would like to span the time scales from the very earliest events associated with bond breaking and formation to completion of the biological response. The temporal window should extend from femtoseconds to seconds, and longer in certain cases.

Briefly, four-wave mixing involves intersecting two coherent beams in a sample so that they form an interference grating. Complex refractive index changes \( \Delta n \) are generated in the bright fringes, forming a physical grating in the material. Scattering of an off-resonant probe beam from this grating allows the observation of the time evolution of the structural dynamics. Short timescale (sub-nanosecond) measurements can be made by observation of the photoacoustic wave that is generated in the sample as a result of the grating excitation process. In the femtosecond to nanosecond window, protein structural relaxation can be inferred through changes in the phase anisotropy (vide infra). On longer timescales, the temporal behaviour of amplitude and phase contributions to the grating will reflect the molecular dynamics of the system.

There are, however, experimental difficulties with four-wave mixing. Signal stability depends critically on the phase relationship between the pump beams. The interferometric nature of the method implies a high degree of sensitivity to spatial jitter of the probe relative to the grating. Furthermore, in femtosecond experiments, when the pump beams cross at an angle, it introduces a loss of temporal resolution as the phase fronts of the two beams do not cross simultaneously at all points.

Recently, the use of diffractive optical elements (DOE’s) has been introduced to circumvent the above limitations. There are a number of advantages to using this method. Because the interference grating in the sample is an image of the relief grating on the optic, the fringe spacing is precisely known and is fixed at half the fringe spacing at the diffractive optic. One of the greatest advantages of using DOE’s is that they provide passive phase stability; they allow the pump and probe beams to traverse the same optics, which minimises phase error due to mechanical vibrations. Furthermore, the DOE produces tilted phase fronts, which eliminates temporal broadening due to the crossing angle and thus permits true femtosecond time resolution even in noncollinear geometries. A further advantage of the use of a DOE is that it allows optical heterodyne detection (OHD) to be easily implemented. For OHD, a steady-state reference field \( E_{ref} \) interferes with the signal field \( E_s(t) \) at the detector. The total signal \( S(t) \) can then be expressed as

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where $\phi_i$ and $\phi_{ref}$ are the phases of the signal and reference fields respectively. The reference field is orders of magnitude larger than $E_i(t)$, and is seen as a DC offset plus an absorption term. In the limit $|E_i(t)| \ll |E_{ref}|$, the signal-only term in Eq. (1) can be neglected, leaving the interference (heterodyne) term. OHD of the four-wave mixing signal has a number of advantages. There is an amplification factor of $2 |E_{ref}/E_i|$ over the direct-detected signal, which considerably enhances signal-to-noise ratio. This is critical to oxygen transport. The phase stability provided by this arrangement is excellent, as can be seen in Fig. 2. For collection of this data, 70 fs (800 nm) pump pulses were used to photoexcite and probe liquid CS$_2$. The fluctuations of the diffracted OHD signal as a function of real time were then monitored, in (a) using a measurement bandwidth of 5 Hz and in (b) over a longer time period with a measurement bandwidth of 0.1 Hz. The phase drift $\delta \phi(t)$ was then isolated from noise due to fluctuations in laser intensity. For comparison, a Michelson interferometer under conditions of approximately the same mechanical stability and environment exhibited multiple waves of phase drift$^5$.

The key question is: what are the most important structural relaxation phases that direct the reaction forces of the iron-ligand active site? By studying dynamic variables (strain, energetics) associated with structural relaxation coupled to the iron-ligand coordinate, the mechanics of the energy transduction process can be determined - at least on a qualitative level. These variables are contained in the real part of the four-wave mixing signal produced by photodissociation of the iron-ligand bond and can now be detected with high sensitivity using heterodyne detection.

**Experimental Studies**

The experimental arrangement for these measurements is shown in Fig. 1. Pump and probe beams are collinearly focused onto the DOE and the first order diffracted beams are imaged in the sample using a spherical mirror$^7$. The inherent symmetry of the system generates both signal and reference probe beams, each with stable phase relationships relative to each another and at the right geometry for Bragg scattering from the material grating. A half-waveplate/polarizer combination is used to set the amplitude of the reference relative to the signal, and the relative phases are adjusted using glass cover slips placed in one of the beams. The heterodyne signal is detected at D1. A second detector D2 records the probe laser noise for subtraction from the heterodyne signal. The probe can be pulsed, for the examination of short-time dynamics, or continuous wave (cw), in which case the time resolution of the experiment originates in the duration of the pump pulses and the diffusional time of the grating (determined by the fringe spacing $\Lambda$).

\[ S(t) = |E_{det}(t)|^2 = |E_{ref}|^2 + |E_i(t)|^2 + 2 |E_{ref}| E_i(t) \cos(\phi_{ref} - \phi_i), \]  

(1)

The dynamics associated with heme proteins such as hemoglobin and myoglobin provide excellent test cases for this new methodology. Both proteins undergo changes in conformation triggered by the binding or dissociation of small ligands such as CO or O$_2$ at the heme site$^7,8$. The tetrameric protein hemoglobin is the cornerstone of our current understanding of molecular cooperativity, having a well characterised structural response to the binding of oxygen. Conformational changes at the quaternary level lead to large changes in oxygen binding; this is critical to oxygen transport. Myoglobin is a single heme protein that has a nearly identical structure to a single subunit of hemoglobin. In this capacity, carboxymyoglobin (MbCO) serves as a model system for understanding the tertiary structural changes that couple the four subunits of hemoglobin through the quaternary coordinate. The pump pulses and the diffusional time of the grating (determined by the fringe spacing $\Lambda$).

The phase stability provided by this arrangement is excellent, as can be seen in Fig. 2. For collection of this data, 70 fs (800 nm) pump pulses were used to photoexcite and probe liquid CS$_2$. The fluctuations of the diffracted OHD signal as a function of real time were then monitored, in (a) using a measurement bandwidth of 5 Hz and in (b) over a longer time period with a measurement bandwidth of 0.1 Hz. The phase drift $\delta \phi(t)$ was then isolated from noise due to fluctuations in laser intensity. For comparison, a Michelson interferometer under conditions of approximately the same mechanical stability and environment exhibited multiple waves of phase drift$^5$.
This study illustrates that DOE’s can provide inherent passive phase stabilisation of up to \( \lambda/300 \) for typical data collection times. Experimental studies of MbCO using 70 fs pulses centered at 400 nm for the excitation and at 800 nm for the probe are shown in Fig. 3. In this experiment, the emphasis was on trying to discern anisotropic mass displacements following bond dissociation – the unequivocal signature of a directed process. The real and imaginary portions of the OHD signal were first determined by using the maximum acoustic modulation to assign the real component. Data was collected for both real and imaginary components of the signal by setting the signal and reference beams to the appropriate relative phase, then recording the diffracted signal level while scanning the time delay between pump and probe pulses. The time axis in the figure refers to this delay. Fig. 3 shows the phase (real) and absorption (imaginary) anisotropy in the signal, as determined by

\[
\frac{r(t)}{E_\parallel(t)+2E_\perp(t)} = \frac{E_\parallel(t)-E_\perp(t)}{E_\parallel(t)+2E_\perp(t)},
\]

for each of the real and imaginary data sets, where \( E_\parallel(t) \) and \( E_\perp(t) \) are the portions of the signal with the pump and probe beams polarized parallel and perpendicular to each other, respectively. The figure indicates that there is a large, time-dependent phase anisotropy in theMbCO signal, having an asymptotic value of \( r_{\text{as}}(t) = 0.37 \pm 0.02 \). After correcting for thermal effects, the most dramatic anisotropy is found to occur within 500 fs. Comparison with phase anisotropy data for carboxy-protoheme (PHCO), which has no protein globin, indicates that the observed anisotropy in the MbCO signal is not attributable to photoselection; the phase anisotropy in PHCO is virtually constant after time zero and is much smaller (\( r_{\text{as}} = 0.13 \pm 0.02 \)). By comparison, deoxymyoglobin signal taken under the same conditions shows no phase anisotropy, as one might expect for diffusive thermal relaxation in the globin where there is no preferential orientation relative to the heme plane.

\[\text{Figure 3. Absorption (real) and phase (imaginary) anisotropy of OHD data for MbCO on the picosecond timescale. Note the large anisotropy in the real signal.} \]

In addition to the anisotropic strain in the protein, there are acoustic volume changes that occur on similar timescales and are likely to be causally connected. The occurrence of these rapid global protein motions, and previous determination of energetics\(^{10}\), indicate that the initial relaxation phase of the protein is dominated by the coupling of the reaction forces to the collective modes of the protein. The larger coupling coefficients to collective motions act to guide the system through the correct path in an otherwise complex coordinate phase space\(^{11}\). The long time (> 1 ns) dynamics are also important in highly associated systems and are often modelled within a glass description. In this approach, equilibrium lowest-energy states are reached by a random walk on a ‘rugged landscape’-hierarchical potential energy surface\(^{13,14}\). In this model, the activation energy for a reaction is represented as a probability distribution rather than a single value. If the structural transitions under discussion follow the dynamics typical of a glassy state, it should be possible to fit the observed dynamic data using a stretched exponential form for the signal decay\(^{15,17}\).

Studies of the long time dynamics used 5 µ of < 30 ns pump pulses at 526.5 nm and a 1064 nm cw probe, with a fringe spacing in the sample of 7.5 µm. Fig. 4 shows the real component of four-wave mixing OHD data of 2.0 mM aqueous solutions of MbCO and deoxymyoglobin at –1.5 °C and at ambient temperature (20 °C). The real component of the deoxymyoglobin dynamics, shown in Fig. 4(a), behaves as one would expect from a purely thermal response. There is no bond broken and all the absorbed photon energy goes into heating the protein and surrounding aqueous bath on picosecond timescales. The decay of this signal fits very well to a single exponential with a 10.4 µs decay time, corresponding to the thermal diffusion time characteristic of the experimental fringe spacing. At –1.5 °C the thermal signal is dramatically reduced, owing to the fact that water undergoes a point at which its index does not change with temperature. Minimising the thermal contribution allows the

\[\text{Figure 4. OHD data for deoxymyoglobin and carboxymyoglobin on the microsecond time scale.} \]

(a) Deoxymyoglobin data

- 1.5 °C data
- 20 °C data
- Fit

(b) Carboxymyoglobin data

- 20 °C

Fit decay time = 10.4 µs.

(b) MbCO at ambient temperature and –1.5 °C. The signal relaxes back to the baseline on longer timescales (ms) through a CO diffusion term (about 10% rise) and full relaxation through CO bimolecular recombination.
The dynamical behaviour of MbCO on this timescale is very different (see Fig. 4(b) and Fig. 5). Both at room temperature and at -1.5 °C, there is the rapid, anisotropic signal discussed previously, followed by a decay feature that is too fast to be caused by thermal diffusion (compare Fig. 4 (a) to Fig. 5). The details of this relaxation component constitute the most interesting new feature in the long time dynamics. At high time resolution, there is a distinct plateau or onset time that gives this inertial functional form and is highly temperature dependent. Following the onset period, the relaxation process is best fit by a single exponential with an offset (decay time 740 ns at 20 °C and 3.0 μm at -1.5 °C) in agreement with our previous findings and related work using homodyne detection. A stretched exponential cannot fit these dynamics. This feature is assigned to CO escape from the protein through a nondiffusive relaxation process. The experiment is sensitive to the global or long length scale motions of the protein. The observation that the data does not fit a stretched exponential indicates that these motions do not involve glass-like behavior. The observation suggests that there is a relatively large, discrete, change in protein strain as the CO leaves the protein – rather than a continuous distribution of relaxation/volume changes.

Segmental relaxation, as mediated by collective modes thermally sampling the relaxation pathway, could produce such unusual dynamics. Such relaxation processes are analogous to segmental motions in quake phenomena. The analogy of “protein quakes” has been made previously in the context of a simple pictorial description of how stress in proteins (produced in this case by reaction forces) is relieved and leads to changes in conformation. The observed dynamic signature suggests that segmental sampling is truly a mechanistic feature of the relaxation process. The energetics governing this relaxation phase are also important. Preliminary analysis indicates that less than 14.7 kJ/mole is dissipated subsequent to the fast inertial relaxation process depicted in Fig. (3). Comparison of this result with earlier studies of the energetics on picosecond timescales illustrates that the initial relaxation phase, propagated by collective mode displacements, is the dominant relaxation mechanism.

The above series of experiments cover a dynamic range spanning nine decades in time. A consistent picture is beginning to emerge in which the collective modes of the protein act as the initial directors of the energy transduction process and appear to manifest their effect over many decades in time. This collective mode coupling mechanism represents a paradigm for understanding the biomechanics of protein structural changes. The small collective motions and anisotropic nature of heme protein relaxation have been resolved through the significantly improved signal to noise and selective access to the real part of the complex index of refraction made possible with the advent of diffractive optics based nonlinear four–wave mixing. This methodology is general and should greatly enhance the capabilities of all forms of photothermal and photoacoustic grating measurements.

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