Periodic and stochastic thermal modulation of protein folding kinetics

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Chemical reactions are usually observed either by relaxation of a bulk sample after applying a sudden external perturbation, or by intrinsic fluctuations of a few molecules. Here we show that the two ideas can be combined to measure protein folding kinetics, either by periodic thermal modulation, or by creating artificial thermal noise that greatly exceeds natural thermal fluctuations. We study the folding reaction of the enzyme phosphoglycerate kinase driven by periodic temperature waveforms. As the temperature waveform unfolds and refolds the protein, its fluorescence color changes due to FRET (Förster resonant Energy Transfer) of two donor/acceptor fluorophores labeling the protein. We adapt a simple model of periodically driven kinetics that nicely fits the data at all temperatures and driving frequencies: The phase shifts of the periodic donor and acceptor fluorescence signals as a function of driving frequency reveal reaction rates. We also drive the reaction with stochastic temperature waveforms that produce thermal fluctuations much greater than natural fluctuations in the bulk. Such artificial thermal noise allows the recovery of weak underlying signals due to protein folding kinetics. This opens up the possibility for future detection of a stochastic resonance for protein folding subject to noise with controllable amplitude. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4887360>]

I. INTRODUCTION

Chemical reactions can be observed by two general approaches. Spontaneous fluctuations are revealed when the molecular ensemble is sufficiently small, such as in single molecule spectroscopy or fluorescence correlation spectroscopy.^{1–3} In the bulk, response to a sudden perturbation can be observed because all molecules move in lock-step, yielding a detectable relaxation response.⁴ In the linear response regime (small fluctuations and/or perturbations from equilibrium), the two approaches are connected by a fluctuation-dissipation theorem and are in principle equivalent.⁵ Generalizations of fluctuation-dissipation have also been developed for larger perturbations out of equilibrium.⁶ The two worlds of perturbation and fluctuation have been combined, for example, to move a single molecule out of equilibrium faster to monitor rapid reactions.⁷

There is another way to combine the worlds of fluctuation and perturbation: apply large artificial fluctuations to the bulk. Frequency-domain fluorescence lifetime detection is a common biophysical application of this idea: instead of initiating a fluorescence decay with a fast light pulse, fluorescence is excited by intensity-modulated light.⁸ The observed phase lag between excitation and fluorescence signal depends on the modulation frequency and fluorescence decay rate, and can be used to extract the fluorescence decay time(s). In contrast, user-supplied modulation has seen much less use in the study of biomolecular reaction kinetics. *In vitro*, Braun and

Libchaber demonstrated detected DNA hairpin folding driven by temperature oscillation,⁹ and Zrelli *et al.* studied DNA hybridization.¹⁰ Schoen *et al.*¹¹ studied DNA hybridization in a live cell by periodic temperature modulation to drive the reaction. Another example is a demonstration of a phase lag in a protein folding reaction driven by a periodic temperature modulation.¹²

Artificially applied fluctuations do not have to be periodic. For example, consider the phenomenon of stochastic resonance: a signal below the detection threshold is lifted above the detection threshold by the addition of random noise.¹³ Too little noise amplitude has no effect, too much buries the signal in the noise, hence an optimum noise level or “stochastic resonance.” Such resonance could be elicited by artificial noise, such as random temperature modulation much greater than ordinary thermal noise. Based on Brownian dynamics simulation of a protein folding free energy surface, Davtyan and Papoian²⁵ propose that protein folding rates could peak (show a resonance) when plotted against driving noise amplitude and average frequency (for colored noise). However, such pseudo-random thermal noise has not been used to drive biomolecular reactions.

Here we describe in detail an instrument that can temperature-modulate a biomolecular reaction with arbitrary waveforms *in vitro* or *in vivo*. Heating rates that are limited by laser power, and cooling rates that are limited by the size and geometry of the sample holder. *In situ* detection of the temperature directly reveals the applied perturbation, irrespective of the laser waveform used to drive the system. We demonstrate the method by applying frequency-swept periodic temperature modulation to detect the folding kinetics of the enzyme phosphoglycerate kinase (PGK). We label PGK with a

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donor and acceptor fluorophore so unfolding/refolding can be detected based on Förster resonant energy transfer (FRET). Modulation of the folding reaction at sufficiently high frequencies allows us to observe a phase-shifted FRET signal as a function of driving frequency characteristic of the folding relaxation rate. The data can be analyzed with a simple model along the lines of the one developed by Lemarchand and co-workers,¹⁴ by adding a temperature-dependent quantum yield of the fluorescent probes to the model.

We expand the modulation approach by adding pseudo-random thermal waveforms to a periodic thermal modulation below the reaction threshold. Pseudo-random noise is not a tool for analysis (which is more straightforward with periodic waveforms), but it has two useful applications. First, we show that artificial thermal noise stimulates the folding/unfolding process. As a control, we compare with higher temperature modulation where the reaction is facile. Second, we propose that artificially enhanced thermal fluctuations can be a useful tool to sample in a Boltzmann-like fashion functional higher energy states of proteins that would normally be visited only rarely under spontaneous thermal fluctuations. All natural systems are subject to thermal fluctuations that allow them to visit excited states, which are often the functional states. Such enhanced experimental sampling with characteristics of thermal noise would be the experimental analog of replica-exchange methods developed for molecular dynamics simulation.^{15,16}

II. METHODS

A. Sample

Our protein construct is based on the destabilized but enzymatically active Y122W/W308F/W333F mutant of yeast PGK.¹⁷ PGK is a multistate folder with rates in the 10^5 – 0.01 Hz range,¹⁸ yielding stretched exponential kinetics. AcGFP1 (the green fluorescent donor D) and a histidine tag were attached at the N-terminus and mCherry (the red fluorescent acceptor A) at the C-terminus to report on the compactness of the FRET-PGK construct. The unfolded protein has a 2–3 times higher D/A fluorescence ratio than the folded protein (Figure 1).¹⁹ For our *in vitro* studies, plasmids containing this construct (Genscript) were transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL cells and grown in lysogeny broth (LB) media to an optical density of 0.6, at which point isopropyl thiogalactopyranoside (1 mM IPTG) was added to induce protein expression. After induction, cells were grown for about 16 h at 30 °C. The cells were then collected by centrifugation and lysed by sonication. Protease inhibitors were added to reduce protein cleavage in this lysate. The cell lysate was run through a nickel-nitrilotriacetic acid (Ni-NTA) column with high affinity for the histidine tag, and purified according to the resin manufacturer's (Qiagen) instructions.

We used a 3 μ m aqueous solution of the protein for *in vitro* unfolding/refolding experiments detected by FRET. The sample chamber was prepared by placing a double-sticky seal of height ~ 120 μ m (Grace, SecureSeal 654006) on a microscope slide and covering with a coverslip. No signs of protein aggregation (changes in FRET) were observed with concen-

FIG. 1. Experimental melting transition of FRET-PGK *in vitro* (circles), and fitted to a two-state Folded $\rightleftharpoons$ Unfolded model (Sec. II). Modulation of the temperature near T_m (horizontal red line) produces modulation of the unfolded and folded protein populations, causing the donor fluorescence D and the acceptor fluorescence A to be modulated, and hence the D/A ratio to be modulated (vertical red lines). The intrinsically temperature-dependent fluorescence quantum yields of donor and acceptor also responds to the temperature modulation (dashed baseline), but on a much faster time scale than the folding reaction.

trations as high as 12 μ M protein over the full temperature range of the experiments (25–45 °C). The protein melts at 38–39 °C, and modulation studies were performed at various temperatures just below the melting transition shown in Figure 1.

B. Apparatus and measurement procedure

The experimental system is built on the same platform as our *in vivo* T-jump imaging instrument.¹⁹ The optical system excites the GFP donor with blue light, and collects green and red fluorescence to measure FRET, while the temperature is modulated by an IR laser (Figure 2). The excitation source is a blue LED (470 nm, 400 mW). An inverted epifluorescence microscope and a 40x objective handled the illumination and collection of fluorescence for FRET (blue excitation) and temperature measurement (amber excitation). A VisionResearch Phantom V12.1 high-speed camera acquired the green and red fluorescence signals simultaneously. We chose a frame rate of about 100 Hz and collected data for > 10 s to focus on the 0.1–10 Hz dynamic range of the PGK folding/unfolding kinetics.

In the experiment, PGK is successively subjected to different frequency temperature modulation waveforms. To allow seamless comparison of data within these frequency sweeps, the sample temperature set point was accurately controlled. The sample chamber was placed on a carrier plate and covered by a heater plate which had a sample access slit on it (21 $\times$ 4 mm) for optical sample heating and detection. This system maintains any temperature from room temperature to 60 °C by using a PID-software-loop (LabVIEW). Thermocouple sensors (Omega 5TC-TT-T-36-36 connected to NI TC01 reader) and heating resistors (Ohmite 810F3ROE) were placed on both sides of the slit in the heater-plate. The resistors were connected to a computer-controlled power-supply (BKPrecision 9130). The resulting average temperature variation at the center of the slit was smaller than 0.1 °C over the full temperature range. Variation along the slit was calibrated to be < 2 °C, and was accounted for in the experiments depending on where fluorescence data were collected.

FIG. 2. Schematic of the thermal modulation instrument. The average sample temperature is held constant by a proportional-integral-derivative (PID) controller driving heating resistors, as long as the temperature is sufficiently above ambient. The programmed temperature waveform is provided by a computer controlled infrared (IR) diode laser. Fluorescence is excited by a blue light emitting diode (LED), collected by a microscope objective, split into separate green and red channels, and detected by a camera that transfers the fluctuating green and red signals to the computer.

The sample temperature was modulated above the set point by an infrared heating laser (m2k Lasers, $\lambda = 2200$ nm, up to 700 mW) coupled to a computer-controlled power-supply (LDC340). The beam was passed through a lens mounted above the sample access slit, and collimated to a ~ 1 mm² spot in the sample chamber. There it directly heated the aqueous sample solution. A set of IR attenuators (Reynard R00380, Thorlabs NENIR) mounted in front of the heating laser was used so the laser could be used in its linear voltage-power operating range. The unattenuated heating laser could modulate the temperature by up to 40 °C, whereas modulation of 2 °C–10 °C was used in actual experiments.

In situ readout of the sample temperature is important because the actual sample temperature as a function of time is a convolution of the laser waveform and the heat dissipation properties of the sample holder. We measured the sample temperature modulation *in situ* to better than 0.25 °C by using the calibrated mCherry quantum yield. The calibration curve of the mCherry quantum yield as a function of temperature is known.¹⁹ Thus mCherry was also excited directly by an amber LED at 580 nm and 190 mW (Thorlabs M590L2) to measure the temperature *in situ*. By switching excitation from blue (FRET) to amber (temperature), the folding experiment and temperature measurement could be carried out on the same sample. The fluorescence signal depends on the absorption pathway of the sample chamber, which could vary from sample to sample or across a single sample. We verified that the fluorescence signal at the center and edges of the sample access slit is uniform to <5%, with a corresponding

uniformity of the temperature readout. As one might expect, the actual temperature modulation we measured is smaller at higher modulation frequency because recoiling is incomplete before the next heating cycle. We corrected for this damping effect by over-compensating the set point and modulation amplitudes to get the desired net temperature waveform. The current geometry has a 3 dB bandwidth of 12 Hz for generation of arbitrary waveforms. Pre-cooling by a thermoelectric cooler (to speed up heat dissipation) and miniaturization would be required to achieve greater bandwidth.

For highest accuracy, green and red FRET data acquired simultaneously were analyzed. However, it is desirable to have accurate timing so multiple experimental runs can be compared. We used the analog output on an NI-6221 board (National Instruments) to drive the laser power supply with the same refresh rate as the camera rate to avoid aliasing. The software triggers the camera first. The camera then emits a hardware trigger, which is processed by a counter with a predefined delay. When the delay expires, the analog output is triggered and the heating waveform is supplied to the power supply. Another counter checks on the ending time and turns the laser off at that point. The experimental protocol, including the opening and closing of shutters, initiating the DC heating, then exposing the sample to different waveforms was fully automated and user-programmable (National Instruments Labview and LabWindows).

The signal loss due to photobleaching was about 10%/min under blue illumination. By starting fluorescence excitation 5 s prior to applying the temperature waveform,

we obtained nearly linear photobleaching profiles. An average slope was subtracted from the fluorescence data to correct for the near-linear bleaching. Software scripts automated the fitting of periodic driving waveforms, and the R^2 of the fit was monitored, and was approximately 0.99 for all the signals we analyzed, or the signal was discarded.

C. Data analysis

The experimental data consist of a green donor fluorescence waveform $D(t)$, a red acceptor fluorescence waveform $A(t)$, and a calibrated temperature waveform $T(t)$. The analysis procedure involves: (1) Extraction of the phase shift from the experimental green and red waveforms. (2) Simulation of the phase shift using a kinetic model. (3) Least squares fitting of model parameters so the modeled phase shift matches experiment.

In the first step, periodic waveforms were least-squares fitted to sine waves $S \sin(2\pi \nu t + \varphi)$, by adjusting the amplitude S and phase φ . The frequency ν was held constant at the known driving frequency. The phases of the two signals (e.g., green and red oscillation) were then subtracted to yield a phase lag vs. driving frequency plot. For pseudo-random sample excitation, the two signal waveforms to be compared were normalized to zero average and unit standard deviation, and then subtracted from one another. This yields a phase-shifted difference waveform. Finally, cross correlation can extract a phase difference for combined periodic and pseudo-random waveforms. The two desired signal waveforms are multiplied together shifted in time by Δt and integrated. The resulting cross correlation $C(\Delta t)$ has a maximum at $\Delta t = 0$ if the two waveforms are identical. If they are not identical, the maximum of the cross-correlation will occur at $\Delta t_{\max} \neq 0$, and the phase shift is defined as $\varphi = 360 \nu \Delta t$ in degrees. In all three analyses, the periodic waveform is sampled experimentally for an exact multiple of its period.

In the next step, the $D(t)$ and $A(t)$ fluorescence waveforms are simulated given the $T(t)$ waveform as an input. The exact same analysis can then be applied to the simulated fluorescence signal as to the data (e.g., fitting to make a phase shift vs. frequency plot) for comparison. We developed a simulation code along the method proposed by Closa *et al.*,¹⁴ or Zrelli *et al.*,¹⁰ similar to the type of data analysis used for many years in frequency-domain fluorescence lifetime measurements (see the supplementary material for code²⁰). We approximated PGK as a two-state system over the frequency interval 0.1–10 Hz: the reaction was simply modeled as

The protein free energy surface is modeled as a double well along the reaction coordinate x , one well for the folded state ($x = 1/2$), and another well for the unfolded state ($x = -1/2$). At the melting temperature T_m the free energies of the two wells are the same and the activation barrier equals $\Delta G^\ddagger$ for both forward and backward reactions. When the temperature is modulated, a term linear in temperature,

$$\Delta G(t) = g_T(T(t) - T_m)x, \quad (2)$$

is added to the free energy, where $\Delta G(t)$ is the free energy difference between unfolded and folded states, T_m is the melting temperature of the protein, and g_T is a fitting parameter. Thus when the temperature T is modulated, the wells move up and down and the unfolded and folded state alternate being lower in free energy. The resulting modulated activation barriers

$$\Delta G_{UF}^\ddagger = \Delta G^\ddagger + \Delta G(t) \quad \text{and} \quad \Delta G_{FU}^\ddagger = \Delta G^\ddagger - \Delta G(t) \quad (3)$$

were used to compute rate coefficients

$$k_{UF}(t) = \nu^\ddagger \exp(-\Delta G_{UF}^\ddagger/RT) \quad \text{and} \quad k_{FU}(t) = \nu^\ddagger \exp(-\Delta G_{FU}^\ddagger/RT) \quad (4)$$

for interconversion between native and unfolded states. We then solved the differential equation

$$\partial [F]/\partial t = -k_{FU}(t)[F] + k_{UF}(t)[U], \quad (5)$$

subject to population conservation $[U] + [F] = 1$ to obtain populations $[F](t)$ and $[U](t)$ in the native and unfolded states. The known D/A ratios of the native and unfolded states and the known temperature dependent quantum yields

$$QY(T) \approx QY_0[1 + m(T - T_0)] \quad (6)$$

of Ac1GFP and mCherry²¹ were then used to calculate fluorescence signals $D(t)$ and $A(t)$.

In the final step, fitting parameters such as g_T , T_m , and $\Delta G^\ddagger$ were adjusted until the experimental and simulated data (e.g., phase vs. driving frequency plot) matched by least-squares. In the experiments, we obtained the highest quality data by plotting the phase difference between $D(t)$ and $A(t)$, which were measured simultaneously.

III. RESULTS

A. Experimental temperature modulation data

We first investigated a protein folding reaction driven by a periodic modulation of the temperature, which produces a periodic modulation of the rate coefficients and equilibrium constant. Phosphoglycerate kinase was labeled with a donor (green, D)–acceptor (red, A) FRET pair so green and red fluorescence intensities could report on the folding. The protein was dissolved in aqueous buffer at a concentration of 3 μM to avoid aggregation. PGK is a multistate folder with a hierarchical distribution of rates in the 10⁵–0.01 Hz range,¹⁸ yielding stretched exponential kinetics. Over a narrow enough time range, it can be analyzed approximately as a two-state folder.¹⁹

The protein solution was heated by a periodic laser waveform to yield a temperature

$$T(t) = T_0 + 1/2 \Delta T \sin(2\pi \nu t + \varphi) \quad (7)$$

driving the folding-unfolding reaction. The observed fluorescence signal has two contributions. When the protein unfolds into state U, green donor fluorescence D increases, and when it folds into the folded state F, red acceptor fluorescence A increases; anticorrelated (180° out of phase) red and green signals thus monitor the chemical reaction. In addition, donor

FIG. 3. Temperature waveform (left vertical axis) and raw experimental fluorescence signals (right vertical axis). The GFP fluorescence (donor = D) is shown in green. The mCherry fluorescence (acceptor = A) is shown in red. ΔT is the temperature above the microscope stage value of $T_0 = 32^\circ\text{C}$. Two driving frequencies are shown. The top panel shows a wide time range; fitting the full time range yields highly accurate phase information after bleaching (not subtracted from the red and green raw fluorescence signals here) is corrected by a linear baseline. The bottom panel shows a single cycle to reveal the small phase shift between red and green fluorescence observed at a low driving frequency, in addition to the phase delay of both fluorescence channels with respect to the driving temperature.

and acceptor quantum yields both decrease approximately linearly with temperature²¹ and respond to temperature changes on a sub-10 ms time scale. In-phase red and green signals thus monitor quantum yield oscillations due to temperature.

Figure 3 shows the experimental data at two modulation frequencies. The top panel shows the signal for many periods. It can be seen that the green and red fluorescence slowly drift downward, due to photobleaching. This is shown explicitly in Figure 3, but is corrected by a linear baseline subtraction (Sec. II) in all other figures. The bottom panel shows a detail view at a lower modulation frequency, revealing phase shifts of red and green fluorescence with respect to temperature (blue) and with respect to each other. At our sampling rate and length of data collection, phase differences between waveforms could be fitted with an uncertainty of $< 0.5^\circ$.

B. Modeling two limits: Reaction only or quantum yield + reaction

To understand the origin of these phase shifts, consider the modeled signal in Figure 4. The temperature-driven kinetic model similar to the one given by Lemarchand *et al.*¹⁴ is described in Sec. II, and simply uses time-dependent rate coefficients $k(T(t))$ in the kinetic master equation for the $F \rightleftharpoons U$ reaction. At the top is a signal without temperature-dependent quantum yield; only the reaction contributes to the phase shift. Red and green are 180° out of phase, as required for a perfect FRET signal. In the example, the driving frequency is comparable to the unfolding/refolding relaxation rate $k_{\text{obs}} = k_u + k_f$. The green signal is therefore shifted towards 90° with respect to the temperature driving waveform (blue), and the red signal

FIG. 4. Simulated signals using the model described in Sec. II. (Top) The temperature-dependent quantum yield of GFP and mCherry is neglected. In that case, the green and red signals are 180° out of phase and purely due to the unfolding/refolding reaction (green increases, while red decreases upon unfolding, and *vice versa*). They phase-shift increasingly with respect to the temperature driving waveform (black line) as the driving frequency increases, while the signal amplitude decreases. (Bottom) Different linearly decreasing quantum yields of the labels are included, as measured in Ref. 21. Now the green and red signals appear nearly in phase, and out of phase with the temperature driving waveform. As the frequency of the driving waveform increases, the folding signal phase shift increases, but its amplitude decreases, yielding the observed maximum in the net phase shift as a function of frequency.

towards 270° . The fluorescence oscillations are attenuated because the folding and unfolding reactions are slow compared to the driving waveform, and cannot go to completion when the temperature drops or rises rapidly at the higher driving frequencies. The conventional Bode plot of phase and amplitude vs. driving frequency (inset in Figure 5) describes this simple situation. At the bottom of Figure 4, we show a signal dominated by the temperature-dependent quantum yield. The quantum yields of red and green fluorophore nearly instantaneously respond to temperature changes (on the sub-second

FIG. 5. Experimentally observed phase shifts between the A = acceptor and D = donor fluorescence as a function of driving frequency. The phase shift goes through a maximum at about 5 Hz. Two average sample temperatures are shown, with modulation up to 33.5°C (blue) and 38°C (red). “p-p” refers to the peak-to-peak amplitude of the driving sine wave. The black lines are fits to the model described in the text. The inset shows the expected phase shift (black) and amplitude (gray) for a sinusoidally driven reaction relative to the driving waveform in the absence of interfering effects such as temperature-dependent quantum yield.

time scale of the experiments), so both green and red fluorescence are approximately 180° out of phase with the temperature waveform (blue). Their amplitudes decrease very little when the driving frequency is increased.

When the folding reaction and quantum yield combine, a phase shift occurs both between fluorescence and temperature (A-T and D-T), as well as between the two fluorescence colors (A-D). We fit the phase difference between the A and D channels to our model because A and D are measured in the same experiment and their relative phase is most accurate. (T is measured by turning off the blue LED and turning on the amber LED and measuring the red channel again.)

C. Frequency-dependent donor-acceptor phase: Experiment and model

Figure 5 shows the experimentally measured phase shifts between acceptor and donor fluorescence of PGK-FRET at 32°C and at 37°C as a function of driving frequency. The phase shifts were obtained from a series of signals as shown in Figure 3 at different driving frequencies. The experimental signal combines temperature-dependent reaction and quantum yield, reaching a maximal phase shift at ≈ 5 Hz. The lower temperature data in Figure 5 has the smaller maximum phase shift because the reaction ($T_m \approx 38^\circ\text{C}$ for PGK) contributes less to the signal. The phase shift maximum at ≈ 5 Hz arises because the phase shift of the folding kinetics increases towards 90° with frequency, but the amplitude of the kinetics decreases when the frequency increases above the reaction rate. The quantum yield signal has a constant phase shift (always 0°), and at very high frequencies it is the only contribution to the observed signal, dragging the phase shift back down to 0° .

Here we limit ourselves to a simple effective two-state analysis of the data. Table I shows the kinetic parameters extracted from the effective two-state kinetic model (Sec. II). The D/A ratios of the native and denatured state, as well as the slopes m of the donor and acceptor quantum yields as a function of temperature were fixed at previously measured values (Ref. 21 and Figure 1). The “speed limit” prefactor of PGK was fixed at $\nu^\dagger = (10 \mu\text{s})^{-1}$, close to the estimate by Sabelko *et al.* for denatured PGK relaxation upon temperature jump in an acidic buffer.²² The fit to the experimental data is shown as a black line in Figure 5. The model describes the experimentally observed phase shift fairly well at both lower and higher average temperatures.

It is worth noting that the combination of prefactor and activation free energy shown in Table I produces a refolding rate about 5–10 times faster than values reported for PGK-FRET in aqueous buffer at similar temperatures using ms T-jumps.^{19,23} We believe that this discrepancy is due to the multi-state folding nature of PGK, which can only be approx-

imated by the two-state assumption; as shown by Osvath *et al.* and Sabelko *et al.*, PGK folds through a hierarchical progression of intermediates, and our frequency window samples a higher range of frequencies than the response function of the T-jump experiment.

D. Modulating reaction kinetics with artificial thermal noise

We also employed stochastic modulation of the PGK folding kinetics. In that case, the heating laser provides artificially enhanced thermal noise compared to the natural thermal noise, which decreases as $\sqrt{N}$ with particle number N . Periodic and random driving waveforms can be combined in any ratio, as seen in the experimental signals in Figure 6,

$$T(t) = T_0 + \frac{1}{2}\Delta T_{\text{periodic}}\sin(2\pi\nu t + \varphi) + \frac{1}{2}\Delta T_{\text{rand}}g_{\text{rand}}(t). \quad (8)$$

Here $g_{\text{rand}}(t)$ is a random waveform of average value 0 and rms value of 1. The function g_{rand} is obtained by computing Gaussian-distributed pseudo-random numbers sampling the same time intervals as the sine wave, and passing the waveform through a first order low-pass filter ($\Delta\nu = 13.3$ cutoff frequency in Figure 6).

For random driving waveforms, the data cannot be fitted to a sine wave to extract phase shifts. Instead, kinetic signals are extracted from the data as follows: the D and A pseudo-random fluorescence waveforms are sampled over an integer number of periods exactly (when there is an underlying periodic signal), or over a long enough period so aliasing becomes unimportant. The red and green signals are then scaled to the same average and standard deviation and subtracted from one another, which eliminates any instantaneous (quantum yield or unresolved fast reaction) components. The difference signal shape and phase can then be compared with the driving temperature waveform.

At low temperature ($T_m - T_0 \approx 4$ K) and low noise level ($<5\%$, Figure 6(a)), no significant kinetic signal is obtained, the D and A oscillations are due to the temperature-dependent quantum yield. At low T but higher noise level (10%, Figure 6(b)), a weak phase-shifted periodic signal due to reaction can be seen. As a control, at higher T and with 30% stochastic modulation, a strong phase-shifted signal due to the folding kinetics is observed (Figure 6(c)), with the same phase shift as the signal induced at low temperature. It can also be seen clearly in Figure 6(c) that larger noise spikes in the temperature (at 0–2 and 6–8 s) produce a larger retrieval of signal.

IV. DISCUSSION

Our experimental results show that models of the type developed by Lemarchand and co-workers¹⁴ can be used to

TABLE I. Effective two-state parameters for PGK unfolding/refolding driven by periodic temperature modulation.

T_m (K)	g_T (kJ/K)	$\nu^\dagger$ (s^{-1})	$\Delta G^\ddagger$ (kJ/mol)	D_F/A_F^a	D_U/A_U^a	m_D^a	m_A^a
310 ± 1	0.104 ± 0.02	10^5 (fixed)	22.3 ± 0.5	$0.5/0.5$ (fixed)	$0.7/0.3$ (fixed)	-0.011 (fixed)	-0.016 (fixed)

^aValues taken from Ref. 21 and held fixed.

FIG. 6. PGK reaction dynamics subject to a sine wave temperature modulation with stochastic component. The bleaching-corrected GFP donor fluorescence (*D*, in green), mCherry acceptor fluorescence (*A*, in red), and their scaled difference (Diff. signal in black, top) are shown, as well as the calibrated temperature (blue). (a) <5% noise, no significant reaction is seen in the difference. (b) Same dynamics subject to a higher noise level (10%), where a small phase-shifted signal is seen (model prediction in red). (c) Control experiment: same dynamics subject to a higher average temperature and a large stochastic component (30%). The green–red difference signal now contains a large, phase-shifted (compare black to green or red curve at vertical dotted line) unfolding/refolding signal component. The phase shift in (b) is similar to the control (c).

extract kinetic and thermodynamic information from protein folding modulation data. In principle, single-step relaxation and modulation techniques extract the same kinetic and thermodynamic information from a reaction. In practice, they can have different advantages in different situations.

The most important potential advantage of temperature modulation, discussed by Lemarchand, and particularly true when arbitrary waveform temperature modulation is available, is that it can allow dissection of more complex mechanisms. Although we are not pursuing a multi-state analysis of PGK folding here, it is evident from our results for PGK-FRET that multiple states are in play: the frequency range used here emphasizes faster rate coefficients than T-jumps done on the same instrument with the same FRET-labeled protein, and yields a faster folding time. The actual mechanism of PGK is thought to be hierarchical with many time scales from microseconds to minutes, and the folding kinetics can be described by logarithmic oscillations superimposed on a stretched exponential or by multiexponential kinetics.¹⁷ (Fitting data with stretched exponentials and logarithmic oscillations is discussed in detail by Klafter and co-workers, Ref. 24.)

The practical advantage of arbitrary waveform modulation for more complex kinetic processes can be appreciated by seeing what information can be extracted from single-step or multi-step jumps in a simple case. For example, consider the two reaction schemes

Both have three states and three rate coefficients, but different mechanisms (parallel paths vs. sequential intermediate). Following a single small step perturbation (e.g., a temperature jump), both reactions have exact analytical solutions that can

be of the type $y \sim Ae^{-kt} - A'e^{-k't}$, where the amplitudes and rate coefficients depend in complex ways on the initial populations of states A, B, and C and on the rate coefficients of individual steps. Thus a single jump, such as a simple temperature jump, cannot distinguish these reactions. However, a rapid sequence of an upward-jump followed by a downward-jump can distinguish the two cases; the first reaction is irreversible, while the second reaction would allow a return to “A” for a large fraction of the population if $k_{BC} \ll k_{AB}$. A temperature modulation is just an arbitrarily controllable sequence of such up-down jumps. An arbitrary waveform therefore could distinguish two arbitrarily complex mechanisms if it is specifically tailored to maximize the experimental difference between two mechanisms to be compared.

The systematic tailoring of reaction-driving waveforms to best distinguish two or more proposed mechanisms remains an unsolved theoretical problem, but hopefully the advent of modulation experiments will stimulate such theoretical developments.

The other interesting use of arbitrary waveforms is that noise, superimposed on oscillations below the detection level of the reaction, can enhance the detected signal. Although we simply observe a signal enhancement here, not stochastic resonance (which requires a signal peak as a function of noise intensity or bandwidth¹³), it has been proposed by Davtyan and Papoian²⁵ that folding reactions driven by periodic or random temperature waveforms may exhibit stochastic resonance. Although live cells are unlikely to make use of temperature fluctuations due to the high heat conductivity over cell dimensions, stochastic fluctuations of crowding, pH or other in-cell environmental variables could play a role in driving reactions. Examples include disordered signaling proteins that fold only when they bind, or formation of rare RNA-protein complexes where stochastic modulation could enhance function. The use

of artificial noise to drive such reactions could shed light on the role of noise in biological processes.

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