Fast-Folding Kinetics Using Nanosecond Laser-Induced Temperature-Jump Methods

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i. Abstract

The development of ultrafast kinetic methods is one of the factors that allowed the research on protein folding to flourish over the last twenty years. The introduction of new optical triggering techniques enabled to experimentally investigate the protein dynamics at the nanosecond to millisecond timescale, allowing researchers to test theoretical predictions and providing experimental benchmarks for computer simulations. In this work the details of how to perform kinetic experiments by the laser-induced temperature jump technique, using the two most commonly used probing techniques (namely infrared absorption and fluorescence spectroscopy) are given, with a strong emphasis on the practical details.

ii. Key words

Protein Folding, Folding Kinetics, Pump and Probe, Temperature jump, Infrared absorption, Fluorescence, Förster Resonance Energy Transfer.

1. Introduction

Protein folding is a central problem in protein science: in order to be able to perform their function proteins have to fold rapidly and reliably (i.e. avoiding misfolding and aggregation) to a specific three-dimensional structure that has been designed by evolution to accomplish that particular task. The theory of protein folding states that the process does not occur following a single well-defined pathway

analogous to elementary chemical reaction, is rather a heterogeneous process in which individual polypeptide molecules can follow very different pathways. Energetically, the problem is described by a hyperdimensional energy landscape, which for well evolved proteins has an overall funnel shape where the unfolded ensemble corresponds to the broader end (large conformational entropy) located at higher energy, whereas the folded state lies at the tip of the funnel, corresponding to the global energy minimum and a minimal number of conformational sub-states[1]. The folding of a protein is then described as the diffusive motion on such energy landscape, which on top of the overall funnel shape contains a rough topography arising by the transient occurrence of non-native interactions that will need to be broken in order to reach the correct folded structure. Roughness plays a crucial role in protein folding kinetics because escape from such local minima can represent the main bottleneck for the process, especially when it is not thermally activated, i.e. when the energy barrier between the folded and unfolded ensembles becomes small or even negligible. Predictions of protein folding kinetics pointed to the existence of fast processes, even complete folding when the global diffusive process goes downhill, that take place below the ms timescale of conventional stopped-flow kinetic experiments. These theoretical predictions were the main inspiration for the development of new ultrafast kinetic experiments that could test them and which have resulted in a major turning point in protein folding research[2].

Time-resolved experiments monitor the relaxation of a protein to its equilibrium condition in response to an external triggering event (perturbation). The triggering event defines the zero time, which is limited by how fast the perturbation can be applied. The first kinetic study on a fast-folding protein used a photochemical trigger to rapidly initiate the folding process, namely the photo-dissociation of carbon monoxide from denatured cytochrome c[3]. Other optically-initiated trigger methods based on photo-induced isomerization[4] or photolysable groups[5] have been proposed. These methods are attractive due to the very high time resolution that is limited only by the onset of the photo-induced reaction, but they

require to modify the protein to introduce the reactive groups for the photo-triggering, with the possibility to introduce structural perturbation to the system under study, thus limiting their use to a small number of proteins.

A class of generalizable optical triggers are the laser-induced temperature jump techniques. The basic concept is to use a pump and probe approach in which two beams are focused on the same spot on the sample; the pump beam induces the perturbation and the probe beam reports about its effect on the sample. As pump beam, the laser-induced temperature jump uses intense infrared laser pulses at a frequency tuned to match the energy of the vibrational modes of the solvent molecules (typically water or deuterated water), thus inducing a local increase in the temperature by vibrational excitation of the solvent, typically in less than 10 ns (when a Q-switched laser is used as pump). The subsequent relaxation of the protein to adapt to the increased temperature is then monitored using a spectroscopic probe, most commonly in this case infrared absorption or fluorescence spectroscopy[6,7]. These methods can be applied to virtually any protein, since the only requirement is that the perturbation of the folding-unfolding equilibrium induced by the temperature jump (which is universal since protein folding involves a significant change in enthalpy) is reflected in a change of the probe spectroscopic signal. Furthermore, the temperature jump technique has an excellent dynamic range, permitting to track the process from the onset of the temperature jump (few ns) up to ms.

1.1. Infrared absorption

Infrared spectroscopy is one of the classical biophysical methods used to characterize the structural features of peptides and proteins. In particular, Fourier Transform Infrared absorption is a popular ensemble technique used to characterize protein unfolding at equilibrium conditions. The amide bond exhibits 9 vibrational modes, identified as amide A, B and I-VII bands: these absorption bands are very sensitive to the protein conformation and from their analysis it is possible to extract information about

the secondary structure of the protein[8]. The most useful band for the analysis of protein secondary structure is the amide I band, which falls in the range 1600-1700 cm^{-1}, and is mainly due to stretching of the C=O bond. The actual value of the amide I resonance depends on the local structure of the amide bond; thus, it is possible to correlate the position of the peak with the secondary structure of the peptide bond, which can be forming α-helices, β-sheets, β-strands, loops, turns or disordered regions. The IR absorption of a typical protein is usually a broad band that results from the summed contributions of all its peptide bonds, each in its particular type of secondary structure. This spectrum can be deconvolved to calculate the average secondary structure contents of the protein.

In the laser-induced temperature jump experiment the goal is not necessarily to measure the whole IR spectrum of the protein, but to characterize the time response of the protein in response to the conformational readjustment upon partial thermal unfolding. Therefore, the probe beam is a laser whose wavelength is tuned to match the maximum of the amide I band of the protein to be studied; the experiment can be repeated at different wavelengths to extract information from different structural properties (keeping in mind that the bands for various secondary structures usually overlap).

1.2. Fluorescence spectroscopy

Fluorescence spectroscopy is a common biophysical technique[9] complementary to IR absorption. The intrinsic fluorescence of proteins is normally due to the presence of tryptophan, the amino acid with the strongest fluorescent quantum yield, whereas other amino acids are either non fluorescent or very weekly fluorescent (tyrosine and phenylalanine). The position and intensity of the tryptophan emission peak is strongly affected by the local environment (i.e. the degree of exposure to the solvent, or interactions with other residues that can act as quenchers), characteristic that can be used as diagnostic of the integrity of the protein tertiary structure. If the protein of interest does not have any tryptophan, it is possible to label it with extrinsic fluorophores that can be used to report the local environment of

the labelled position, for example by studying fluorescence quenching, or to measure the distance between two specific protein locations from the Förster resonant energy transfer (FRET) efficiency between two suitable extrinsic fluorophores.

FRET arises from a dipole-dipole interaction between a donor fluorophore in its excited state and an acceptor fluorophore in its ground state. The main requirement is that the fluorescence spectrum of the donor overlaps with the absorption spectrum of the acceptor (i.e. the overlap integral should be non-null). If this condition is accomplished, radiation-less transfer of excitation from the donor to the acceptor occurs via a dipole-dipole interaction. The transfer efficiency depends on the spectral overlap, on the relative orientation of the transition dipoles of the two dyes and on their distance. The distance dependence is of particular interest for biophysical applications . Due to the dipole-dipole nature of the interaction, the FRET efficiency scales as the inverse of the sixth power of the inter-dye distance according to the expression:

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (1)$$

where E is the FRET efficiency, r is the inter-dye distance and R_0 is the characteristic Förster radius, i.e. the distance at which the efficiency is 0.5 for a specific donor/acceptor pair. Thanks to this relation, by experimentally measuring E it is possible to determine the average distance between the two fluorophores. For typically used fluorophores couples the accessible distance range is 2-8 nm, making FRET a particularly suitable “spectroscopic ruler” to detect protein conformational transitions.

The implementation of fluorescence in laser-induced temperature jump experiments requires a probe beam tuned to excite either the intrinsic fluorescence of the protein (i.e. the tryptophan absorption maximum at 280nm) or of the donor fluorophore for FRET applications.

2. Materials

2.1. Instrumentation

A typical setup for an IR absorption laser induced temperature jump instrument is shown in Figure 1. The pump beam originates from a pulsed infrared laser run at a low frequency (1-10 Hz) (see **Note 1**). Typically a Q-switched Nd:YAG is used for this purpose. Because the fundamental of this laser (1,064 nm) is not in resonance with the vibrational modes of D_2O (the solvent used for protein IR spectroscopy), the beam is converted to longer wavelengths taking advantage of the stimulated Raman emission effect (a Raman cell filled with high-pressure H_2 is used for this purpose). The output from the Raman cell contains the fundamental plus different Stokes and anti-Stokes conversions. This heterogeneous output is dispersed by a Pellin-Broca prism, and the beam corresponding to the first Stokes beam at 1,907 nm is chosen for D_2O excitation. The protein sample dissolved in D_2O is placed on a IR transmission spectroscopic cell (e.g. two $CaCl_2$ windows separated by a Teflon spacer) of typically 50 μm pathlength. The 1,907 nm pump beam is then focused onto the sample (with a spot of about 1 mm diameter), where it induces the rapid increase in temperature (the thin spacer thickness facilitates relatively uniform heating along the pathlength with only one pump beam coming from the front). The probe comes from a continuous wave quantum cascade laser that is tunable within the range of the amide I band. This beam is focused to the center of the heated volume to maximize the magnitude and homogeneity of the temperature jump. The transmitted probe beam is collimated and then focused on a fast mercury-cadmium-telluride (MCT) detector. The output of the detector is analysed by an oscilloscope that is synchronised with the pump beam.

A typical setup for a spectrally-resolved fluorescence laser induced temperature jump instrument is shown in Figure 2. The configuration of the pump beam is similar, with only two differences: (i) the Raman converter is filled with D_2 in order to obtain a first Stokes beam at 1,561nm that is in resonance with the absorption spectrum of regular H_2O; (ii) the pump beam is separated in two halves, using one

beam to reach the sample from the front and the other one from the back (see **Note 2**). The probe beam is emitted by a laser with an appropriate wavelength to excite the singlet state of the fluorophore in the protein of interest. In the scheme depicted in Figure 2 the fourth-harmonic of a Nd:YAG at 266 is used to directly excite tryptophan (see **Note 3**). If the protein is labelled with extrinsic fluorophores other excitation sources are needed, e.g. the second or third harmonic of a Nd:YAG laser or other kind of pulsed lasers. In this setup the pulsed probe laser is run at the same frequency of the pump laser and triggered using a pulse delay generator, so that the time delay between the pump and probe pulses can be carefully set and changed automatically following a predefined sequence. The sequence of time delays generates the full kinetic experiment (each probe pulse delayed by a specific time relative to the pump pulse provides a given time point in the kinetic decay of the protein). The probe beam is then coupled to the front pump beam with a dichroic filter, making them collinear, and is focused to a diffraction limited spot on the sample and in the centre of the pump beams. The fluorescence emitted by the sample is collected at 90° (see **Note 4**), and focused to the entrance slit of a spectrograph connected to a CCD camera that records the spectrum. A kinetic experiment in this type of setup is conducted by performing a sequence of pump-probe pulses in which the time between pump and probe is varied between 1 ns and 10 ms by the time delay generator. After the probe pulse is fired, fluorescence emission is collected, resolved spectrally via a spectrograph and recorded by the CCD camera. Each recorded fluorescence spectrum corresponds to a snapshot of the properties of the protein at a specific time in the reaction, as determined by the delay between the two pulses (see **Note 5**). The system is run at a constant rate (1-10 Hz) set so that it is sufficiently slow to ensure full heat dissipation and return to the initial temperature (before the temperature jump) before the next cycle. A programmed sequence of time delays is then performed at that rate to generate a set of time-resolved fluorescence spectra (each spectrum could be from a single shot or from an average of multiple shots).

2.2. Chemicals and reagents

Buffer solutions should be prepared with the highest possible purity: MilliQ water or HPLC-grade water should be used. For the infrared experiments, NMR-grade D_2O should be used. All the chemicals needed to prepare the buffers should be at the highest available purity, especially for the fluorescence experiments where the presence of impurities can give unexpected background. N-acetyl-tryptophanamide (Sigma), or another reference fluorophore that can be excited at the probe wavelength and has strongly temperature-dependent quantum yield, is needed to calibrate the temperature jump in the fluorescence experiments. Samples for tryptophan fluorescence temperature jump experiments should be prepared in a 2% v/v solution of carbon disulphide (CS_2, Sigma), which acts as an efficient tryptophan triplet quencher to minimize triplet buildup that can result on decreased and/or delayed fluorescence.

2.3. Other materials

Laser burn paper is used to record the position of the pump laser spot.

Temperature-sensitive liquid crystal sheets are used to locate the far-infrared probe beam of the infrared experiments.

Sample cuvette holders with Peltier temperature controllers.

Optical power and energy meters.

3. Methods

3.1 Sample preparation for infrared absorption measurements

- Protein production. Different strategies can be used to obtain the protein object of the study. The most common technique uses cell culture (bacteria, yeast or eukaryotic cells) to over express recombinant proteins[10]. This approach renders high yields and is generally applicable to

any protein. Other synthetic approaches are solid-phase peptide synthesis[11] (which is typically limited to protein of less than 100 amino acids) or cell-free *in vitro* protein synthesis[12], which may be preferable for proteins difficult to express recombinantly, but at the expense of lower production yields.

- Protein purification. Depending on the specific protein and on the method used to produce it, different purification strategies can be used. For example, his-tag, ionic exchange, reverse phase, size-exclusion or other types of affinity chromatography methods are available. If the final concentration after purification is insufficient, a step in which the protein sample is concentrated by ultrafiltration might be required.
- Protein deuteration. H_2O has strong absorption in the IR region overlapping with the amide I band. This is a major limitation for measuring the amide I band of proteins in solution. However, the absorption of D_2O is shifted to lower wavenumbers relative to H_2O so that it does not overlap with the amide I band anymore. For IR measurements in aqueous solution (as opposed to dry films) it is therefore necessary to substitute H_2O by D_2O as solvent for sample preparation. The labile amide protons of the protein must be deuterated, and suitably deuterated buffers should also be used. To achieve uniform deuteration of protein amide protons it is necessary to repeat several cycles of lyophilization to remove H_2O followed by dilution in pure D_2O (see Note 6). Typically, three cycles are enough to achieve the complete deuteration of the protein. As a final step, the effectiveness of the deuteration process should be evaluated by measuring the IR absorption spectrum of the sample resuspended in D_2O with the final deuterated buffer.
- Sample preparation. The final sample should be prepared in deuterated buffers using chemicals which don't have any absorption in the IR region of interest. The final concentration of the protein should be in the range 0.5-2.5 mM, depending on the amount of secondary structure of

the actual protein. Not being able to reach these high protein concentrations is often times the main limitation for IR temperature jump measurements. On the other hand, the required volume of protein solution needed for a single measurement is very small (less than 20 µL), then the sample can be prepared at the minimal volume required to measure its pH using an ultrathin pH electrode (similar to those use for NMR sample preparation).

- pH adjustment. For these experiments it is important to realize that the readout of a glass electrode used to measure pH is affected by D_20, resulting in an isotopic shift relative to electrode reading in pure water[13]. The addition of salts and/or denaturants in very high concentrations also affect electrode-based measurements of pH[14]. These effects are usually accounted for by applying a simple linear correction to the glass electrode reading.
- Mounting the sample in the cell holder. The sample cell is constituted by two windows of CaF_2, or of any other material that does not absorb in the range of the pump and probe wavelengths. Typical windows are round windows with a diameter of 12 mm and a thickness of 2 mm. The sample solution is sandwiched between the windows with a Teflon spacer with thickness that defines the optical path length, as depicted in Figure 3, left. Typical spacer thickness is about 50 µm to minimize the decay of the temperature jump along the path length due to the solution's absorbance of the pump beam. The cell should be mounted manually, clamped together (see Note 7), and held in a customized sample holder that is temperature controlled (for example using a Peltier thermoelectric system) to accurately define the initial temperature of the experiment (before the temperature jump).

3.2 Sample preparation for fluorescence measurements

- Protein production. If the protein to be studied is intrinsically fluorescent (i.e. has tryptophan residues), the same production strategies described in the section 3.1 can be used. If the protein

is not fluorescent, fluorescent labels need to be attached to the protein using standard protein chemistry procedures. The addition of one extrinsic fluorophore enables measurement of fluorescence quenching and/or photon-induced electron transfer. Adding two different fluorophores enables Förster resonant energy transfer (FRET) measurements in which one fluorophore act as donor and the other as acceptor of the resonant energy transfer process. The easiest way to achieve this goal is to modify the protein sequence inserting one or two cysteines that will be used for the subsequent labelling[15]. If the protein has natural cysteines that are not essential for the biological function and/or stability of the protein, they should be mutated out. The new mutants should be tested to verify that the point mutations do not alter the structure, stability and activity of the wild type protein. Other strategies have been proposed, for example the incorporation of unnatural amino acids that allow click chemistry with orthogonal reactivities to achieve site selectivity and specificity[16]. The labelling with the fluorescent dyes should be done following the instructions of the manufacturer for the specific reactive group. See chapter on FRET labelling for more technical details on how to label proteins with extrinsic fluorophores.

- Protein purification. The same purification protocols cited in section 3.1 are to be used for obtaining proteins for fluorescence temperature jump experiments. For samples for FRET experiments, the protocols should be optimized to ensure that the sample only contains protein molecules simultaneously labelled with both donor and acceptor (see chapter on FRET labelling).
- Sample preparation. The sample should be prepared in the required buffer at a final concentration in the range 1-10μM. For tryptophan measurements, 2% v/v C_2S can be added to the sample to minimize tripled buildup.
- Mounting the sample in the cell holder. For fluorescence experiments, since the detection path is perpendicular to the excitation path, the whole sample cell needs to be completely

transparent both in the UV-VIS (for the probe beam and the fluorescence spectrum) and in the NIR (for the pump beam). Practically, one can use UV-silica grade cuvettes with a sample thickness in the range of 100-500 μm. These cuvettes are constituted by two rectangular plates. One is carved at the required pathlength thickness to contain the sample. The other plate is set on top of the carved plate filled with the sample solution (using 1.5 times the void volume), starting from one end and setting the plate carefully (by slowly dropping the other end of the top plate until it touches the bottom plate) to avoid formation of bubbles. The top plates are then pushed together applying gentle pressure so that the excess liquid is removed and the two plates become stuck by capillarity. See for example the sample cell depicted in Figure 3, right.

3.3 Infrared temperature-jump kinetic measurements

- Set-up optimization. The probe beam needs to be aligned onto the MCT detector, which is normally done by maximizing the readout signal. This can be done by inserting a mechanical chopper on the probe beam path so that an easily recognizable pattern can be observed on the oscilloscope and maximized in amplitude. Once the probe beam is aligned onto the detector, the pump beam must be aligned by adjusting the last mirror to steer the pump beam until the probe beam is centered onto the larger probe beam. To maximize the overlap of the two beams, the position of the pump beam should be scanned along the X-Y axes looking to maximize the change in transmission of a sample with only buffer (no protein) taking place concomitantly to the pump pulse (about 8 ns). This sudden change in transmission reflects the jump in temperature induced by the pump beam on the sample (Figure 4, left). Therefore, this sudden change in transmission can be used to optimize the alignment since it will be maximal when the probe beam is exactly placed in the center of the pump beam (which has a Gaussian beam

profile). This step in transmission is also used to determine the magnitude of the temperature jump.

- Temperature jump calibration. Before making the actual measurements, the amplitude of the temperature jump must be calibrated. As first step one must measure what is the conversion factor between the amplitude of the detector readout (which depends on the specific setup and alignment) and the transmitted intensity as a function of temperature. The sample cell should be filled with just the buffer, and the magnitude of the step in transmission needs to be measured at different temperatures covering the required temperature range, as this change in transmission depends on the initial temperature. The signal measured before the pump pulse is fired is proportional to the intensity of the transmitted light at the initial temperature (base temperature at which the whole sample holder is kept). A plot of the magnitude of the step in transmission as a function of the base temperature will give rise to a linear correlation in which the slope provides the conversion factor from buffer transmission to temperature. One can then set the pump pulse energy to achieve the desired temperature jump. For example, using 8 ns pump pulses of 30 mJ it is usually possible to obtain temperature jumps of about 10-12 K.
- Data collection and pre-analysis. The actual experiment consists on a measuring a series of time traces at different initial temperatures. The buffer and the sample containing the protein must be measured in exactly the same temperature conditions. A single time trace is normally very noisy so that it is usually necessary to acquire hundreds of traces and average them. This can be simply done by setting the oscilloscope to acquire directly the average of the appropriate number of traces (the instrument is run at a constant rate of 1-10 Hz). One can also acquire single traces directly, which can then be averaged at the time of analysis. This second strategy is more cumbersome since it requires longer acquisition times and produces much larger datasets that need to be post-processed, but it allows for a more accurate data analysis. This is because

the pulsed energy of the pump laser is not constant and thus the temperature jump exhibits pulse-to-pulse variability that introduce uncertainty in the experiment. The availability of single traces allows to select traces within a certain temperature-jump threshold and discard the rest (rather than average all of them). Recording single traces also allows to identify traces with clear signs of cavitation, which is the major sources of artifacts in laser-induced temperature jump experiments, especially at higher temperatures (see Note 8).

- The time-resolved IR absorption kinetic decays. The kinetic trace reflecting the time-dependent changes in amide I band absorption of the protein in response to the jump in temperature starting from the initial temperature are obtained from the averaged time traces of the sample with only buffer and the sample containing the protein as:

$$\Delta A(t) = -Log\frac{V_{sample}}{V_{buffer}} \quad (2)$$

where V_{sample} and V_{buffer} are the measured traces for the sample and the buffer respectively. The resulting decay can be fitted with the appropriate decay function that describes the system under study. An example of a typical infrared T-jump kinetic decays is shown in Figure 4.

3.4 Fluorescence temperature-jump kinetic measurements

- Set-up optimization. In these experiments the most critical aspect of the alignment is to ensure the required overlap of the three excitation beams (the front and back pump beams and the probe beam) onto the sample. In this case the optimal alignment of the three beams needs to be carried out daily because the exact position of this multi-focal point is strongly sensitive to minor changes in the detailed alignment of all components. The position is defined by the probe excitation beam, that should be focused to approximately the center of the sample cell. The

front and back pump beams need to be aligned so that their center coincides with the probe beam (which is typically diffraction limited, and thus much smaller in diameter at the focal point than the ~1 mm diameter of the pump beams). A first coarse alignment of the pump beams is carried out using burn paper to signal the position of the infrared pump beam and adjusting the last mirror in the beam path to set its approximate position relative to the probe beam. After the coarse alignment is done for both front and back pump beams, it needs to be optimized by maximizing the amplitude of the temperature jump (see below). The position of the fluorescence collection optics needs to be optimized as well. The collection optics alignment is performed by placing in the sample cuvette a solution of a reference dye, recording its spectrum with the spectrograph/CCD camera, and maximizing the overall intensity as a function of the optics position.

- Temperature jump calibration. The temperature jump is determined from the decrease in fluorescence intensity induced by the pump probe in a reference sample with a well characterized temperature dependent quantum yield. For example, the fluorescence intensity of tryptophan decreases by about 1% for each degree of increase in temperature[17]. The temperature jump is determined from the differences in intensity between two spectra: one acquired right after the temperature jump is completed (maximal temperature), which practically means exciting the sample at a time delay slightly longer than the duration of the pump pulse (e.g. 15 ns); and a second spectrum corresponding to the base temperature, which is recorded by exciting the reference sample at a time previous to the pump pulse period, and thus previous to the temperature jump.
- Data collection. In this type of fluorescence temperature-jump instrument a kinetic experiment consists on a series of fluorescence spectra recorded after firing the ~5 ns pulsed probe laser (here the probe is a pulsed UV laser rather than the CW laser used for infrared experiments) at

different times relative to the pump pulse. The spectrum at each time delay corresponds to a snapshot of the fluorescence properties of the protein sample under study at that given time from the heating pulse. A complete experiment consists in cycling through a series of suitable time delays and recording a matrix of fluorescence spectra as a function of time (see Note 9). Similarly to the IR experiments, it is usually convenient to average several spectra for each delay time to minimize shot-to-shot variability and fluctuations in fluorescence intensity. In addition, care must be exerted to ensure that the pulse energy of the probe excitation laser is kept low enough to avoid photobleaching and blinking of the fluorophores. Alternatively, one can use photoprotection cocktails added to the sample solution to minimize the photo-degradation of the sample, or employ a customized cuvette fitted with entry and an exit ports to permit the circulation of fresh sample during the experiment.

- Data analysis. The resulting three-dimensional dataset (fluorescence intensity vs. wavelength vs. time) is best analyzed using singular value decomposition (SVD) procedures[18], which is an efficient method to further reduce noise (using the entire matrix of time-resolved fluorescence spectra) and identify relevant components that represent the temporal evolution of specific spectral features. The first SVD component (the one with highest singular value) corresponds to the average spectrum and its amplitude reflects its variation as a function of time (e.g. changes in quantum yield). Additional non-random SVD components (usually the very first few as the SVD components are ranked according to their singular values, or contributions) report on spectral changes and their amplitudes on how they change with time. These spectral changes can be either shifts of an emission maximum, or anti-correlated changes in intensity such as the ones that take place for a donor and an acceptor FRET pair. Some non-random components may also correspond to artefacts with a specific time dependence (for example a slow oscillation of the probe excitation laser energy output). Components representing well understood signal

artifacts can be easily discarded, and keep only those components that represent the changes in fluorescence properties of the sample that are of interest. The changes in amplitude as a function of time of each of the selected components can then be fitted to an appropriate time decay function (i.e. single, double, multi- or stretched exponential). Figure 5 shows an example of fluorescence temperature-jump kinetic data obtained with this type of instrument and a sample labelled with a donor/acceptor FRET pair. In this case the second component is an anti-correlation between the emission intensity of donor and acceptor fluorophores, which is proportional to the changes in FRET efficiency.

4. Notes

1. The pulse frequency should be low enough to allow for the temperature perturbation to be completely quenched when the following pulse arrives, avoiding accumulation effects that can results in a drift in the experimental conditions.

2. For the fluorescence temperature jump experiment the sample is normally placed in a UV-grade quartz cell of 0.2-0.5 mm pathlength to increase the number of molecules that are excited at a given protein concentration. The unwanted implication of the longer pathlength is that the heating profile from a single beam decays exponentially along the optical path due to the absorption of the solvent according to Lambert-Beer's law. A simple way to remedy this problem is to split the pump beam into two and bring them to the sample cell from its both sides in an counter propagating configuration (dual front-back excitation). The key here is to ensure that the two beams are as collinear as possible and focused to the centre of the sample. This symmetrical excitation setup reduces the gradient of temperature along the optical path, which is non negligible for fluorescence temperature jump experiments. In our hands the dual front-back excitation allows to achieve an almost constant temperature jump along the whole sample thickness.

3. Laser pulses at 266 nm are not optimal for tryptophan excitation because its maximum absorption is at 280 nm. An even more serious problem is that the high energy associated with 266 nm facilitates crossing the photoionization barrier from the singlet state, thus causing significant photodegradation of the sample during the experiment. There are no many existing options of ns pulsed lasers in the 280-290 nm range that is ideal for repeated excitation of tryptophan fluorescence, and the ones available are very expensive. However it is possible to convert the fourth harmonic of a Nd:YAG laser from 266 to 288 nm using stimulated Raman emission. This can be achieved by selecting with a Pellin-Broca prism the first Stokes from the combined beam exiting from a Raman cell filled with CH_4 at high pressure (500 psi).
4. The collection of fluorescence is carried out at a 90° angle despite the short pathlength of the cell (0.2-0.5 mm) to minimize the amount of stray light from the excitation probe beam as well as the two pump beams that reaches the detector. Any residual stray light can be easily removed using bandpass optical filters.
5. To improve the precision of the kinetic measurements, it is better to directly measure the delay between the pump and probe pulses rather that rely on the time set by the delay generator because the electronics controlling the laser triggering contain a certain degree of jitter in the nanosecond timescale, which is particularly problematic for the shortest time delays in the experiment. One can easily achieve this by inserting a microscope slide at a 45° angle in the beam path (for the pump beam before it is split into front and back beams and for the probe beam before the focusing lens) so that a small fraction of the light is reflected and sent to a fast (ns) photodiode that is sensitive in the required spectral range (IR or UV). The electrical signals from the two photodiodes are then sent to a digital counter that measure the actual delay between the two pulses. The photodiodes should be placed in a position such that the length of the optical path between the beam splitter and the

photodiode is identical to the length between the beam splitter and the sample (e.g. a 10 cm longer optical path corresponds to an extra delay of 0.333 ns).

6. To reach a complete deuteration of the protein amide bonds for IR absorption measurements it could be necessary either to heat the sample solution above the melting point of the protein to expose the hydrophobic core of the protein and make it more accessible to the solvent (this strategy is viable only for protein that do not suffer from misfolding or aggregation), or to perform even more cycles of lyophilization.
7. The cell assembly process starts with one window and the Teflon spacer placed on top. A sufficient volume of the sample solution is placed on top of the window's center (added volume should be about twice as much as the void volume of the mounted cell, defined by the cylinder with radius and length as determined by the spacer) to avoid the appearance of bubbles. Finally, the second window is placed on top, and the assembly is placed on the sample holder with two plastic O-rings on top and below to protect the windows from scratches or breaking. Finally, the whole assembly is clamped together by applying gentle and uniform pressure by tightening the set screws in the cell holder. A relatively high pressure needs to be applied to seal the cell assembly and limit the evaporation of the sample during the experiments (especially at the higher temperatures).
8. One of the major sources of artefacts in laser-induced temperature jump experiments is the production of photo-acoustic (shock) waves that lead to cavitation[19]. The sudden (<10 ns) and steep raise in temperature induced by the pump laser pulse produces a shock wave that propagates centrifugally from the focal point of the pump in the sample. The shockwave propagates through the sample at 343 m/s, resulting in a pressure drop from the center of the heated volume. If the pressure drop reaches a certain magnitude, the liquid cavitates producing a bubble that results in a void in the solution that strongly deflects the probe beam, resulting in an artifactual time trace. This phenomenon is stochastic, but it increases in probability as the initial temperature increases (the

water density decreases) or as the pump robe energy increases (this is what practically restricts the temperature magnitude to about 15 degrees or lower) so it becomes more problematic in experiments at higher initial temperatures. Tracers with signs of cavitation should not be used for further analysis, as the trace distortion is very marked. When traces are averaged directly with the oscilloscope, the presence of a few cavitation events may go unnoticed, resulting in distortions that affect the entire measurement. The acquisition of single traces permits to eliminate those with signs of cavitation during post-acquisition analysis. In parallel, it is good practice to try to minimize photoacoustic cavitation effects by removing the possible nucleation centers for cavitation from the solution, for example by thoroughly degassing the sample before the experiments.

Since in this type of instrument the delay times are set by the delay generator that triggers the probe laser, it is possible to perform sophisticated acquisition experiments. The delay generator is controlled by a computer in which is possible to set up the experiment so that the delays are extended in a logarithmic rather than linear timescale. This strategy enables to observe complex kinetic relaxations that extend over many time decades in a single experiment. It also allows to randomize the order of the time delays during the experiment and then reorder them to get the relevant time series. This feature is extremely useful to minimize contributions from artifactual fluctuations in fluorescence emission, such as the shot to shot drift of most pulsed lasers, or photodamage induced in the sample after so many excitations.

5. References

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Figure captions

Figure 1. Schematic representation of the apparatus for infrared T-jump measurements installed at IMDEA Nanociencia in Madrid. (abbreviations used for the components: mirror (M), lens (L), Pellin-Broca prism (PB), beam blocker (BB)).

Figure 2. Schematic representation of the apparatus for fluorescence T-jump measurements installed at IMDEA Nanociencia in Madrid. (abbreviations used for the components: mirror (M), lens (L), Pellin-Broca prism (PB), beam blocker (BB), beam splitter (BS), photodiode (PD)).

Figure 3. Typical types of sample cells used to perform T-jump experiments in the infrared (left) and in fluorescence (right). For the IR experiments, the sample solution is sandwiched between two round windows of a material transparent to both the pump and probe beams (e.g. CaF_2) with a 50μm Teflon spacer. For the fluorescent experiments, an all glass (UV-silica to be transparent at the wavelengths of both the lasers) cuvette is filled with the sample solution.

Figure 4. Typical data obtained for a kinetic experiment in the infrared. In the left panel the time traces of the protein sample and of the buffer needed as a reference are reported. In the right panel the resulted transient absorption decay is represented, with a fit to an exponential decay function.

Figure 5. Typical data obtained for a fluorescence experiment in which FRET is studied. The experimental data are a series of fluorescence spectra recorded at different delay times (left panel). Performing a singular value decomposition of the experimental data it is possible to identify the components that describe the overall experiment (central panel) and to analyze the temporal evolution of the component reporting for the FRET efficiency (right panel).