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Dual time-resolved temperature-jump fluorescence and infrared spectroscopy for the study of fast protein dynamics

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ABSTRACT

Time-resolved temperature-jump (T-jump) coupled with fluorescence and infrared (IR) spectroscopy is a powerful technique for monitoring protein dynamics. Although IR spectroscopy of the polypeptide amide I mode is more technically challenging, it offers complementary information because it directly probes changes in the protein backbone, whereas, fluorescence spectroscopy is sensitive to the environment of specific side chains. With the advent of widely tunable quantum cascade lasers (QCL) it is possible to efficiently probe multiple IR frequencies with high sensitivity and reproducibility. Here we describe a dual time-resolved T-jump fluorescence and IR spectrometer and its application to study protein folding dynamics. A Q-switched Ho:YAG laser provides the T-jump source for both time-resolved IR and fluorescence spectroscopy, which are probed by a QCL and Ti:Sapphire laser, respectively. The Ho:YAG laser simultaneously pumps the time-resolved IR and fluorescence spectrometers. The instrument has high sensitivity, with an IR absorbance detection limit of <0.2 mOD and a fluorescence sensitivity of 2% of the overall fluorescence intensity. Using a computer controlled QCL to rapidly tune the IR frequency it is possible to create a T-jump induced difference spectrum from 50 ns to 0.5 ms. This study demonstrates the power of the dual time-resolved T-jump fluorescence and IR spectroscopy to resolve complex folding mechanisms by complementary IR absorbance and fluorescence measurements of protein dynamics.

1. Introduction

The three-dimensional structure of proteins is not static; flexibility is necessary in order for proteins to function properly. Many methods have been developed to study the functional dynamics of proteins, but are limited in their ability to monitor fast dynamics with structural specificity. Experimental techniques such as X-ray crystallography, nuclear magnetic resonance and cryogenic electron microscopy can detect equilibrium fluctuations in protein structures with high structural resolution, but these populations are stochastic and such methods do not usually provide dynamics of the structural fluctuations [1]. Even single-molecule and stopped-flow methods, which have millisecond resolution at the cost of reduced structural resolution, are too slow to capture important dynamics [2,3]. Submillisecond resolution is necessary to monitor important protein processes including early events in protein folding, fast folding/unfolding of secondary structures, or fast unfolding for degradation and regulation [4,5]. Photo-triggers can access faster times, but cannot be generally applied to all proteins [6]. Laser-induced T-jump has arisen as a powerful method for investigating fast protein dynamics [7]. The free energy of a protein in solution depends on many factors including temperature, pressure, pH, and other solvent conditions. Manipulating one of these factors, temperature in the case of T-jump, changes the relative free energies of multiple alternative structural states, resulting in a new equilibrium distribution among these states. A spectroscopic probe monitors the system as it relaxes to the new equilibrium under the new conditions. Fluorescence is one of the most convenient T-jump probes, because it requires a relatively small amount of material (μM), has a high signal-to-noise ratio and the intrinsic fluorophore tryptophan (Trp) is sensitive to environmental changes. The structural specificity of fluorescence spectroscopy depends on the location of the side chain reporter, which may report on local or global structure, or both. IR spectroscopy of the amide I mode is a complementary method that offers improved structural resolution. It directly probes changes in the protein backbone, and different vibrational modes can be assigned to specific secondary structural elements.

With the advent of the widely tunable quantum cascade laser (QCL) operating in the mid-IR it is possible to efficiently probe multiple frequencies in order to monitor dynamics of multiple protein secondary structure elements. Before the availability of QCL sources, continuous wave lead salt diode lasers that were partially tunable across the

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amide I′ spectral region were used to obtain time-resolved T-jump IR transients [8]. QCLs offer several advantages over diode lasers that make it possible to probe the temporal response as a systematic function of the probe wavelength. Because commercially available lead salt diode lasers do not possess an external cavity and thus no tuning element, they can only be tuned by varying the temperature and current. As a result these lasers exhibit nonlinear tuning, mode-hopping and the tuning parameters change over time. The QCL by contrast has an external cavity and dispersive tuning element that can be computer controlled in a linear and reproducible manner, yielding finer, more rapid and reproducible control of the output wavelength. QCLs also have a broader tuning range, usually between 100 and 200 cm^{-1}. QCLs have improved amplitude stability, they are less prone to mode-hopping and they are less sensitive to temperature variations [12]. QCLs have higher output power than diode lasers, which eases alignment and improves measurement sensitivity [13]. The QCL beam diverges much less than diode lasers, and the high power allows the use of liquid crystal paper as a heat sensor to locate, focus and align the beam. The higher power also enables transmission through more optically dense samples, which in turn improves signal levels because higher sample concentrations and longer path lengths are accessible. Finally, the ability to measure multi-wavelength IR absorbance kinetics using the QCL allows us to sort complex, multi-step reactions.

T-jump has been coupled to broadband mid-IR methods, for example step-scan FTIR and 2DIR [9,10]. The advantage of broadband IR methods over single wavelength methods is that the entire spectral region of interest is collected simultaneously at a specific time following pump laser excitation, but the associated challenge is that acquisition of broadband data is relatively slow. In order to speed measurements, step-scan probed T-jump measurements are collected at a relatively low spectral resolution, typically 4–8 cm^{-1} compared to 0.0007 cm^{-1} accessible by QCL lasers [9]. High spectral resolution is desirable to detect changes in different populations; for example there is <5 cm^{-1} difference between the frequency for single and double stranded β-sheets [11]. 2DIR spectra are often only collected at select time points during broadband 2DIR measurements, and other nonlinear methods are used to collect the kinetic measurements. 2DIR measurements have several additional inherent technical difficulties including synchronization of the nanosecond T-jump laser and the femtosecond laser system, decreased signal-to-noise ratio from low repetition rate data acquisition and maintaining optical alignment as changes in index of refraction are induced by the T-jump [10].

Here we have developed a dual T-jump apparatus that is capable of simultaneously pumping transient fluorescence and IR spectroscopy experiments from a single source (Fig. 1). Eigen and Hammes pioneered the T-jump technique based on a rapid capacitance discharge to heat a solution held between capacitor plates [14]. However, the temporal resolution of this approach was limited to the microsecond timescale by the slow capacitive discharge and optical access to the solution was not possible. These limitations were alleviated by the use of a Q-switched laser characterized by its short pulse to optically heat the solution, with a T-jump response on the order of nanoseconds [15]. Typically the T-jump is achieved using a 10 Hz pulsed Q-switched Nd:YAG laser, which has a fundamental output wavelength at 1.064 μm. In order to achieve effective heating this fundamental wavelength must be shifted to match a vibrational absorbance of the solvent. D_2O is chosen as the solvent for IR spectroscopy of the protein amide I′ band, because its background absorbance is much lower in this spectral region compared to H_2O. The broad D_2O overtone absorption at 1.9 μm is selected for optical heating with a short laser pulse to produce the T-jump. Frequency shifting is often accomplished using a Raman shifter with one Stokes shift in 200 psi H_2 gas [16]. Recently, we and others have demonstrated that a Q-switched Ho:YAG laser, which has a fundamental output wavelength at 2.09 μm, can be used as an IR T-jump source [17,18]. The advantages of the Ho:YAG laser over the more widely used Nd:YAG laser/Raman shifter are its stability, beam quality and safety. There are non-linear processes in Raman shifting that result in shot-to-shot fluctuations of the pump pulse of ~15% [8,15]. Furthermore, the Raman shifter output has hot spots and is nonuniform due to the nonlinearity of the process and instability of the gas medium. By eliminating the Raman shifter in the Ho:YAG apparatus this variability decreases. The output mode of the Ho:YAG is a Gaussian TEM 00 mode, which produces a stable and uniform heated volume. The T-jump is stabilized by minimizing shot-to-shot fluctuations in the T-jump magnitude and

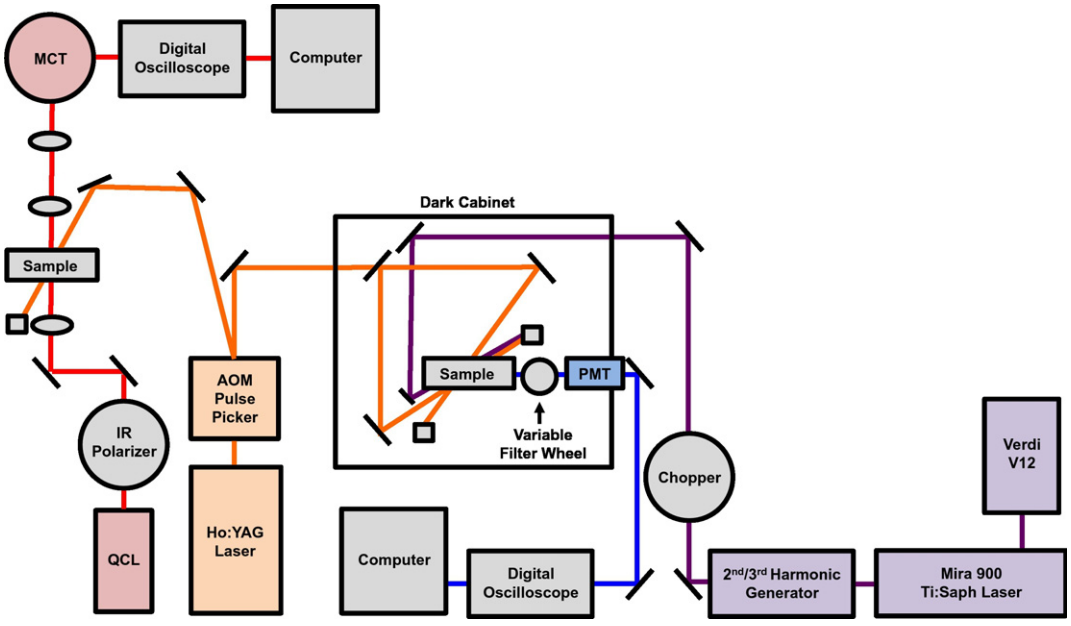

Fig. 1. Schematic of the dual time-resolved T-jump fluorescence and IR spectrometer. An AOM pulse picker reduces the repetition rate of the 50 Hz pulsed Q-switched Ho:YAG laser and produces the 2.09 μm pump radiation that is the source for both the IR and fluorescence T-jump. By using multiple quantum cascade lasers, the total frequency range spanned by the IR system is 1000–2250 cm^{-1}. Fluorescence is excited using a mode-locked, frequency tripled Ti:Sapphire laser and emission is collected with a photomultiplier tube and an appropriate band pass filter.