New light on protein folding: Unraveling folding and unfolding mechanisms using time-resolved and two-dimensional vibrational spectroscopy
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Citation for published version (APA):

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Experimental investigation of protein folding dynamics require time-resolving the structural rearrangements of the protein during the (un)folding transition, which occur typically on time scales ranging from nanoseconds to microseconds. In this chapter, we describe the experimental methodologies used in the acquisition of equilibrium and non-equilibrium protein folding data. In the first part we focus on the methods used for equilibrium investigations of protein folding, and introduce the basic concepts behind the equilibrium experiments as applied in this thesis, including UV circular dichroism and two-dimensional infrared spectroscopy in the frequency-domain. The second part of this chapter describes the experimental realization of the time-resolved infrared measurements that were performed to investigate the structural dynamics of different peptides and proteins studied in this thesis. In all our time-resolved experiments, initiation of the folding and unfolding dynamics is achieved by a rapid perturbation of the thermodynamic equilibrium by means of a laser-induced temperature-jump, and the subsequent re-equilibration is followed by transient infrared spectroscopy.

2.1 Equilibrium measurements

2.1.1 Circular dichroism spectroscopy

The circular dichroism (CD) response of the polypeptide backbone, arising from the differential interaction of asymmetric peptide bonds with left- and right-handed circularly polarized radiation during electronic excitation, is probably the most commonly used spectroscopic probe of secondary structure in protein folding studies. The amide chromophore of the peptide bond is optically active in the far-UV
Experimental methods

Figure 2.1: Characteristic UV-CD patterns arising from an α-helix (orange), β-sheet (blue), and random coil conformation (red).

(170–250 nm), and absorption in this spectral region originates from a weak but broad $n \rightarrow \pi^*$ transition centered around 220 nm and an intense $\pi \rightarrow \pi^*$ transition at about 190 nm [1–4].

The CD response of a (protein) molecule is defined as the difference in extinction coefficient $\Delta \epsilon$ between right- and left-handed polarized light. As the absorbance ($A$) usually obeys Beer’s law, the difference can be expressed as [1, 2]:

$$\Delta \epsilon = \epsilon_L - \epsilon_R = \frac{A_L - A_R}{c l} \quad (2.1)$$

where $A_L$ and $A_R$ denote the absorbance of left- and right-handed circularly polarized light, respectively, $c$ the molar concentration of amino acid residues (in mol L$^{-1}$), and $l$ the pathlength of the light (in cm). In general, CD spectra are recorded in degrees of ellipticity ($\theta$) as a function of wavelength, which is related to the measured difference in absorbance by $\Delta A = \theta/32.98$. The molar ellipticity ($[\theta]$) is related to the difference in extinction coefficients by $\Delta \epsilon = [\theta]/3298$, where $[\theta]$ is expressed in cm$^2$ deg mol$^{-1}$ [1, 3, 5].

In the case of proteins and peptides, the difference in absorbance (or ellipticity) is not simply an intrinsic property of the polypeptide backbone, but is also determined by the highly asymmetric secondary structure motifs of its folded conformation. Depending on the spatial orientation of the peptide bonds relative to each other when involved in secondary structure conformations, the electronic transitions of the amide chromophores may interact. As a consequence, different secondary structural elements produce characteristic UV-CD patterns [6]. Fig. 2.1 illustrates the specificity of UV-CD spectroscopy for secondary structure, showing UV-CD spectra typical for an α-helix, β-sheet, and random coil structure. Amide chromophores in an α-helix show a strong characteristic negative CD signal at 222 nm due to the backbone $n \rightarrow \pi^*$ transition, and a negative and positive couplet corresponding to the parallel and perpendicular components of the $\pi \rightarrow \pi^*$ transition at approximately 208 nm and 192 nm, respectively [4, 5]. The former is associated with the strong hydrogen-bonding environment of the α-helical configuration and is relatively independent of the length of the helix [5]. Proteins with
mainly a $\beta$-sheet conformation exhibit distinct negative and positive CD signatures at around 216 nm and 195 nm, respectively, originating from the backbone $\pi \rightarrow \pi^*$ transition. However, because the position and magnitude of these $\beta$-sheet CD bands is slightly variable, an accurate estimate of $\beta$-sheet content is more difficult than for $\alpha$-helices by CD spectroscopy [2, 5]. When the protein backbone loses its highly ordered structure upon unfolding, the amide chromophores become randomly oriented with respect to each other and the characteristic UV-CD patterns change. The UV-CD signature of a disordered or random coil conformation is characterized by a strong negative band at 195 nm due to the backbone $n \rightarrow \pi^*$ transition, and a weak positive band at 212 nm arising from the $n \rightarrow \pi^*$ transition. Because the actual shape and relative intensities of the UV-CD bands of a protein are determined by the relative contributions of each of its individual secondary-structure elements, UV-CD spectra provide an accurate determination of its conformation. The changes in CD intensities can therefore be used to determine the thermodynamics of unfolding by probing protein denaturation curves, which can be initiated thermally or chemically.

**Measuring thermal unfolding**

The thermal unfolding transition of proteins and peptides can be investigated either by collecting complete UV-CD spectra (Fig. 2.2a) or by following the change in ellipticity at a single characteristic wavelength (Fig. 2.2b) as a function of temperature. The changes in the UV-CD response of the polypeptide backbone as a function of temperature will be proportional to the change in concentration of the folded (F) and unfolded (U) state populations. For a reversible two-state transition ($F \rightleftharpoons U$), the fraction of the unfolded-state population at temperature $T$ can

![Figure 2.2: Temperature-dependency of the UV-CD intensities of a typical $\alpha$-helical peptide. (a) UV-CD spectra at increasing temperature. The isosbestic point is indicative of a two-state unfolding process. (b) The dependence on temperature of the mean residue ellipticity probed at 222 nm fitted to a two-state model (Eq. 2.2).]
be related to the enthalpy $\Delta H_{U-F}$ and entropy $\Delta S_{U-F}$ of unfolding by [2]:

$$\frac{[U]}{([U] + [F])} = \frac{\exp\left\{(-\Delta H_{U-F} + T\Delta S_{U-F})/RT\right\}}{1 + \exp\left\{(-\Delta H_{U-F} + T\Delta S_{U-F})/RT\right\}}$$  \hspace{1cm} (2.2)

where $\Delta S_{U-F} = \Delta H_{U-F}/T_m$, with $T_m$ denoting the melting temperature in Kelvin. It should be noted that for proteins with a nonzero change in heat capacity $\Delta C_p$, the $\Delta C_p$ terms need to be added to Eq 2.2 (see Eq. 17.1 and 17.2 in Ref. [2]). However, we find that all thermal unfolding data reported in this thesis can be described by assuming no change in the specific heat upon folding ($\Delta C_p = 0$) during the folding transition, as is to be expected since the solvent exposure generally does not change significantly upon unfolding of small protein systems or peptides. In addition to UV-CD, the fraction of the unfolded state population can also be experimentally determined using other temperature-dependent spectroscopic methods, including Fourier-transform infrared (FTIR) spectroscopy of the conformationally sensitive amide I transition of the polypeptide backbone (see section 1.3.1).

### 2.1.2 Two-dimensional infrared spectroscopy

Two-dimensional infrared (2D-IR) spectroscopy provides structural information of a system by observation of the interaction between coupled vibrational modes [7, 8]. Vibrational couplings can be measured by using a narrow ($\sim 10$ cm$^{-1}$) mid-IR pump pulse with frequency $\nu_{\text{pump}}$ to selectively excite a particular vibrational mode, and a broadband ($\sim 200$ cm$^{-1}$) mid-IR probe pulse to investigate the response of the system at a different frequency $\nu_{\text{probe}}$. A difference IR absorption spectrum ($\Delta A$) is generated by subtracting the unpumped ($A_{\text{pump}}$) from the pumped ($A_{\text{pump}}$) absorption signals of the system:

$$\Delta A(\nu) = A_{\text{pump}}(\nu) - A_0(\nu)$$  \hspace{1cm} (2.3)

where $\nu$ refers to the frequency-dependence of the IR absorption spectrum. 2D-IR spectra are obtained by measuring the vibrational response as a function of both frequencies $\nu_{\text{pump}}$ and $\nu_{\text{probe}}$, and by scanning $\nu_{\text{pump}}$ across the resonances of the involved oscillators. When two vibrational modes are coupled, the selective excitation of one mode causes a spectral response at the frequency of the second mode, which is observed in the 2D-IR spectrum as an off-diagonal signal or cross peak.

To illustrate the principle of 2D-IR spectroscopy and the information content of a 2D-IR spectrum, Fig. 2.3 shows the vibrational energy-level diagram of a system with two coupled oscillators, modes A and B with $|\nu_A \nu_B|$ the vibrational states of the combined system (with $\nu_A$ vibrational quanta in mode A and $\nu_B$ vibrational quanta in mode B), and its corresponding FTIR and 2D-IR spectrum. Oscillator A is resonantly excited with a spectrally narrow pump pulse $\nu_{\text{pump}}$, leading to a population transfer from the ground state $|00\rangle$ to the first vibrationally excited state $|10\rangle$ (indicated by the magenta arrow in Fig. 2.3a). After excitation of mode A, the
broadband probe pulse probes different vibrational transitions when it interacts with the molecule, resulting in distinct spectral features in the 2D-IR spectrum, each of which consists of a negative (blue) and positive (red) part (see Fig. 2.3b). Because the population of the ground state |00⟩ is depleted and a fraction of the population is promoted to the |10⟩ state by the pump, the negative ΔA signal 1 observed in the 2D-IR spectrum at (ν_{probe}, ν_{pump}) = (ν_A, ν_A) is caused by bleaching of the ground state (absence of absorption of the |00⟩ → |10⟩ transition) and stimulated emission of the |10⟩ state (|10⟩ → |00⟩ transition). The positive ΔA signal 1′ arises from excited-state induced absorption (the |10⟩ → |20⟩ transition) and appears at ν_{probe} = ν_A − Δ, where Δ denotes the anharmonic shift caused by the anharmonicity of the potential energy of the oscillator (E_{|00⟩→|10⟩} > E_{|10⟩→|20⟩}). The resulting 1/1′ couplet observed at the diagonal of the 2D-IR spectrum sometimes shows a tilt along the diagonal due to inhomogeneous broadening, while the anti-diagonal width is caused by the homogeneous linewidth of the oscillator [8]. When modes A and B are vibrationally coupled, excitation of mode A also influences the vibrating bonds of oscillator B, causing a change in its transition frequency and leading to a response in the off-diagonal region of the 2D-IR spectrum at (ν_{probe}, ν_{pump}) = (ν_B, ν_A) (indicated by the 2/2′ couplet in Fig. 2.3b). Vibrational coupling gives rise to cross-anharmonicity and lowers the energy of state |11⟩, in which both oscillators are in their first vibrationally excited states ν = 1 [8]. Thus, the energy of state |11⟩ is lower in case of coupling than the

![Figure 2.3: Principle of 2D-IR spectroscopy [9, 10]. (a) Schematic energy diagram of two coupled vibrational oscillators A and B with |ν_Aν_B⟩. (b) Corresponding FTIR (upper panel) and 2D-IR spectrum (lower panel). Negative signals are indicated in blue and positive signals are indicated in red. The labeled transitions in (a) corresponds to the labels of the 2D-IR spectrum. Redrawn from Ref. [10].](image)
total energy of the two individual |10⟩ and |01⟩ states (E_{11} < E_{10} + E_{01}). Since the |10⟩ state is significantly populated, the probe pulse senses not only the overtone |10⟩ → |20⟩ but also the |10⟩ → |11⟩ transition, resulting in the positive peak 2'. Because both 2 and 2' involve the promotion of oscillator B from its ground to its first-excited state, the 2 and 2' transition frequencies would occur at exactly the same energies if modes A and B were not coupled. In that case, the off-diagonal absorption changes 2 and 2' in the 2D-IR spectrum would cancel each other and no cross peaks would be visible. Therefore, the existence of cross peaks in a 2D-IR spectrum unambiguously demonstrates the coupling between different vibrational modes. The intensity of a cross peak is a direct measure for the coupling strength between the two oscillators [8]. The diagonal peak 3/3' and the cross peak 4/4' in Fig. 2.3b appear when oscillator B is resonantly excited, and the corresponding vibrational transitions are analogous to the 1/1' and 2/2' transitions, respectively.

**Experimental realization**

In this thesis, 2D-IR spectra are acquired by scanning the center frequency of a narrow-band pump pulse ω_pump across the desired spectral range, while the response of the system is recorded with a broadband probe pulse ω_probe which covers the whole spectral range of interest. Thus, the 2D-IR measurements reported in this thesis were performed in the frequency domain:

\[
\Delta A(\nu_1, \nu_2, t) = A_{IR}(\nu_1, \nu_2, t) - A_0(\nu_2, t) = -\log_{10}\left(\frac{T_{IR}(\nu_1, \nu_2, t)}{T_0(\nu_2)}\right)
\]

(2.4)

where \(A_{IR}\) and \(A_0\) denote the pumped and unpumped IR absorbance, \(T_{IR}\) and \(T_0\) the transmission of the probe pulse in the presence and absence of the IR pump pulse, and \(\nu_1\) and \(\nu_2\) the frequencies of the pump and probe, respectively.

A detailed description of the femtosecond pump-probe setup used for the 2D-IR experiments covered in this thesis is given elsewhere [9–11]. In brief, we use a commercial mode-locked Ti:sapphire oscillator (Mantis, Coherent) and Ti:sapphire regenerative amplifier (Legend Elite, Coherent) combination to generate pulses of 800 nm wavelength (1 kHz, 3.5 mJ, 35 fs FWHM). The output beam is used to pump a β-Barium Borate (BBO) based optical parametric amplifier (OPeraA Solo, Coherent) to generate signal and idler pulses with a combined energy of 700 µJ and a pulse duration of <100 fs (BBO crystal thickness 1.2 mm). Subsequent difference-frequency generation (DFG) of signal and idler using a type II Silver Gallium Sulphide (AgGaS_2) crystal (thickness 1.2 mm, cut angle \(\theta = 39^\circ, \phi = 45^\circ\)) results in mid-IR pulses with a duration of <100 fs centered at 1580 cm\(^{-1}\), a FWHM bandwidth of \(\sim 150\) cm\(^{-1}\), and an energy of \(\sim 20\) µJ. The mid-IR pulse obtained from the DFG is split into pump, probe and reference pulses by means of a beamsplitter (see Fig. 2.4). The probe and reference pulses (solid and dashed lines, respectively in Fig. 2.4) are obtained by reflection off the front and back surfaces of a wedged BaF_2 window, each of which consists of \(\sim 5\)% of the total
The intensity of the mid-IR beam obtained from the DFG. The remainder of the mid-IR beam is used as a pump beam and is passed through a Fabry-Perot etalon to narrow its bandwidth, resulting in pump pulses with a bandwidth of 10 cm$^{-1}$ FWHM and an energy of approximately 2 $\mu$J. The center frequency of the pump beam is varied by adjusting the distance between the parallel mirrors of the etalon using a feedback-controlled piezoelectric mount. Because the light transmitted by the etalon can contain low-intensity side bands due to the broad power spectrum of the mid-IR beam, long- and/or short-pass filters are placed in the pathway of the pump pulse before the sample in order to avoid the undesirable excitation of other vibrational modes (for example the amide II mode). The pump, probe and reference pulses are focused in the sample (S in Fig. 2.4) using a 100 mm off-axis (30°) parabolic mirror. The mid-IR probe beam has a focal diameter of typically 200 $\mu$m. The pump and probe pulses are spatially overlapped in the sample, while the reference pulse passes through the sample in an area that is not influenced by the pump pulse. The frequency-dependent absorption changes induced in the sample by the pump pulse are monitored by the probe pulse at various delay times after the pump. The time delay between the pump and mid-IR probe pulses is controlled by adjusting the path length between them using a motorized delay stage. Before passing through the sample, the polarization of the pump pulse is rotated by 45° with respect to the mid-IR probe pulse using a MgF$_2$ zero-order $\lambda/2$ plate. The polarization of the measured spectrum is selected by

Figure 2.4: Schematic representation of the experimental setup used to measure 2D-IR spectra in the frequency domain. The pump, probe, and reference pulses are separated using a wedged BaF$_2$ window. The reference beam is depicted by the dashed line. Figure from Ref. [10].
placing a wire-grid polarizer directly after the sample to a $0^\circ$ (parallel spectrum) or $90^\circ$ (perpendicular spectrum) angle with respect to the polarization of the pump pulse. After passing through the sample, pump, probe, and reference pulse are re-collimated by means of a 140 mm off-axis parabolic mirror ($45^\circ$). A third off-axis parabolic mirror ($f = 100$ mm, $30^\circ$) is used to focus the mid-IR probe and reference beams into the spectrograph (MS260i, Newport Oriel), where the frequencies of both beams are dispersed and imaged onto a liquid-nitrogen-cooled $2\times32$ HgCdTe (MCT) array detector (Infrared Associates). The spectrum of the pump pulse during optimization of the etalon is monitored by focusing it after it has passed the sample onto the probe array of the MCT detector. To this purpose, pump and mid-IR pulses are overlapped on an adjustable CaF$_2$ window, which is used to couple the pump beam into the spectrograph. Since the pump and probe beams are focussed on the same pixels of the MCT array, a bi-positional beam block is placed such that it blocks the probe and reference pulses during optimization of the etalon, while it blocks the pump pulses during data acquisition. To obtain 2D-IR spectra, we determine the IR absorbance in the presence ($A_{IR}$) and absence ($A_0$) of the pump pulse (see Eq. 2.4). To determine $A_{IR}$(\(\nu_1, \nu_2, t\)) and $A_0(\nu_2, t)$, the pump beam is optically chopped at half the pulse repetition frequency of the mid-IR probe beam (500 Hz). Parallel ($\Delta A_{\parallel}$) and perpendicularly ($\Delta A_{\perp}$) polarized 2D-IR spectra are obtained by repeatedly collecting signals with parallel and perpendicularly polarized pump and probe pulses.
2.2 Time-resolved temperature-jump measurements

In temperature-jump (T-jump) spectroscopy, the thermodynamic equilibrium of a protein system is disturbed on the nanosecond time scale and the structural changes that take place as the system proceeds to its new equilibrium position are probed (see section 1.3.2). We use a near-IR laser source to excite the overtone transition of the OD-stretching vibration of the solvent (D$_2$O), inducing a T-jump of $\sim$10 K on the nanosecond time scale. The T-jump pulse is generated using a $\beta$-Barium Borate (BBO)-based optical parametric oscillator (OPO) pumped by the second harmonic ($\lambda = 532$ nm) of a 20-Hz, Q-switched Nd:YAG laser (Quanta-Ray INDI, Spectra-Physics). The idler pulse ($\lambda = 1.98 \mu$m, 12.5 mJ, 5 ns FWHM) is focused ($f = 75$ mm, CaF$_2$) to 500 $\mu$m diameter at the sample, leading to a temperature-rise within the pulse width of the laser ($\sim$5 ns). As we will describe in the following two subsections, the temperature-induced re-equilibration dynamics of the different peptides and protein systems investigated in this thesis are followed with transient linear IR absorption experiments (Fig. 2.5, section 2.2.1) as well as with transient dispersed IR pump-probe (t-DPP) spectroscopy (Fig. 2.8, section 2.2.2) in the amide I’ spectral region (1600–1700 cm$^{-1}$).

2.2.1 Temperature-jump amide I infrared-probe experiments

During T-jump linear IR absorption experiments, the frequency-dependent absorption changes induced in the sample are detected at various time delays ($\Delta t$) after the T-jump by mid-infrared (mid-IR) probe pulses (see Fig. 2.5) using an optical setup described in detail elsewhere [12]. In brief, we use the 800 nm output of a commercial Tisapphire laser (Spectra-Physics Hurricane, 1 kHz, 1 mJ, 100 fs FWHM) to pump a BBO based optical parametric amplifier (OPA-800C, Spectra-Physics). Subsequent difference-frequency generation of signal and idler in AgGaS$_2$ results in mid-IR pulses with a duration of $\sim$150 fs centered at 1620 cm$^{-1}$, and

![Figure 2.5: Schematic picture of the experimental T-jump IR-probe setup. A near-IR laser pulse induces a nanosecond T-jump in the protein sample, and the conformational re-equilibration is subsequently followed by mid-IR pulses at several time delays ($\Delta t$) after the T-jump pulse.](image-url)
with a FWHM bandwidth of $\sim$200 cm$^{-1}$, and an energy of 1 $\mu$J. The mid-IR pulse is split into probe and reference pulses by means of a 50/50 beamsplitter, which are subsequently focused in the sample by means of a 100 mm off-axis parabolic mirror.

To obtain transient IR spectra, we determine the absorption changes $\Delta A_{TJ}(\nu, t)$ in response to the nanosecond $T$-jump:

$$\Delta A_{TJ}(\nu, t) = A_{TJ}(\nu, t) - A_0(\nu) = -\log_{10} \left( \frac{T_{TJ}(\nu, t)}{T_0(\nu)} \right)$$  \hspace{1cm} (2.5)

where $T_{TJ}$ and $T_0$ refer to the transmission of the IR-probe pulse in the presence and absence of the $T$-jump pulse, respectively, $\nu$ to the probe frequency, and $t$ to the delay between the $T$-jump and IR-probe pulse. We correct for small pulse-to-pulse fluctuations in the intensity of the IR-probe pulses by simultaneously measuring the intensity of a reference pulse (split off from the probe pulse before the sample) that passes through the sample in an area that is not influenced by the $T$-jump pulse. Transient absorption changes are measured using frequency-dispersed detection of the probe and reference pulses using a liquid-nitrogen-cooled 2×32 HgCdTe (MCT) array detector (Infrared Associates). Spatial overlap between the probe and $T$-jump pulse is found by optimizing the transmission of the two beams through a 250 $\mu$m pinhole (beam waist of 250 $\mu$m and 500 $\mu$m for probe and $T$-jump pulse, respectively). The spatial overlap is optimized by maximizing the change in absorption of the probe pulse through D$_2$O. The polarization of the $T$-jump and mid-IR pulse are perpendicular with respect to each other.

The initial temperature of the sample is controlled using a thermostatted cell-holder with a circulating heat bath, and is calibrated with an IR camera (FLIR ThermaCAM E2). Reference samples containing the D$_2$O buffer solution were measured under identical conditions and used to correct for contributions of the solvent to the transient signals. The transient absorbance change of the D$_2$O buffer solution also provides information about the magnitude of the $T$-jump, and was used to calibrate the size of the $T$-jump using the steady-state absorbance change of the buffer with temperature (which are only due to changes in D$_2$O). The IR response of D$_2$O can be used as an internal thermometer due to its nearly linear response with temperature in the amide I’ spectral region [13].

**Timing of the $T$-jump experiment**

To achieve accurate time delays between the $T$-jump and mid-IR laser systems, a variable, computer-controlled time delay between the two laser outputs is achieved in a similar manner as reported by other groups [14, 15]. Synchronization between the two laser outputs is based on the electronic configuration as shown in Fig. 2.6. A fast photodiode detects the residual output from the Ti:sapphire oscillator (80 MHz), which is amplified and frequency divided to produce a 1 kHz signal. This signal triggers a pulse generator, which provides the timing for (1) the YLF pump laser and Pockels-cell driver of the regenerative Ti:sapphire amplifier used
2.2 Time-resolved temperature-jump measurements

Figure 2.6: Electronic configuration used to synchronize the Ti:sapphire and Nd:YAG laser systems. The solid lines represent optical pulses, while the dashed lines represent electronic pulses. Based on the electronic setup described elsewhere [12].

to generate the IR probe pulses, (2) the electronic gated integrator used to amplify and detect the signals of the MCT array, (3) a computer-controlled digital delay and pulse generator (SRS, Stanford Research Systems Model DG535). The latter is clocked by a 10 MHz signal and provides the triggering for the lamp and Q-switch of the Nd:YAG laser. Clocking the SRS is necessary to ensure that the internal delay value is synchronous with the Ti:sapphire oscillator. The delay time $t_0 = 0$ between $T$-jump pulse and mid-IR probe pulse is set to the point where the change in absorption in D$_2$O is half of its maximum.

In the results presented in this thesis, the signal at each delay value is the average of typically about 1000 independent $T$-jumps. Given the repetition frequency of the $T$-jump laser (20 Hz) and the mid-IR laser source (1 kHz), care is taken to ensure that the sample has relaxed to its initial temperature before the subsequent IR probe pulse passes through the sample for $T_0$ determination. Because the system generally requires longer than 1 ms to recover from the $T$-jump disruption, the subsequent mid-IR probe shots are not used in the data acquisition until the sample has relaxed to its initial state using an inhibit signal. Fig. 2.7 shows the pulse scheme of the inhibit counter box, which is triggered from the 20 Hz input (see Fig. 2.6). After the $T$-jump and the delayed mid-IR probe pulse, it raises the output high before the next mid-IR probe shot comes in and starts counting the low→high transitions of the 1 kHz input (Fig. 2.7). All mid-IR shots are ignored
for the $T_0$ determination when the inhibit signal is high. When the sample has relaxed to its initial temperature (after $n$ counts, being typically 30), the inhibit output is low until the next 20 Hz trigger from the $T$-jump laser.

2.2.2 Transient infrared dispersed pump-probe spectroscopy

Dispersed pump-probe (DPP) IR spectroscopy measures the absorption changes of a mid-IR probe pulse following vibrational excitation by a broad-band ($\sim 200 \text{ cm}^{-1}$) mid-IR pump pulse [16]. The DPP signal measured is equivalent to the 2D-IR projection spectrum on the probe axis ($\nu_{\text{probe}}$), which is obtained by integration of the 2D-IR response over the pump axis ($\nu_{\text{pump}}$) [8, 16]. Therefore, polarization-dependent DPP experiments offer additional structural resolution by allowing the observation of cross-peak interactions separately from the diagonal response of the system. This method will be used in chapters 7 and 8. In order to time-resolve the DPP relaxation in response to a $T$-jump, we extend the $T$-jump IR-probe experiment (Fig. 2.5) to include a broad-band IR pump pulse (see Fig. 2.8). The transient DPP (t-DPP) spectrum, $\Delta \Delta A(\nu, t_{TJ}, t_{IR})$, is the difference between the DPP spectrum after a $T$-jump, $\Delta A_{TJ,IR}(\nu, t_{TJ}, t_{IR})$, and the DDP spectrum in absence of a $T$-jump, $\Delta A_{IR}(\nu, t_{IR})$:

$$\Delta \Delta A(\nu, t_{TJ}, t_{IR}) = \Delta A_{TJ,IR}(\nu, t_{TJ}, t_{IR}) - \Delta A_{IR}(\nu, t_{IR}) \quad (2.6)$$

$$\Delta \Delta A(\nu, t_{TJ}, t_{IR}) = A_{TJ,IR}(\nu, t_{TJ}, t_{IR}) - A_{TJ}(\nu, t_{TJ}) - (A_{IR}(\nu, t_{IR}) - A_0(\nu)) \quad (2.7)$$

where $A_0(\nu)$ is the absorption of the sample when neither pump pulse is present, $\nu$ is the probe frequency, $t_{TJ}$ the delay between the $T$-jump and mid-IR pulse pair,
and $t_{\text{IR}}$ the delay between the IR-pump and IR-probe pulses (see Fig. 2.8).

**Experimental realization of the transient DPP experiment**

For the t-DPP experiments we use a commercial Ti:sapphire laser (Coherent Legend Elite, 3.5 mJ, 35 fs FWHM) synchronized with the Nd:YAG laser system used for generating $T$-jump pulses (Quanta-Ray INDI Spectra-Physics). Using the amplified 800 nm output of the Legend and the same optical setup described in section 2.1.2, we obtain mid-IR pulses with a duration of $\sim$150 fs, a bandwidth of 200 cm$^{-1}$ and an energy of 20 $\mu$J. Probe and reference pulses are obtained from the mid-IR light by reflection off the front and back surfaces of a wedged BaF$_2$ window. The polarization of the IR pump pulse is set at 45° with respect to that of the probe pulse using a MgF$_2$ zero-order $\lambda/2$ plate. Subsequently, the polarization of the measured spectrum is selected using a polarizer situated directly after the sample set at either 0° (parallel spectrum) or 90° (perpendicular spectrum) with respect to the pump polarization.

The $T$-jump pulse ($\lambda = 1.98 \mu$m, 12.5 mJ, 5 ns FWHM) is focused ($f = 75$ mm, CaF$_2$) to 500 $\mu$m diameter at the sample, leading to a temperature-rise within the pulse width of the laser. The changes induced in the sample are monitored by the two mid-IR probe pulses, which are spatially overlapped with the pump beam, at various time delays between the $T$-jump and mid-IR pulses (see Fig. 2.8). The mid-IR reference beam passes through an area of the sample not influenced by the pump. The mid-IR pump, probe and reference beams are focused through the sample by means of an $f = 100$ mm off-axis parabolic mirror. At the sample, the mid-IR beam diameter is 200 $\mu$m. Transient absorption changes are measured by frequency-dispersed detection of the mid-IR pulses using a $2 \times 32$ HgCdTe (MCT)

![Diagram](image)

**Figure 2.8:** Schematic picture of the transient dispersed pump-probe (t-DPP) setup. The $T$-jump-induced changes in the sample are monitored by the two mid-IR pulses, which are spatially overlapped with the $T$-jump pulse, at various time delays between the $T$-jump and mid-IR pulses.
array detector (Infrared Associates).

We use the timing scheme shown in Fig. 2.9 to obtain the signals necessary to construct $\Delta \Delta A$ as shown in Eq. 2.7: the sample is probed at a repetition rate of 1 kHz. The mid-IR pump pulse is optically chopped at a frequency of 250 Hz such that two sequential pulses are allowed to pass and the following two sequential pulses are blocked, effectively pumping the sample at 500 Hz. The $T$-jump laser runs at a repetition rate of 20 Hz, which is a factor of 500 Hz but not a factor of 250 Hz, so that the $T$-jump pulse alternately coincides and does not coincide with a mid-IR pump pulse. In this manner, the following absorption spectra required for the generation of the t-DPP spectrum can be recorded: the ground state absorption, $A_0$, is measured at each laser shot where both $T$-jump and IR pumps are off (occurring at 490 Hz); the vibrationally excited state absorption, $A_{IR}$ at a certain pump frequency and delay time, is measured at each shot where only the IR pump pulse (and no $T$-jump) acts on the sample (occurring at 490 Hz); the electronic excited state absorption, $A_{TJ}$ at a certain delay time, is measured at each shot where only the $T$-jump pulse acts on the sample (occurring at 10 Hz); the vibrationally excited absorption of the electronic excited state species, $A_{TJ,IR}$ at a certain IR-pump frequency and delay time, is measured at each shot where both IR and $T$-jump pumps act on the sample (this occurs at 10 Hz).

![Figure 2.9](image-url): Electronic timing of a pump-pump-probe experiment [17]. The colored bars represent the probe (red), pump (orange), and $T$-jump (blue) laser pulses. The green line in the middle graph shows the electronic "pump present" signal which encompasses two subsequent pump pulses.
References


