Excited-State Dynamics of Pyrimidine DNA Bases Revealed by Ultrafast Vibrational Spectroscopy

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Introduction

The American Cancer Society estimates nearly 60,000 new cases of, and over 8,000 deaths from, melanoma skin cancer in 2007.[1] Along with genetics, the major risk factor for melanoma development is exposure to the sun.[1] Light absorption by DNA can lead to deleterious photochemistry, followed by mutations and ultimately carcinogenesis. Much about this complex pathway is unknown and so to better understand the link between light absorption and skin cancer, we study DNA photophysics; the earliest events that occur after DNA has absorbed light. The likelihood of contracting light-induced skin cancer depends critically on these earliest events. The last 10 years have seen a resurgence of interest in DNA photophysics.[1]

It is the nucleobases of DNA, running through the center of the double helix, that absorb UV light. The five DNA and RNA bases are either purine or pyrimidine derivatives (see Fig. 7). Absorption of a UV photon promotes the nucleobases from their lowest-energy (ground) electronic state to a higher-energy (excited) electronic state given the label $^1\pi\pi^*$ due to its electronic character. The majority of photoexcited bases return to their ground state (S_0) via internal conversion, a nonradiative process that converts chemically-useful electronic energy into comparatively-inert vibrational energy. The initially-excited $^1\pi\pi^*$ state of all five canonical nucleosides decay with a lifetime of 1 ps (1×10^{-12} s) or less in solution.[2,3] This rapid deactivation of potentially damaging energy is often speculated as the basis for DNA's photostability.

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Recent work, however, has shed new light on this picture. For example, 2006 marked the discovery, in pyrimidine bases, of slower decay pathways through $^3\pi\pi^*$ (or triplet) and $^1n\pi^*$ excited states.[4,5] These long-lived excited states leave open the potential for DNA-damaging chemistry and therefore require further study and characterization. Unfortunately, the $^1n\pi^*$, $^1\pi\pi^*$, $^3\pi\pi^*$ and S_0 states of single pyrimidine bases have strongly-overlapping electronic absorption spectra which significantly complicates study of their dynamics using typical techniques.

Vibrational absorption spectra, more so than electronic absorption, are unique to a given species or electronic state. We therefore seek unique vibrational marker bands for the relevant electronic states of pyrimidine bases using femtosecond time-resolved IR spectroscopy (TRIR). This technique uses a UV light pulse, ~ 200 fs (2×10^{-13} s) in duration, to excite the DNA bases to the $^1\pi\pi^*$ state and an IR light pulse, also about 200 fs in duration, that is absorbed by the excited molecule. By systematically changing the relative arrival times (time delays) of the UV and IR pulses, the IR absorption by the excited states can be measured as a function of the time, providing the kinetics of the excited states. In principle the different electronic states of the molecule absorb different frequencies of IR light and so these unique marker bands for each different state allow their dynamics to be followed independently, even when their electronic spectra may significantly overlap. TRIR techniques have been fruitfully applied to the study cyclobutane dimer formation in thymine oligonucleotides.[6]

Here we report TRIR studies of the excited-state dynamics of the pyrimidine bases shown in Fig. 1, thymine and 1-cyclohexyluracil, in the region where C=O (carbonyl) stretching vibrations absorb (1500-1800 cm^{-1}). These vibrations were chosen because they provide the strongest signals. Acetonitrile was chosen as a solvent because the $^1n\pi^*$ and $^3\pi\pi^*$ states of pyrimidine bases

are significantly populated in organic solvents.[4] Measurements in methanol-d_1 were also made for comparison.

We identify unique marker bands for both the $^1n\pi^*$ and $^3\pi\pi^*$ states. The $^1n\pi^*$ band maximum lies at higher frequency than nearby ground-state C=O bond vibrations and is independent of solvent. We suggest that this band belongs not to a C=O vibration but involves a stretching of the C4-C5 bond. Triplet marker bands are consistent with recent nanosecond TRIR measurements as well as theoretical calculations.[7] The dynamics of the $^3\pi\pi^*$ (triplet) band is consistent with recent conclusions about the triplet formation rate in these systems.[4]

Methods

Thymine and 1-cyclohexyluracil were used as received from Aldrich. Acetonitrile, acetonitrile-d_3 (99.8% D), and methanol-d_1 (99% D) were used as obtained from Aldrich. When dissolved in methanol-d_1, the N3 position of CHU deuterates to form CHU-d_1. Because this fact has no significant effect on the results presented here will generally be neglected in the following analysis and discussion.

Mid-IR transient absorption was measured with femtosecond temporal resolution using pump-probe techniques. Femtosecond pulses at 800 nm produced by a Ti:sapphire oscillator and amplified by a 1 kHz regenerative amplifier system (Coherent, Inc.) pumped independent optical parametric amplifiers (Coherent, Inc.) to create pump (excitation) and probe pulses. Pump pulses at 270 nm were generated by second harmonic generation after sum-frequency mixing of signal and idler pulses. The pump pulses were chopped at 500 Hz with a mechanical chopper (New Focus) and had pulse energies of 5 μJ at the sample. Mid-IR probe pulses centered at 1600 cm^{-1} were generated via difference-frequency mixing of signal and idler beams. Probe pulses had spectral bandwidths of ~125 cm^{-1} (FWHM) and pulse energies of < 1 μJ at the sample. Pump and

probe polarizations were set at magic angle (54.7°). Pump and probe spot sizes were 500 μm at the sample.

The sample was flowed through a 1 mm path length cell (Harrick Scientific) with a 2 mm thick BaF_2 front window and 2 mm thick CaF_2 back window. After the sample, probe pulses were dispersed by a spectrometer and detected on a shot-by-shot basis with a 32-element linear MCT detector array (Infrared Systems Development Corporation). A reference beam, split from the probe beam and directed through the sample, was monitored on a second 32-element array and used to remove probe pulse fluctuations. At each time delay, reference-normalized spectra recorded with and without the pump pulse were averaged separately and ratioed. Induced absorbance changes (ΔA) in the transient absorption difference spectra were calculated as the negative logarithm of the ratioed spectra. The spectral resolution was ~8 cm^{-1}. To prevent absorption by water vapor the entire mid-IR optical path was purged with dry air.

Steady-state IR spectra were obtained with a Perkin-Elmer Spectrum 2000 FTIR spectrometer. All uncertainties are reported as two standard deviations resulting from unweighted fits.

Results

Ground-State Spectra. The steady-state IR spectra of CHU in methanol-d_1 (black), and acetonitrile (light grey) as well as thymine in acetonitrile-d_3 (dark grey), in the 1575 – 1800 cm^{-1} region, are shown in Fig. 2. The steady-state spectra are assumed to contain contributions from only the ground state. All spectra contain at least two strong bands due to C=O stretch vibrations. For thymine in acetonitrile-d_3, there are two strong bands at 1683 and 1724 cm^{-1} which are assigned to C4=O and C2=O stretching vibrations (see Fig 1 for atom numbering), respectively, following Hare et al.[7] and others.[8-12]

For CHU in acetonitrile these peaks shift to 1691 and 1709 cm^{-1}. Similar shifts have been observed comparing thymine and thymidine.[7] In methanol-d_1, the two most intense bands in the CHU spectrum shift to 1655 and 1674 cm^{-1}. Hydrogen-bond donating solvents are known to shift carbonyl stretching frequencies by as much as 50 cm^{-1}.[13] Weaker bands in the spectra are due to carbon-carbon stretches and other skeletal vibrations.[8-12]

TRIR Signals: CHU. Transient IR spectra obtained for CHU in acetonitrile are shown in Fig. 3. Positive bands result from new absorption created by photoexcitation, i.e., transient excited states. Negative bands result from absorption removed by photoexcitation (bleaches), i.e., the ground state. The negative spectrum would exactly match the ground-state spectrum if there were no overlap with positive bands. The most prominent features in Fig. 3 are ground-state bleach bands at 1688 and 1713 cm^{-1}. The bleach signals decay with two components, one with a lifetime of 16 ± 2 ps and another with a lifetime of 3-6 ns. While these bleach frequencies are in excellent agreement with the ground-state spectrum, the shape of the negative bands and the ground-state spectrum are noticeably different. This suggests that a transient species also in this region. However, the most prominent transient (positive) band is observed at 1760 cm^{-1} with a FWHM of 33 cm^{-1}. The band decays with a single lifetime of 3.1 ± 0.8 ns.

Transient spectra obtained for CHU in methanol-d_1 are shown in Fig. 4. The results are qualitatively similar to those in acetonitrile but the decay of all transient features is accelerated and residual offsets are not seen at long time delays. Bleach bands are observed at 1705 and 1673 cm^{-1}, respectively, and decay with 10.8 ± 1.6 ps and 0.52 ± 0.06 ns lifetimes. The positive band at 1760 cm^{-1} decays with a single lifetime of 0.51 ± 0.09 ns.

TRIR Signals: Thymine. Fig. 5a shows a transient spectrum obtained for thymine in actonitrile-d_3. To improve signal-to-noise, the spectrum was generated by averaging spectra obtained at

time delays between 0.03 and 3 ns. Negative ground-state bleach bands are observed at 1683 and 1726 cm^{-1}. Positive bands are observed at 1603 and 1714 cm^{-1}. The latter band is partially obscured by the nearby bleach bands. This spectrum is in excellent agreement with the nanosecond TRIR and calculated transient spectra present in Fig. 5b and 5c, respectively.

Transient kinetic decays at 1602 cm^{-1} and 1628 cm^{-1} are shown in Fig. 6. At delays between 0 and 10 ps, the signal is dominated by a broad positive absorption band initially centered at ~1640 cm^{-1}. This band subsequently narrows, shifts to higher frequencies and decays by 30 ps. Due to these complex spectral dynamics, the transient absorption signal at 1628 cm^{-1} rises to a maximum at 5 ps and decays to zero by 30 ps, while the signal measured at 1602 cm^{-1} rapidly decays from its initial amplitude to a small offset by 10 ps. This offset it due to the presence of another much weaker band centered at 1600 cm^{-1}. This band shows no change in intensity or spectral shape, within signal-to-noise, from 10 to 2800 ps.

Discussion

Ultrafast Decay Channel. The major decay channel for single bases following 270 nm excitation to the $^1\pi\pi^*$ state is rapid internal conversion to the ground state.[2,3] The $^1\pi\pi^*$ lifetime for CHU in methanol and acetonitrile is 0.14 and 0.20 ps, respectively.[4] Because our time resolution is 0.45 ps, we could not detect any positive features that we can definitively assign to the $^1\pi\pi^*$ state. However, evidence for this ultrafast decay channel is provided by the bleach recovery signals, which monitor the return of excited molecules to the ground state. At 1688 cm^{-1} the bleach of CHU in acetonitrile recovers with two time constants, 16 ps and ~4 ns.

A recovery time exactly matching the $^1\pi\pi^*$ lifetime is not observed because, for this decay channel, return to the ground-state minimum is rate-limited by vibrational cooling within the ground-state vibrational manifold.[14] Vibrational cooling occurs because internal conversion con-

verts the excited-state electronic energy into ground-state vibrational energy (> 30 000 cm^{-1}). This is equivalent to heating the base to a temperature of roughly 2000 K.[14] This "hot" ground state has a broader and lower-energy absorption spectrum compared to its room-temperature spectrum. The room-temperature ground-state spectrum recovers as the hot ground state dissipates this thermal energy to the surrounding solvent molecules, and it is the rate of this process, vibrational cooling, that we observe in our experiment. However, the vibrational cooling can only be kinetically resolved if the initial internal conversion occurs at a rate significantly faster than the vibrational cooling rate. Therefore observation of a vibrational cooling signal is evidence that some fraction of excited bases return to the ground state on an ultrafast timescale, as previously observed for all DNA bases.[2-4,14,15]

The vibrational cooling time for thymidine in acetonitrile measured with UV transient absorption is 9.1 ps,[4] in good agreement with the 16 ps bleach-recovery component we observe for CHU in the IR. The fast recovery component for CHU in methanol-d_1 is 11 ps. The vibrational cooling time for CHU in methanol measured with UV transient absorption is 3.7 ps. Vibrational cooling of DNA bases is strongly solvent dependent and is accelerated in protic solvents.[14] The presence of the ~4 and 0.52 ns recovery components in acetonitrile and methanol-d_1, respectively, is evidence of additional decay channels through intermediate, longer-lived excited states.

Marker Band for the $^1n\pi^*$ State. The most notable feature in the transient spectra of CHU is the positive band centered at 1760 cm^{-1} in acetonitrile and methanol-d_1. The band appears within our time resolution of 0.45 ps and decays with a 3.1 ns lifetime. The lifetime is in excellent agreement with the 3.2 ns lifetime of the $^1n\pi^*$ state in acetonitrile measured via UV-visible transient absorption.[4] The band decays in methanol-d_1 with a 0.51 ns lifetime, while the lifetime measured in methanol with UV-vis transient absorption is 0.110 ns. The origin of this difference

is under investigation. However, given the agreement in acetonitrile and the consistent trend with solvent, we assign this band to the $^1n\pi^*$ state.

As the notation suggests, the electronic character of the $^1n\pi^*$ involves promotion of a non-bonding electron to an anti-bonding electron should, in general, weaken bonds and shift excited-state vibrations to lower frequencies. Indeed, this holds true in many cases.[16,17] However, there are no significant ground-state vibrations whose frequency could reasonably be expected to shift down to 1760 cm^{-1}. The $^1n\pi^*$ band must therefore arise from a lower-frequency ground-state vibration. The two most likely candidates are C=O and carbon-carbon stretch vibrations.

Carbonyl stretching frequencies can shift to higher frequencies as a result of solute-solvent hydrogen-bond breaking.[18] This explanation can be ruled out because the $^1n\pi^*$ band is observed at the same frequency in both acetonitrile and methanol-d_1 which have very different hydrogen-bonding properties. High-level quantum calculations on pyrimidine-base excited states predict that the C4-C5 bond contracts by 0.075 Å (~5%) in the $^1n\pi^*$ state.[19] This suggests that the 1760 cm^{-1} band belongs to a vibration involving the C4-C5 stretching coordinate. High-quality theoretical calculations of the $^1n\pi^*$ vibrations would aide in a definitive assignment.

Marker Band for the $^3\pi\pi^*$ State. High-quality normal mode calculations have already been performed for the lowest-energy $^3\pi\pi^*$ state.[7] Together with nanosecond TRIR measurements,[7] the calculations allow for definitive assignment of positive bands seen in Fig. 5. The band observed at 1603 cm^{-1} is assigned to the C4=O stretch. The band observed at ~1714 cm^{-1} is assigned to the C2=O stretch. This latter band is also apparent in the transient spectra for CHU in acetonitrile at long delay times, see Fig. 3.

Unfortunately, the rise of the triplet bands is obscured by larger amplitude bands at delay times below 10 ps, making it impossible to precisely determine the rate of triplet formation (also

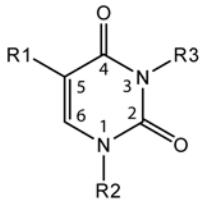
called intersystem crossing). The larger amplitude bands narrow, shift to higher frequency, and decay by 30 ps. This behavior is a well-known signature of vibrational cooling[20] and these bands are assigned to the hot ground-state C4=O stretch.

The IR experiments also show that the triplet state is fully formed on a timescale significantly faster than the $^1n\pi^*$ lifetime. This result suggests that intersystem crossing from the $^1n\pi^*$ state to the $^3\pi\pi^*$ state occurs before the excited population reaches the minimum on the $^1n\pi^*$ potential surface, supporting previous conclusions from UV-visible transient absorption measurements.[4]

Conclusions

These studies demonstrate the advantages of TRIR for elucidating the nature and dynamics of pyrimidine-base excited states. Because vibrational frequencies are sensitive to the electronic and structural properties of the molecule, these spectra additional provide specific criteria for evaluating the accuracy of the high-level quantum mechanical calculations being performed on pyrimidine bases. The identification of unique marker bands for the $^1n\pi^*$ and $^3\pi\pi^*$ states will allow for further studies on these systems. This will be particular important for studies on DNA oligomers where there is increased spectral congestion in the UV-visible region due to the presence of additional excited states.[21-24] In future studies, the marker bands identified here will be used to determine the extent that these possible photochemical precursors are populated in DNA polymers - important and necessary steps toward a better understanding of UV-induced skin cancer.

Figures



Thymine	R1 = CH_3,	R2 = H,	R3 = H
CHU	R1 = H,	R2 = C_5C_6,	R3 = H
CHU-d_1	R1 = H,	R2 = C_5C_6,	R3 = D

Figure 1. Structures and numbering for thymine, CHU and CHU-d_1.

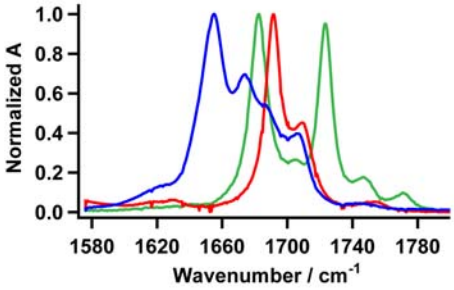


Figure 2: Steady-state IR spectra of CHU-d_1 in methanol-d_1 (blue), CHU in acetonitrile (red) and thymine in acetonitrile-d_3 (green).

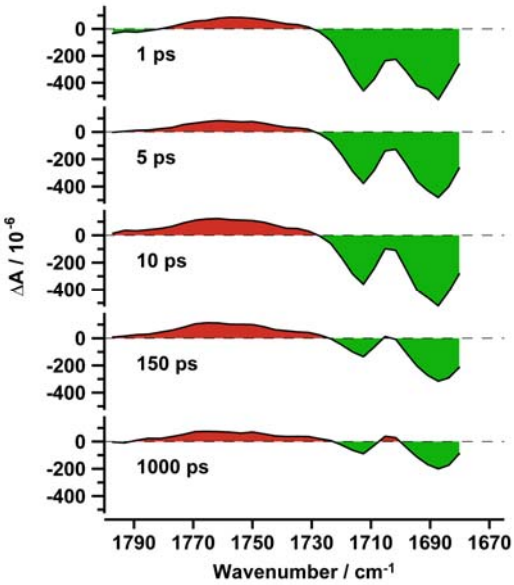


Figure 3: Transient mid-IR spectra of CHU in acetonitrile following excitation at 270 nm. Spectra are labeled with their respective time delays.

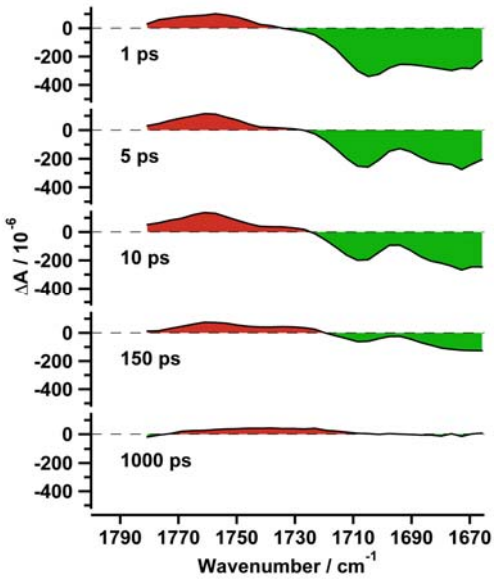


Figure 4: Transient mid-IR spectra of CHU in methanol-d_1 following excitation at 270 nm. Spectra are labeled with their respective time delays.

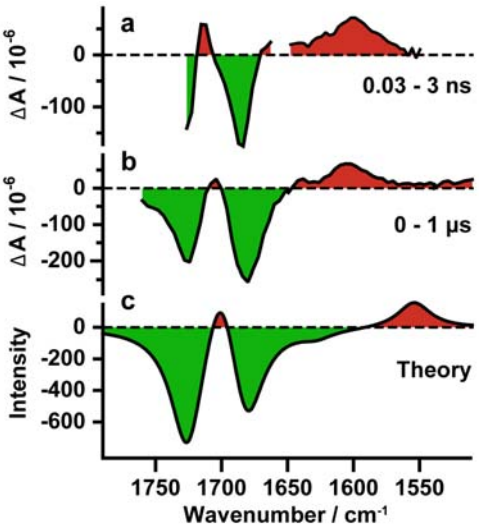


Figure 5: Transient mid-IR spectra of thymine in acetonitrile-d_3. Top: fs-TRIR spectrum averaged over 0.03 - 3 ns. Middle: ns-TRIR spectraum averaged over 0 - 1 µs (adapted from Ref. 1). Bottom: Simulated transient spectrum calculated at the B3LYP/6-31++G** level (adapted from Ref. 1, see original article for details).

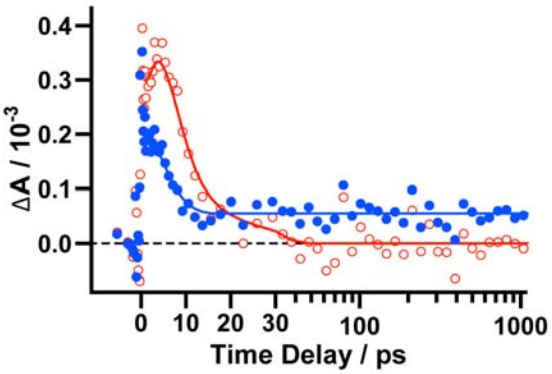


Figure 6: Transient kinetics of thymine in acetonitrile-d_3 at 1602 cm^{-1} (solid circles) and 1628 cm^{-1} (open circles). Delay times after 30 ps are shown on a logarithmic scale. Sold lines are provided to guide the eye.

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