Solution conformation of 2-aminopurine dinucleotidet determined by ultraviolet two-dimensional fluorescence spectroscopy

Julia R Widom\textsuperscript{1,2}, Neil P Johnson\textsuperscript{2}, Peter H von Hippel\textsuperscript{2} and Andrew H Marcus\textsuperscript{1,2,3}

\textsuperscript{1} Oregon Center for Optics and Department of Chemistry, University of Oregon, Eugene, OR 97403, USA
\textsuperscript{2} Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, OR 97403, USA
E-mail: ahmarcus@uoregon.edu

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Abstract. We have observed the conformation-dependent electronic coupling between the monomeric subunits of a dinucleotide of 2-aminopurine (2-AP), a fluorescent analogue of the nucleic acid base adenine. This was accomplished by extending two-dimensional fluorescence spectroscopy (2D FS)—a fluorescence-detected variation of 2D electronic spectroscopy—to excite molecular transitions in the ultraviolet (UV) regime. A collinear sequence of four ultrafast laser pulses centered at 323 nm was used to resonantly excite the coupled transitions of 2-AP dinucleotide. The phases of the optical pulses were continuously swept at kilohertz frequencies, and the ensuing nonlinear fluorescence was phase-synchronously detected at 370 nm. Upon optimization of a point–dipole coupling model to our data, we found that in aqueous buffer the 2-AP dinucleotide adopts an average conformation in which the purine bases are non-helically stacked (center-to-center distance $R_{12} = 3.5 \pm 0.5$ Å, twist angle $\theta_{12} = 5^\circ \pm 5^\circ$), which differs from the conformation of such adjacent bases in

\textsuperscript{3} Author to whom any correspondence should be addressed.

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The conformational manipulation of nucleic acid bases within functioning ‘macromolecular machines’ is a central feature of DNA replication and RNA transcription reactions. In many cases, these manipulations involve the unwinding of DNA–DNA (or DNA–RNA, or RNA–RNA) duplexes at single-stranded–double-stranded (ss–ds) replication forks, or at primer–template junctions. Such unwinding processes provide access for the relevant DNA or RNA polymerases to the duplex ‘interior’, and expose ss DNA or RNA sequences that serve as templates for replication and transcription. Thermally driven conformational fluctuations (i.e. ‘breathing’) within duplex DNA play a prominent role in these processes, although it has been difficult to directly probe these motions and to determine their influence on the activities of protein–nucleic acid complexes. In this work, we introduce two-dimensional fluorescence spectroscopy (2D FS) in the ultraviolet (UV) regime to measure the electronic coupling between the subunits of a dinucleotide of the fluorescent nucleic acid base analogue 2-aminopurine (2-AP) [1, 2]. These experiments establish the feasibility to measure the relative separation and orientation of a selected pair of 2-AP bases that has been site-specifically substituted for designated canonical adenine (A) bases within a DNA molecule. Such analogue dinucleotide-substituted DNA constructs have previously been studied using linear spectroscopic methods to probe local equilibrium and steady-state conformational changes of defined DNA bases within functioning protein–nucleic acid complexes [3, 4]. Nevertheless, these earlier experiments did not permit the interpretation of linear spectroscopic signals in either structural or dynamic terms.

2D FS belongs to a class of two-dimensional Fourier transform spectroscopies widely used to analyze molecular structure and dynamics [5, 6]. Such experiments measure correlations between field-induced quantum transitions, which are excited by sequences of phase-related electromagnetic pulses. The resulting spectra are functions of two independent frequency variables, and can provide information about the couplings between excitation transition dipole moments. For example, 2D NMR is a well established structural tool for solution phase studies [5]. More recently, 2D IR experiments have provided structural and dynamic information about local vibrational modes in proteins [7, 8] and DNA [9]. 2D electronic spectroscopy
(2D ES) is sensitive to correlations between electronic transition dipole moments, and has been used to study mechanisms of energy redistribution in photosynthetic protein–pigment complexes [10, 11], semiconductors [12] and conjugated polymers [13]. The 2D FS method we apply here is a fluorescence-detected variation of 2D ES, which we recently used to elucidate the conformation and characterize the excited state population transfer of an electronically coupled porphyrin dimer in a biological membrane [14, 15].

In order to apply 2D FS to study nucleic acid base conformation and excited state dynamics in these systems, it was necessary to extend the capabilities of this method to excite the lowest energy UV transitions of nucleotide base analogue probes. Although considerable progress has been made toward performing 2D ES at UV and shorter wavelengths [16–20], a significant challenge is due to the occurrence of a large non-resonant background, which limits signal-to-noise (S/N) when pulses are temporally overlapped [18, 20]. For example, Raleigh scattering, which scales as the fourth power of the frequency, is especially severe for experiments using multiple UV pulses. This problem can be addressed using 2D FS. As a fluorescence-based method, 2D FS is an effective strategy to elevate signal levels by enhancing contrast, because Stokes-shifted fluorescence can be effectively separated from non-resonant background and scattered laser light. Moreover, because of the inherent sensitivity of fluorescence, 2D FS can be employed under low signal conditions (e.g. low sample concentration, low laser power, moderate extinction coefficient, etc). These considerations make 2D FS an appealing approach for solution phase structural studies of macromolecular complexes, which are very often only marginally stable at physiological concentrations (≤1 μM).

Most 2D optical experiments are based on four-wave mixing (FWM), in which three non-collinear pulses coherently excite a third-order polarization in the sample. The FWM signal is phase-selectively detected using wave vector matching and optical homodyne interferometry. The phase-selectivity of 2D ES allows for different nonlinear signal contributions to be spatially isolated according to their wave vector matching conditions. The so-called non-rephasing (NRP) signal satisfies \( k_{S}^{\text{NRP}} = k_1 - k_2 + k_3 \), the rephasing (RP) signal \( k_{S}^{\text{RP}} = -k_1 + k_2 + k_3 \) and the double quantum coherence (DQC) signal \( k_{S}^{\text{DQC}} = k_i + k_2 - k_3 \), where \( k_i \) with \( i \in \{1, 2, 3\} \) is the wavevector of the \( i \)th pulse, and \( k_S \) is that of the signal field [21].

2D FS uses a sequence of four collinear pulses to excite fourth-order populations [14, 15, 22–24], which are detected by monitoring a suitable action signal (e.g. fluorescence). Isolation of the desired fourth-order signal terms (i.e. NRP, RP and DQC) is accomplished by phase-cycling [23] or, in the case of 2D FS, by phase-modulation [22]. In 2D FS, the phases of the excitation pulses are swept at kilohertz frequencies such that the NRP, RP and DQC signals uniquely oscillate at modulation frequencies that satisfy energy conservation. Thus, the nonlinear signals of interest can be distinguished according to their phase-modulation frequencies: i.e., \( S_{S}^{\text{NRP}} \propto \expi(\Omega_1 - \Omega_2 + \Omega_3 - \Omega_4) \), \( S_{S}^{\text{RP}} \propto \expi(-\Omega_1 + \Omega_2 + \Omega_3 - \Omega_4) \) and \( S_{S}^{\text{DQC}} \propto \expi(\Omega_1 + \Omega_2 - \Omega_3 - \Omega_4) \), where \( \Omega_i \) with \( i \in \{1, 2, 3, 4\} \) is the phase imparted to the vibronic wave function upon interaction with the \( i \)th laser pulse. For the experiments presented below, we focus on the NRP and RP signals, which we write \( S_{S}^{\text{NRP}} \propto \expi(\Omega_{43} + \Omega_{21}) \) and \( S_{S}^{\text{RP}} \propto \expi(\Omega_{43} - \Omega_{21}) \), respectively, where \( \Omega_{ij} = \Omega_j - \Omega_i \) is the relative phase-modulation frequency associated with the \( i \)th and \( j \)th pulses. In our current experiments, we have set \( \Omega_{21} = 5 \) kHz and \( \Omega_{43} = 8 \) kHz, such that the NRP and RP signals oscillate at the ‘sum’ and ‘difference’ frequencies 13 and 3 kHz, respectively. By using a lock-in amplifier to phase-synchronously detect these nonlinear signals, the measurement achieves a S/N ratio of approximately 50:1.
2. Experimental methods

We performed 2D FS experiments on a dinucleotide of 2-AP, a fluorescent nucleic acid base analogue of adenine (A) (see figure 1(a)). The 2-AP ribonucleoside (r(2-AP)) has peak absorbance at $\lambda_{\text{max}}^{\text{Abs}} = 303$ nm, although this is significantly red-shifted to the value $\lambda_{\text{max}}^{\text{Abs}} = 315$ nm upon incorporation into ss or ds DNA constructs (1). 2-AP fluorescence has a large Stokes shift, with peak emission at $\lambda_{\text{max}}^{\text{FL}} = 370$ nm. The fluorescence quantum yield of the free probe in solution is $Q = 0.68$, although this is significantly reduced upon incorporation into DNA constructs [25]. 2-AP is a useful probe of local nucleic acid base environment, since it may be readily substituted for A in various DNA sequence contexts. The chemical structure of the 2-AP base is similar to A, and it selectively forms a two-hydrogen-bonded base pair with thymine (T) (see figure 1(b)), although we note that one of the hydrogen bonds of the (2-AP)–T base pair involves a different set of hydrogen bond donors and acceptors than in the canonical Watson–Crick A–T base pair [26].

Recent studies have shown that exciton coupling between 2-AP dinucleotide substituted DNA constructs can be used to spectroscopically monitor local base conformation in protein–DNA complexes [2–4]. These experiments take advantage of the electronic interaction between adjacent 2-AP residues, which is sensitive to the magnitude, separation, and relative orientation of the 2-AP electric dipole transition moments (EDTMs). A model that we used to simulate dimer conformation-dependent linear absorption and 2D FS spectra is described in detail elsewhere [14, 15]. This model uses the point–dipole approximation to describe electronic coupling between the EDTMs of the monomeric 2-AP chromophores. Here, the coupling is given by

$$V_{12} = \frac{1}{4\pi \epsilon_0 R_{12}^3} \mu_1 \cdot \left( 1 - 3 \frac{R_{12}^2}{R_{12}^3} \right) \cdot \mu_2,$$

where $\mu_{1(2)}$ is the EDTM of the first (second) 2-AP residue, $R_{12}$ is the vector connecting their centers, and $4\pi \epsilon_0$ is the vacuum permittivity. Although more accurate electronic coupling models can be readily implemented, the point–dipole approximation is a useful starting point for our calculations.

In figure 1(c), we illustrate the energy levels of two EDTMs with relative angle $\theta_{12}$. In the absence of coupling, the two EDTMs have degenerate electronic transition energy $\epsilon_1$. The effect of the electronic coupling is to create a four-level system consisting of a common ground state $|g\rangle$, two non-degenerate singly excited states $|e_{\pm}\rangle$ with energies $\epsilon_{\pm} = \epsilon_1 \pm V_{12}$, and a doubly excited state $|f\rangle$ with $\epsilon_f \approx 2\epsilon_1$. The coupling strength $V_{12}$ is determined by the EDTM magnitude $|\mu|^2$ and dimer conformation according to equation (1). In our modeling for the 2-AP dinucleotide, we used the known magnitudes and directions of the EDTMs within the 2-AP molecular frame [27]. The singly excited states are related to the monomer site basis according to $|e_{\pm}\rangle = (1/\sqrt{2})[|g_1\rangle \pm |g_2\rangle]$, where $g_{1(2)}$ label the ground and excited states, respectively, of monomer 1 (2). These symmetric and anti-symmetric states have wave functions delocalized among the chromophore sites. The collective transition dipole moments between ground and singly excited states are given by $\mu_{\pm} = (1/2)[\mu_1 \pm \mu_2]$, such that the intensities of ground state accessible transitions depend on the relative orientation of the EDTMs in the dimer. For example, a side-by-side H-type conformation (i.e. $\uparrow\uparrow$) with $\theta_{12} = 0$ results in a purely blue-shifted absorbance spectrum. On the other hand, a head-to-tail J-type conformation (i.e. $\rightarrow\rightarrow$) results in a purely red-shifted spectrum. The effect of non-zero $\theta_{12}$ is to partition intensity to the otherwise dipole forbidden (dark) transition, according to
Figure 1. (a) The molecular structure of the 2-AP dinucleotide. The lowest-energy EDTMs are indicated as blue arrows. (b) The molecular structures of 2-AP-T and A–T base pairs (bp). Note that while Watson–Crick base pairing is maintained, the 2-AP–T bp involves a different hydrogen-bonding pattern than that utilized in A–T. (c) Energy level diagram of two chemically identical two-level molecules. (Inset) An example configuration with relative transition dipole angle \( \theta_{12} \). Electronic interactions result in an exciton-coupled four-level dimer with a single ground state, two singly excited states, and a single doubly excited state. Multi-pulse excitation can excite transitions between ground, singly excited and doubly excited state manifolds. The conformation of the dimer determines the energy level spacing and the strengths of the transitions. For an H-dimer, which has a side-by-side arrangement of transition EDTMs, the red-shifted transition will be weak, while the blue-shifted transition will be strong. (d) The lowest-energy absorption band of 2-AP dinucleotide is shown (black) along with the optimized spectrum obtained by simultaneously fitting the linear absorption and 2D FS spectra to the point-dipole model (green) (described in the text). The energies of the underlying exciton transitions (\( \varepsilon_+ = 32742 \text{ cm}^{-1} \) (305.4 nm) and \( \varepsilon_- = 31258 \text{ cm}^{-1} \) (319.9 nm)) are indicated by red dots and a dashed blue line. The value obtained for the optimized monomer transition energy is \( \varepsilon_1 = 32000 \text{ cm}^{-1} \) (312.5 nm). The laser spectrum used in the 2D FS measurements (center frequency 30960 cm\(^{-1}\) (323 nm)) is shown as a dashed blue curve.
On the right side of figure 1(c), we depict an energy level diagram for an H-type dimer—the case in which the 2-AP residues are at least partially stacked. The side-by-side configuration results in blue-shifted absorbance. Furthermore, fluorescence self-quenching of the H-dimer occurs due to rapid internal conversion of the initially excited population in the $|\varepsilon_+\rangle$ level to the lowest excited $|\varepsilon_-\rangle$ level. Because the $|\varepsilon_-\rangle$ state carries little oscillator strength, slow relaxation to the ground state occurs predominantly by internal conversion.

In figure 1(d) are shown the experimental linear absorption spectrum of the 2-AP dinucleotide, and the simulated spectrum obtained by performing an optimization of the point–dipole model to the linear and 2D FS data (see section 3). Also shown is the laser spectrum used in these experiments with center energy at 30 960 cm$^{-1}$ (323 nm). The optimization procedure we performed was similar to that used for porphyrin dimers in membranes [14, 15]. As we discuss further below, our results indicate that the 2-AP dinucleotide adopts an H-type conformation, with bright and nearly dark transitions occurring, respectively, at the energies $\varepsilon_+ = 32 742$ cm$^{-1}$ (305.4 nm) and $\varepsilon_- = 31 258$ cm$^{-1}$ (319.9 nm) (indicated by red dots), and with monomer transition energy $\varepsilon_1 = 32 000$ cm$^{-1}$ (312.5 nm). We note that the fluorescence intensity of the 2-AP dinucleotide in solution is known to be quenched 20-fold relative to that of the monomeric 2-AP nucleoside (2), which is the expected outcome for an H-type dimer conformation.

Linear absorption and 2D FS measurements were performed using 50 µM 2-AP dinucleotide in aqueous buffer containing 20 mM HEPES (pH 7.5), 150 mM KOAc, 10 mM Mg(OAc)$_2$, 0.1 mM EDTA and 1 mM DTT. Control measurements were performed using the fluorescent dye POPOP in cyclohexane.

2.1. Two-dimensional fluorescence spectroscopy in the ultraviolet regime

The principles of 2D FS are described in detail elsewhere [14, 15, 22]. Here we briefly outline the approach for experiments in the UV. The collinear four-pulse sequence with interpulse delays and phase-modulation frequencies is depicted in figure 2(a). A schematic of the instrument is shown in figure 2(b). A 250 kHz regenerative amplifier (Coherent RegA 9000, 9 µJ pulse$^{-1}$, 790 nm) was used to simultaneously drive two collinear optical parametric amplifiers (Coherent OPA 9400). The output of each OPA was tuned to 646 nm (∼18 nm bandwidth), and directed through a pair of fused silica prisms using double-pass geometry for pre-dispersion compensation. Each beam entered a Mach–Zehnder interferometer (MZI), where an acousto-optic Bragg cell (Gooch and Housego R46200-2.2-.25-.7-LTD-XQ) was placed within each interferometer arm to impart a continuous phase-sweep to the incident pulses. After the Bragg cells, the pulses were frequency doubled using beta barium borate (BBO) crystals (0.1 mm thickness), and recombined at the exit beam-splitters of each MZI to form collinear trains of 323 nm pulse-pairs.

The two independently generated pulse-pairs were combined downstream to form a sequence of four collinear pulses that were focused into the sample using a 5 cm focal length fused silica lens. A pair of dichroic mirrors (Semrock FF409-Di03) was placed in the beam path just prior to sample incidence to remove residual 646 nm light. Retro-reflective mirrors (PLX 533511) mounted onto computer-controlled delay stages (Aerotech ALS130-050, 20 nm step size) were used to control the inter-pulse delay times. We note that after the BBO crystals, all mirrors used UV-enhanced aluminum coatings, all lenses were made from fused silica, and all transmissive optics used UV anti-reflection coatings.
Phase selective detection of the nonlinear signals was carried out using the 2D FS phase-modulation technique [22]. Each of the four Bragg cells were driven continuously at unique frequencies, which were detuned from their resonances (∼175 MHz) such that a relative phase sweep was applied between the first and second pulses at 5 kHz, and between the third and fourth pulses at 8 kHz. Replicas of the output pulse-trains from each MZI were directed to separate monochromators (Acton SpectraPro 150 and SpectraPro 2150i, 1200 grooves mm⁻¹ grating blazed to 500 nm, <0.4 nm resolution), and the spectrally filtered time-varying pulse intensities (5 kHz at 326 nm, 8 kHz at 326 nm) were detected using photomultiplier tubes (PMT, Hamamatsu R1527), which were mounted in air-cooled housings (Products for Research PC202CE). The photocurrent signals were pre-amplified and filtered to produce high quality ac references for lock-in detection, as described below.

Pulse widths were characterized on a daily basis using the two-photon absorption (2PA) autocorrelation method of Tian and Warren [28] (see figure 3). A silicon photodiode (Thor Labs DET10A) was placed at the sample position where a train of phase-modulated pulse pairs was incident. The signal was detected using a lock-in amplifier (Stanford Research Systems SR830), which was referenced to the ac carrier signal matching the excitation pulse-pair. The 2PA response was measured as a function of inter-pulse delay by locking to the photodiode signal at twice the pulse pair modulation frequency (i.e. using second harmonic detection). This procedure was carried out in turn for pulses 1 and 2, for pulses 3 and 4, and for pulses 2 and 3 to

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**Figure 2.** (a) UV–2D FS pulse sequence. (b) Schematic of the 2D FS instrument for experiments at UV wavelengths.
Pulse duration = 32.8 fs

(a) (b)

0 50 100-50-100
0.5
1.0
1.5
2.0
2.5
t21 (fs)

Intensity

0 50 100-50-100
t21 (fs)

1PA ACF

FWHM = 79.2 fs
2PA ACF

FWHM = 53.0 fs

λL = 346 nm

Figure 3. (a) A typical pulse spectrum, and (b) 2PA intensity auto-correlation function are shown for the case with laser center wavelength λL = 346 nm. The pulse duration is 33 fs, the pulse spectrum FWHM is 5.7 nm. The time–bandwidth product is 0.47. (c) The 2PA autocorrelation is narrower than the one-photon absorption (1PA) autocorrelation, which demonstrates that our measurements isolate the 2PA signal, and not simply leakage of the 1PA signal.

determine the pulse widths and stage positions at which the pulses were maximally overlapped (i.e. the zero-time delays). Upon optimization of the prism compensators, 2PA autocorrelation pulse-widths on the order of ∼33 fs were obtained, which corresponds to approximately 1.1 times the value for Fourier transform-limited pulses. Autocorrelation signals detected at twice the carrier frequency were significantly narrowed relative to those detected at the pulse pair modulation frequency, which confirms that our measurements isolated the 2PA signal.

For our 2D FS experiments, we placed a sample containing 2-AP dinucleotide in a fused silica flow cell at the sample position, and the fluorescence was collected at a right angle using a fused silica lens (6 cm focal length, 2 inch diameter). The fluorescence was filtered from scattered laser light (Semrock, 364LP) and imaged onto an air-cooled PMT (Hamamatsu R3896). The photocurrent signal was pre-amplified and filtered before it was split into two signals. The NRP and RP contributions to the fluorescence signal were separately detected using two identical lock-in amplifiers, which were referenced, respectively, to sum- and difference-frequency waveforms. These reference waveforms were constructed from the 5 and 8 kHz pulse pair modulation signals using a custom-built waveform mixer that generated the sum (13 kHz) and difference (3 kHz) frequency sidebands [22]. The time delays t21 (between pulses 1 and 2), and t43 (between pulses 3 and 4) were scanned from 0 to 200 fs, while setting t32 = 0. The cosine and sine projections of the NRP and RP signals were simultaneously measured at each time point. Fourier transformation of the resulting signal interferograms, with respect to t21 and t43, yielded the 2D fluorescence spectra.

We emphasize that the fluorescence-detected population signal contains NRP, RP and DQC contributions, as well as majority contributions from linear population terms. In our current experiments, we measured the NRP and RP terms by separately locking to the sum and difference frequency side bands, Ω43 ± Ω21, respectively. We note that in principle, DQC signals could also be measured using this instrument with minor adjustment to the electronic filters to create a reference waveform with modulation frequency (Ω4 + Ω3) − (Ω2 + Ω1). The selection of phase-matching condition is accomplished through the electronic synchronization of a modulated signal with its corresponding reference waveform. Thus, calibration of the signal...
phase is a simple matter of adjusting the lock-in detector to maximize the cosine projection and minimize the sine projection at the time origin (i.e. \( t_{21} = t_{32} = t_{43} = 0 \)) \[22\].

An important advantage of UV–2D FS is that the fluorescence-detected nonlinear signal is free of non-resonant background contributions from the solvent response, as recently noted by West et al [18] and West and Moran [20]. In FWM approaches to 2D ES, a large non-resonant background degrades S/N when UV pulses are temporally overlapped (e.g. for \( t_{32} = 0 \)), which limits the time-resolution of the measurement. In figure 4(a), we present control 2D FS data obtained from a sample containing the UV chromophore POPOP, where we have set the interpulse delay \( t_{32} = 0 \) fs and we used the same laser spectrum as that shown in figure 1(d). In these measurements, we observed only a single resonant feature in the RP and NRP 2D line shapes, which were peaked at the positions of the laser spectrum. The appearance of just a single resonant feature in the 2D spectrum is consistent with the behavior of an uncoupled two-level molecule. Total correlation function (TCF) spectra were constructed by combining RP and NRP spectra, as is customary \[29\]. In figure 4(b), we show the \( t_{32}\)-dependence of the absolute value of the RP and NRP signals (for fixed \( t_{21} = t_{43} = 0 \)). In contrast to FWM \[18\], the 2D FS signal is free of non-resonant contributions for inter-pulse delays close to \( t_{32} = 0 \).

3. Discussion

In figure 5(a), we present the UV–2D FS measurements that we performed on the 2-AP dinucleotide. The first electronic transition of 2-AP residues, when incorporated into various DNA constructs, is close to 315 nm (1), which allows it to be selectively excited in protein–nucleic acid complexes because the natural nucleotides and amino acids are transparent at these wavelengths. It has been shown using circular dichroism (CD) spectroscopy that when two adjacent base residues in a DNA construct are substituted with 2-AP, interaction between the chromophores leads to exciton-coupling of the electronic states and splitting of the energy levels (2). This exciton-induced energy level splitting is clearly evident in the experimental 2D FS data shown in figure 5(a). Both real and imaginary 2D spectra exhibit a strong resonant feature lying approximately on the diagonal, close to the laser center frequency at \( \omega_\tau = \omega_s = 31\,000\ \text{cm}^{-1} \). The presence of exciton coupling gives rise to an additional weak resonant feature that lies just below the diagonal at \( \omega_\tau = 31\,000\ \text{cm}^{-1} \) and \( \omega_s = 30\,600\ \text{cm}^{-1} \). This qualitative pattern in the 2D FS line shapes, in addition to the blue-shifted linear absorbance of the dinucleotide relative to the monomer (shown in figure 1(d)), suggests that the dinucleotide adopts an average side-by-side H-type arrangement of coupled transition dipoles, as expected for a base-stacked conformation [15]. In figure 5(b), we show the simulated 2D FS spectra corresponding to the optimized base-stacked conformation most consistent with our data (discussed further below). These simulated 2D spectra exhibit the same intense diagonal and weak cross-peak features evident in the experimental 2D spectra. For comparison, we also show in figure 5(c) simulated 2D spectra corresponding to the J-dimer conformation, in which the laser and linear absorbance spectra are assumed to be the same as in figure 1(d). The J-dimer exhibits only a single diagonal feature, and does not resemble the experimental 2D spectra.

As was previously demonstrated for membrane-supported MgTPP dimers [14, 15], 2D FS can be used to find a globally optimized solution for the relative conformation of the two coupled probe residues. Here we apply a similar approach to solve for the conformation of the 2-AP dinucleotide. These experiments demonstrated that excellent S/N was obtained for this system. Scattered laser light and non-resonant background were effectively removed from
the signal using two stacked long-pass emission filters. Control measurements, in which the fluorescent sample was replaced with buffer, yielded no detectable signal.

We followed a procedure similar to one we used previously for porphyrin dimers \cite{14, 15} to perform a geometry optimization using the combined linear absorption and 2D FS
Figure 5. (a) Experimental and (b) simulated UV–2D FS spectra of 2-AP dinucleotide in aqueous buffer. The experimental 2D spectra exhibit a diagonal peak and a cross-peak below the diagonal, indicating the existence of exciton splitting in the dinucleotide. Horizontal and vertical dashed lines indicate the center frequency of the laser 30 960 cm\(^{-1}\) (323 nm). The simulated 2D spectra are the result of an optimization of the point–dipole model to the experimental 2D FS and linear absorbance data shown in figure 1(d). The structural parameters so obtained are the inter-base distance \(R_{12} = 3.5 \pm 0.5\) Å, and relative twist angle \(\theta_{12} = 5^\circ \pm 5^\circ\) between EDTMs of the 2-AP residues. The top row shows NRP spectra, the middle row shows RP spectra, and the bottom row shows TCF spectra. (c) Simulated UV–2D FS spectra corresponding to a J-dimer conformation with laser and linear spectrum the same as in figure 1(d).

In the current work, the laser spectral bandwidth was significantly narrower than the spectral bandwidths of the underlying exciton features (see figure 1(d)). Furthermore, the laser spectrum was positioned near the low-energy shoulder of the 2-AP absorption band in order to emphasize the weaker red-shifted exciton. The features in the resulting 2D FS signal were observed to be red-shifted relative to the linear absorption maximum, and slightly blue-shifted...
relative to the laser maximum (see figure 5(a)). To account for the effects of the finite laser
bandwidth, the laser detuning from the absorption maximum, and the observed peak shifts in our
data, we included in our model corrections based on the spectral overlap between the laser and
absorption spectrum. We modeled the laser spectrum \( g(\omega) \) and the individual spectral features
\( a_{nm}(\omega) \) as Gaussians

\[
g(\omega) = \exp \left( -\frac{(\omega - \omega_L)^2}{2\sigma_L^2} \right)
\]

and

\[
a_{nm}(\omega) = \exp \left( -\frac{(\omega - \omega_{nm})^2}{2\sigma_1^2} \right),
\]

where \( \omega_L \) is the center frequency of the laser spectrum, \( \sigma_1 \) is the standard deviation of the exciton
line shape (\( = 658 \) cm\(^{-1}\), obtained from optimization), and \( \sigma_L \) (\( = 277 \) cm\(^{-1}\)) is the standard
deviation of the laser spectrum. For a given transition, the simulated 2D FS spectrum was
determined from the spectral overlap function \( g(\omega) \ast a_{nm}(\omega) \). The transition frequency was thus
adjusted to

\[
\omega_{nm} \rightarrow \tilde{\omega}_{nm} \equiv \frac{\omega_L \sigma_1^2 + \omega_{nm} \sigma_L^2}{\sigma_1^2 + \sigma_L^2},
\]

and the weight of the transition determined by the laser amplitude was adjusted to

\[
\alpha(\omega) \rightarrow \tilde{\alpha}(\omega) = \exp \left( -\frac{(\omega - \omega_L)^2}{2(\sigma_L^2 + \sigma_1^2)} \right).
\]

We have implemented the above model to describe a wide range of experimental 2D FS
data for a membrane-supported porphyrin dimer in which the subunits were connected by
a flexible linker. In these studies, the laser center frequency was systematically tuned across
the absorption band, and we obtained very good agreement between our experimental results
and the corrections predicted by equations (4) and (5) when the laser was centered near the
absorption maximum. This work will be published elsewhere. When the laser was detuned
further from the absorption maximum, the model reproduced the relative intensities of peaks in
the 2D FS spectra very well, but slightly underestimated the extent to which the peak positions
were red-shifted toward the laser’s peak position. To account for this inaccuracy, we included in
our fit an additional shift parameter that allowed for the 2D FS peak positions to be optimized
beyond the predictions of the above model. Our optimized peak positions were thus further
red-shifted relative to the prediction given by equation (4): \( \tilde{\omega}_{nm} - 191 \) cm\(^{-1}\).

Using the above model, we simultaneously fit the linear absorption spectrum (results shown
in figure 1(d)) and the RP and NRP 2D FS spectra (results shown in figure 5(b)). Our results
yielded a coupling \( V_{12} = 742 \) cm\(^{-1}\) between 2-AP residues and an angle \( \theta_{12} = 5^\circ \pm 5^\circ \) between
EDTM s. The lowest-energy EDTM of 2-AP has magnitude \( |\mu| = 2.55 \) D [27]. For a side-to-side
EDTM geometry, these parameters yielded an average distance \( R_{12} = 3.5 \pm 0.5 \), between the
2-AP bases of the dinucleotide, which we obtained by inversion of equation (1). These values
for \( \theta_{12} \) and \( R_{12} \) are to be compared to the known twist angle 36\(^\circ\) and distance 3.4, between
adjacent bases in B-form duplex DNA. Previous CD studies of the 2-AP dinucleotide suggested
that the molecule adopts a right-handed helical conformation with moderate base stacking (2).
The CD intensity of the lowest-energy transition of the 2-AP dinucleotide is five times weaker
than in the corresponding 2-AP dinucleotide-substituted dsDNA construct. When the sample
was heated or placed in a denaturing solvent, the exciton-coupling contribution to the CD signal was observed to decrease. Although these data were originally interpreted to indicate that the dinucleotide is partially un-stacked, the structural parameters determined in this work ($R_{12} = 3.5 \pm 0.5 \text{ Å}$, and $\theta_{12} = 5^\circ \pm 5^\circ$) suggest that the weak CD signal of the dinucleotide may be the result of its reduced chirality in comparison to dsDNA. In addition, the dinucleotide fluorescence is quenched 20-fold relative to the monomeric nucleoside (2), as expected for an H-coupled dimer, thus providing further evidence that there is significant base stacking in this system. The combination of these results with the previous CD and fluorescence quenching experiments suggests that at room temperature, in aqueous solvent, there is significant base stacking in the dinucleotide with relatively small twist angle.

These results are significant in the context of the energetics of base stacking in duplex DNA. Our observations imply that optimal nearest-neighbor stacking of a dinucleotide in
aqueous solution may involve as much surface occlusion of the bases as possible, consistent with the small twist angle that we measure (see figure 6(a)). This is the expected outcome for the dinucleotide, provided that the hydrophobic forces favoring stacking exceed the opposing strain that such maximal stacking may induce in the connecting sugar-phosphate backbone [30, 31]. The reduced stacking (surface occlusion) seen between neighboring bases in longer dsDNA (twist angle $\sim 36^\circ$ in B-form dsDNA, see figure 6(b)), where of course both ‘faces’ of the bases are significantly removed from contact with the aqueous solvent by stacking, may reflect the energetic requirement to accommodate the stiffness of the sugar-phosphate backbones by simultaneously maximizing base stacking and minimizing backbone strain, resulting in the formation of base-stacked double helical structures of duplex DNA of the type introduced by Watson and Crick (see figure 6(c)). We note that because the 2D FS measurement is sensitive to the absolute value of the twist angle, and not the sign of the chirality, the relatively small magnitude for $\theta_{12}$ we observe cannot reflect a cancellation between signal contributions from either a pair of static or dynamic structures of B-form geometry and opposing chirality, as might be the case for a comparable CD result.

4. Conclusions

We have determined the average solution conformation of the dinucleotide of the fluorescent nucleic acid base analogue 2-AP, which is a useful probe of local nucleic acid base structure. This was accomplished by implementing the 2D FS method in the UV regime, which allowed us to directly observe the effects of conformation-dependent exciton coupling between the 2-AP bases of the dinucleotide. 2D FS presents important advantages for experiments in the UV, such as the relatively high sensitivity associated with fluorescence measurements and the straightforward separation of signal from non-resonant background. Future work will involve altering the pulse polarization sequences used for 2D FS measurements to enhance cross peaks, and using 2D FS to determine the local conformations of DNA and RNA within protein–nucleic acid complexes.

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