Early Events of DNA Photodamage

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Abstract

Ultraviolet (UV) radiation is a leading external hazard to the integrity of DNA. Exposure to UV radiation triggers a cascade of chemical reactions, and many molecular products (photolesions) have been isolated that are potentially dangerous for the cellular system. The early steps that take place after UV absorption by DNA have been studied by ultrafast spectroscopy. The review focuses on the evolution of excited electronic states, the formation of photolesions, and processes suppressing their formation. Emphasis is placed on lesions involving two thymine bases, such as the cyclobutane pyrimidine dimer, the (6-4) lesion, and its Dewar valence isomer.

1. INTRODUCTION

Around 1930, the physician Frederick L. Gates conducted experiments on the bactericidal action of ultraviolet (UV) light (1–3) and measured the wavelength dependence of the effect. With regard to these action spectra, he stated (1, p. 480)

> The close reciprocal correspondence between the curve of bactericidal action and the curves of absorption of UV energy by these nuclear derivatives not only promotes the probability that a single reaction is involved in the lethal action of UV light, but has a wider significance in pointing to these substances as essential elements in growth and reproduction.

Concerning the biological relevance of "these nuclear derivatives," deoxyribonucleic acid (DNA) in modern parlance, this statement was prophetic. Nowadays, it is of course established that DNA is the molecular basis for genetics. Its damage by UV light has implications for medicine, biotechnology, and our understanding of the very early phases of evolution (4–9).

Starting with the isolation of the thymine dimer as a UV irradiation product of DNA in 1958 (10–12), numerous photoproducts have been discovered (13). Some structures of these photolesions are discussed in this review, together with their formation kinetics and the photophysical properties of DNA.

DNA consists of a sugar phosphate backbone to which the four bases carrying the genetic information are attached by glycosidic bonds. The four DNA bases—adenine, cytosine, guanine, and thymine—exhibit absorption bands peaking in the range 250–270 nm (**Figure 1*c***), whereas the backbone absorbs only at wavelengths below ∼220 nm.

The absorption peaks of DNA bases fall into the UV-C (200–280 nm), which is very harmful to biomolecular systems. Yet the UV-C is strongly absorbed by ozone and other protective molecules in the atmosphere and reaches Earth's surface only in very small doses. Its lethal action is used for sterilization purposes via artificial UV sources (14). The long-wavelength part of DNA absorption has some overlap with the UV-B (280–315 nm), where solar irradiation is not negligible (**Figure 1*a***), and results in the direct excitation of DNA. The UV-A (315–400 nm) most often acts on DNA via sensitizing molecules (13).

For all DNA bases, the peak absorption coefficient ε ranges from 8,000 to 15,000 $M^{-1}cm^{-1}$ (**Figure 1*c***). This magnitude is in accordance with singlet $^1\pi\pi^*$ transitions being responsible for the bands (15, 16). The dynamics induced by the absorption of UV photons via the $^1\pi\pi^*$ state in monomeric DNA bases have extensively been described in review articles (17–20). Luminescence experiments on aqueous solutions of the bases themselves, of nucleosides (bases attached to deoxyribose), and of nucleotides (base carrying deoxyribose and phosphate) revealed fluorescence quantum yields Φ_{fl} of the order of 10^{-4} (21). As evidenced by femtosecond UV/visible absorption (22) and fluorescence spectroscopy (21) on single bases, these low yields result from efficient nonradiative processes depleting the $^1\pi\pi^*$ state within 200 fs to 1 ps. Internal conversion to the electronic ground state, generating vibrationally hot molecules, dominates excited state depletion in single bases (see **Figure 2*a***). The vibrationally hot ground state cools on a 1–10-ps timescale by heat transfer to the surrounding medium. The transfer of vibrational energy in the DNA base and to the solvent has been studied by multidimensional infrared (IR) spectroscopy (23–25). Conical intersections mediating these transitions have been identified by quantum chemical methods (19, 26–31). For the pyrimidine bases, there have been indications of competing internal conversion processes populating longer-lived excited states with $^1n\pi^*$ character (18, 32). The quantum yields of these states are of the order of a few percent. Intersystem crossing transitions to triplet states with $^3\pi\pi^*$ character were also observed (4, 33–35). For instance, for a monomeric thymidine

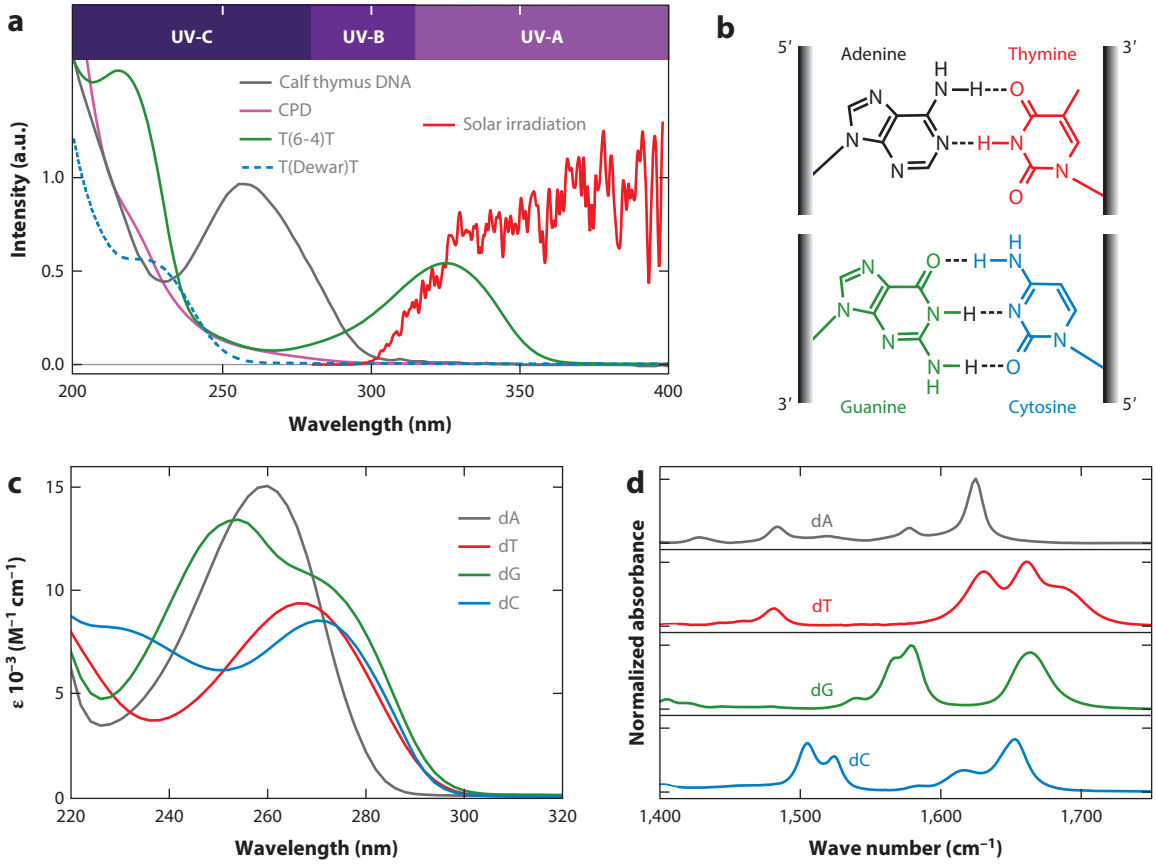

Figure 1

(*a*) Overview of the UV absorption spectrum of natural calf thymus DNA (*solid gray line*) and of the most prominent photolesions, together with the short-wavelength part of the solar emission spectrum determined for the Munich area (*solid red line*). (*b*) Scheme of DNA, including hydrogen-bonding interactions for Watson-Crick base pairs. (*c*,*d*) UV and IR absorption spectra of the DNA nucleosides adenosine (dA), thymidine (dT), guanosine (dG), and cytidine (dC). Abbreviation: CPD, cyclobutane pyrimidine dimer.

(thymidine monophosphate) in water, the triplet state is populated with a quantum yield Φ_{ISC} of ~1% that varies strongly with wavelength, from 0.4% at 280 nm to approximately 3% at 250 nm. For diluted water solutions, the lifetime of $^3\pi\pi^*$ states is of the order of 10 μs but rapidly decreases with O_2 or thymine concentration (4, 35, 36).

As stated above, all single bases dissipate electronic excitation predominantly (>90%) via very rapid (<1 ps) decay channels. This is in stark contrast to many other closely related heterocyclic compounds with much longer $^1\pi\pi^*$ lifetimes (19). Fast nonreactive deactivations lower the probability for photoreactions. Thus, this property has been discussed in relation to the selection of these bases and the RNA base uracil in the early stages of evolution (for a recent discussion, see 9).

In single- and double-stranded DNA, the bases are held in close proximity (**Figure 3**, left). The intrastrand distance between bases is approximately 3.5 Å (i.e., they are close to van der Waals contact). At these distances, stacking interactions organize the structures and yield nearly parallel arrangements of the heterocyclic ring systems of the nucleobases. In double-stranded DNA, the bases of a Watson-Crick pair are connected by two (A···T) or three (G···C) hydrogen

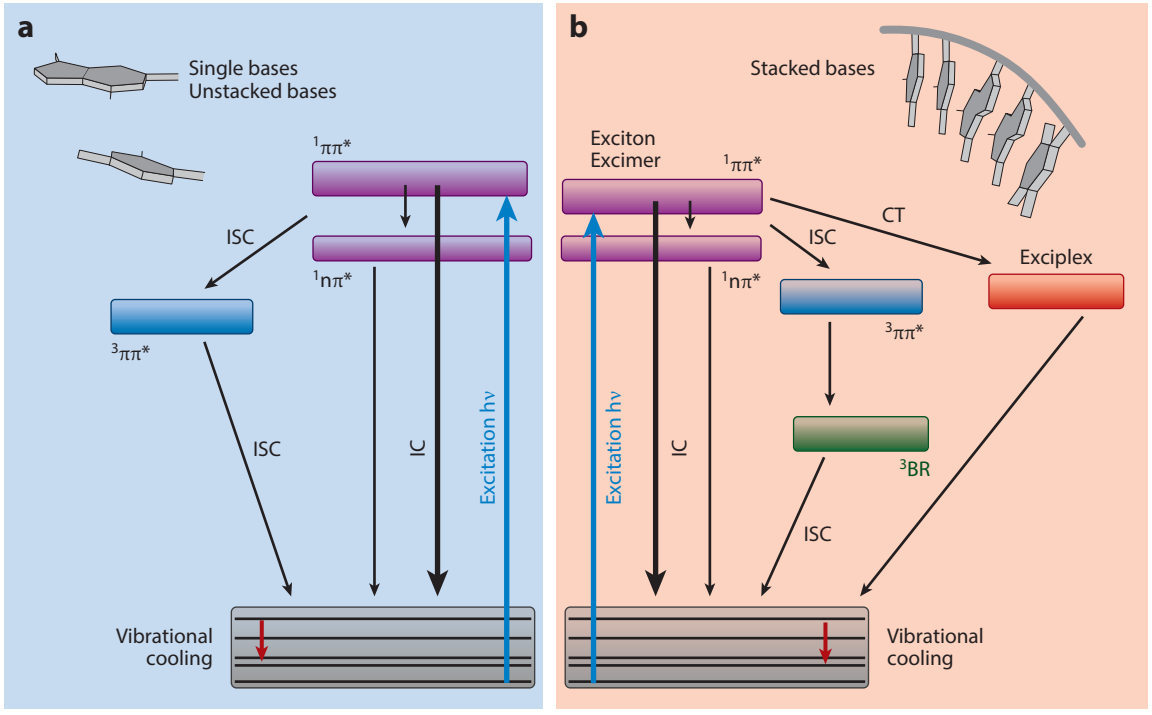

Figure 2

Photophysical processes after the UV excitation of (*a*) isolated DNA bases and (*b*) single- and double-stranded DNA. All nonradiative transitions [internal conversion (IC), intersystem crossing (ISC), and charge transfer (CT)] are followed by vibrational cooling.

bonds. The spatial and energetic proximities of the $^1\pi\pi^*$ states delocalize the excitation over several bases or base pairs and complicate the photophysics of DNA (see **Figure 2*b***) (17, 18, 37–39). Borrowing from solid-state physics, these excitations are often referred to as excitons. To a certain extent, these excitons decay by processes similar to those mentioned for isolated bases. However, additional decay or reaction routes become important. The close proximity of bases may facilitate light-induced reactions, generating photolesions directly from the originally excited singlet or via the subsequently produced $^1n\pi^*$ or triplet states. In addition, exciplexes with strong charge transfer character (18, 37, 40) and photoinduced proton transfer over the hydrogen bond of bridged molecules have also been discussed (41–43). Some of these processes result in long-lived excited states with considerable occupation probabilities (approximately 50%), which may play an important role in lesion formation or damage repair.

For photochemistry studies, the spectroscopic and photophysical properties of DNA pose a number of challenges: The absorption bands of the four bases overlap, and the selective excitation of one type of base, let alone a specific base in the sequence, is not possible for native DNA (see **Figure 1*a*,*c***). The photophysical processes occurring on timescales from 100 fs to microseconds may mask the photochemical ones. The different electronic states accessed are potential precursors for the photoproducts. Furthermore, the quantum yields of the photoreactions are low. Even for the most important lesions, the formation yields are only of the order of 0.01 (4, 13, 44). Finally, photoproducts or intermediates toward these products often exhibit a higher degree of saturation than the bases, and their absorption is shifted to shorter wavelengths (**Figure 1*a***). For instance, upon the formation of the most abundant DNA photolesion, the cyclobutane pyrimidine dimer

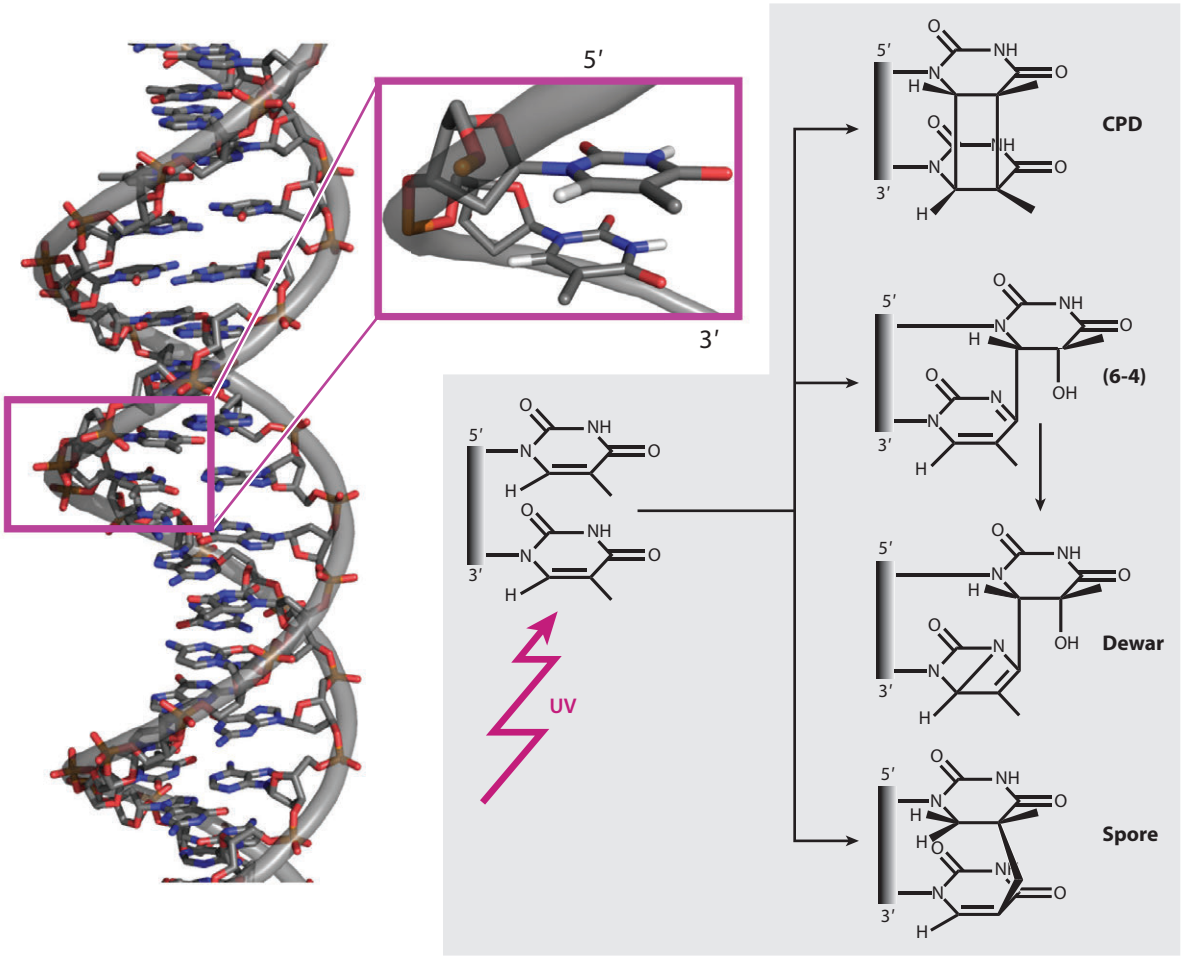

Figure 3

Molecular structures of the DNA photolesions discussed in this review. (*Left column*) Stick representation (rendered using PyMol) providing the structure of double-stranded DNA in the most common B form. The gray tubes are overlaid on the sugar phosphate backbone. The violet box highlights two adjacent thymine bases on one strand. (*Middle column*) A simplified scheme of this precursor of the photolesions compared with the stick representation. (*Right column*) Chemical structures of the photolesions. Note that the structures illustrate the chemical connectivities; the actual geometries differ substantially.

(CPD), two double bonds transform into single bonds. The photoproduct absorbs at shorter wavelengths (see **Figure 1*a***) (45), and the absorption may overlap with the backbone. The spectroscopic identification of the lesion by UV spectroscopy becomes difficult, if not impossible.

Time-resolved experiments intended to investigate the kinetics of DNA photochemistry have been designed to take these issues into account: The experiment ought to cover a large temporal range, ideally from ~100 fs to milliseconds. The spectroscopic technique also has to be sensitive to the signatures of the photoproducts. The technique should allow for a high dynamic range to record patterns of the initial photoexcitation, as well as those of the photoproducts. The latter will exhibit signals typically two orders of magnitude smaller than the former. As discussed below, (ultrafast) UV pump/IR probe spectroscopy fulfills these requirements (46–53). A comparison of

steady-state UV and IR spectra of the bases (**Figure 1*c*,*d***) already points to the advantages of IR probing. Many characteristic resonances are resolved in the IR, which allows one to trace the photoproducts.

Sample selection is also crucial. From the perspective of biological relevance, working with native DNA seems most appropriate. The studies summarized below adopted a reductionist's approach. Samples often contain only one type of base. Another strategy is the use of specific sequences, designed to allow selective excitation, to highlight a specific reaction or to monitor certain product states.

Of the numerous products resulting from the UV excitation of DNA (for recent overviews, see 13 and 20), only a few are discussed here. These dimeric products may form most frequently if two thymine bases are adjacent on a DNA strand (**Figure 3**). Following UV excitation, the two bases may transform into a CPD. In the CPD, two single C–C bonds interconnect the two bases. Under native conditions, this is the lesion with the highest abundance (13). UV irradiation can also yield the (6-4) lesion. Herein, the two bases are linked by a single bond between the C6 atom of one heterocyclic ring and the C4 atom of the other one. Its abundance is smaller by an order of magnitude than that for the CPD (13). The (6-4) lesion contains a pyrimidinone moiety. Photoexcitation of this moiety triggers another reaction, resulting in a Dewar valence isomer. For solar irradiation, most (6-4) products undergo this secondary reaction (13). These three lesions are found in DNA of all life-forms. A lesion specific to bacterial spores is the spore photoproduct (54). In this product, the methyl substituent of one thymine transforms into a methylene linker between the two rings. All these photoproducts are stable under physiological conditions. In living cells, their elimination requires the action of specialized enzymes (55–60).

2. DNA EXCITED STATES

2.1. Excited State Dynamics in Single-Stranded DNA

DNA contains a linear sequence of bases as bits of information, in which the close distance between adjacent bases induces interactions of the electronic system for well-arranged, stacked geometries. The interactions in strands significantly modify the temporal evolution of the excited electronic states, as can be seen from time-resolved absorption and fluorescence experiments (18). Whereas long-lived states are formed in monomeric bases with quantum yields of less than 10%, much higher amplitudes occur in single-stranded DNA. Time-resolved absorption spectroscopy has shown that the amplitudes of long-living components are related to the probability of base stacking (37, 61–63). The molecular nature of these long-living states has been discussed extensively in the literature (18, 20). Time-resolved UV absorption spectroscopy of different DNA strands presented a picture in which the initially excited excitons form excimers or exciplexes with charge transfer character. The exciplexes themselves disappear by charge recombination on a subnanosecond timescale. Because exciplex states with strong charge transfer character commonly have low radiative rate constants, they give only very small contributions to the fluorescence emission. Recently, details of the molecular nature of these states were identified by time-resolved IR spectroscopy (37, 51, 64, 65). DNA oligomers have been designed to allow the selective excitation of one base. Selectivity for specific molecular states was obtained by probing the evolution of the IR absorption spectra. With reference experiments and quantum chemical calculations, it was possible to identify IR marker bands of cationic and anionic states of the different bases in the recorded spectra. At early times, the absorption signal displayed the decay of the initially populated $^1\pi\pi^*$ state and the cooling of the vibrationally hot ground state. These signal components disappeared within 10 ps. The remaining long-living component revealed the following trends (37):

larger signal amplitudes for base combinations representative of increased base stacking, cationic and anionic marker bands originating from charge transfer along the strand, and charge transfer directed according to the oxidation potential of the participating bases. Additionally, in longer oligomers, there were indications of charge transfer over several bases and charge delocalization. Finally, charge recombination depends on the type of base. Time constants between 20 and 500 ps were found, with a qualitative correlation with the energetics deduced from ionization potentials and electron affinities. Similar correlations have been reported for recombination rates from UV experiments (62).

The molecular processes leading to the observed IR transients in single-stranded DNA may be described as follows. The single strands contain regions with stacked and interacting bases, as well as unstructured parts. The UV excitation of a base in the unstructured part leads to the well-known rapid decay of the $^1\pi\pi^*$ state, as in monomeric samples. Excitation in a stacked part may be delocalized (excimer). Charge transfer traps the excitation in charged radicals for many picoseconds. These long-lived radicals may cause further reactions, such as oxidative or reductive damage formation or damage repair, not considered presently in DNA photochemistry (65).

2.2. Excited State Dynamics in Double-Stranded DNA

In cells, DNA is organized for the longest part of the life cycle in the double-helical structure of the B-DNA (**Figure 3**, left). This organization leads to base stacking in each strand with a parallel arrangement of the heterocycles of neighboring bases. Base pairing via Watson-Crick hydrogen bonds (**Figure 1**) combined with electronic interstrand interactions secures the stability of the double helix. Upon the formation of the double helix, the UV and the IR spectra change because of the altered stacking interactions and the newly formed hydrogen bonds. The double-helical organization is the origin of interesting features of natural DNA. Planar aromatic molecules can be intercalated in the base stack. The close distance between the bases facilitates energy as well as charge transfer along the strand (66, 67) and allows applications such as DNA electronics. The close packing keeps adjacent bases in arrangements in which dimeric photolesions can be formed. Conversely, the DNA structure may disfavor lesion formation.

In the double-helical structure, pairing interactions lead to a higher complexity of the ultrafast dynamics. Experiments have been performed for different types of paired bases: for single hydrogen bridged base pairs in the gas phase or in solutions, for well-ordered sequences such as polyA or poly GC of different lengths, for short double strands for which the structure was induced by a hairpin turn, and for long natural DNA strands. In time-resolved experiments on single model base pairs, an accelerated excited state decay was reported as compared to monomers (68–70). For longer double strands, time-resolved experiments in the UV revealed subpicosecond components together with slow absorption dynamics of large amplitudes, extending to the 100-ps range (61). These observations were interpreted in terms of delocalized intrastrand excitons with some charge transfer character. A pairing dependence of the recombination rate was explained by a modulation of the energetics by the interstrand hydrogen bonds. In this model, base stacking was assumed to control the decay of the long-lived excited electronic state. In time-resolved fluorescence experiments, several different kinetic components were observed, extending from subpicoseconds to nanoseconds, with larger amplitudes of the short-lived components (63, 71–73). The fast kinetics were assigned to the transition to dark states. From anisotropy measurements, the authors concluded that there is a very rapid initial energy transfer in natural and model duplexes (63). The anisotropy of the long-lived components should originate from delayed $^1\pi\pi^*$ emission induced via longer-lived charge transfer states. Until now, the question of whether charge and excitation transfer remain intrastrand processes is still controversial.

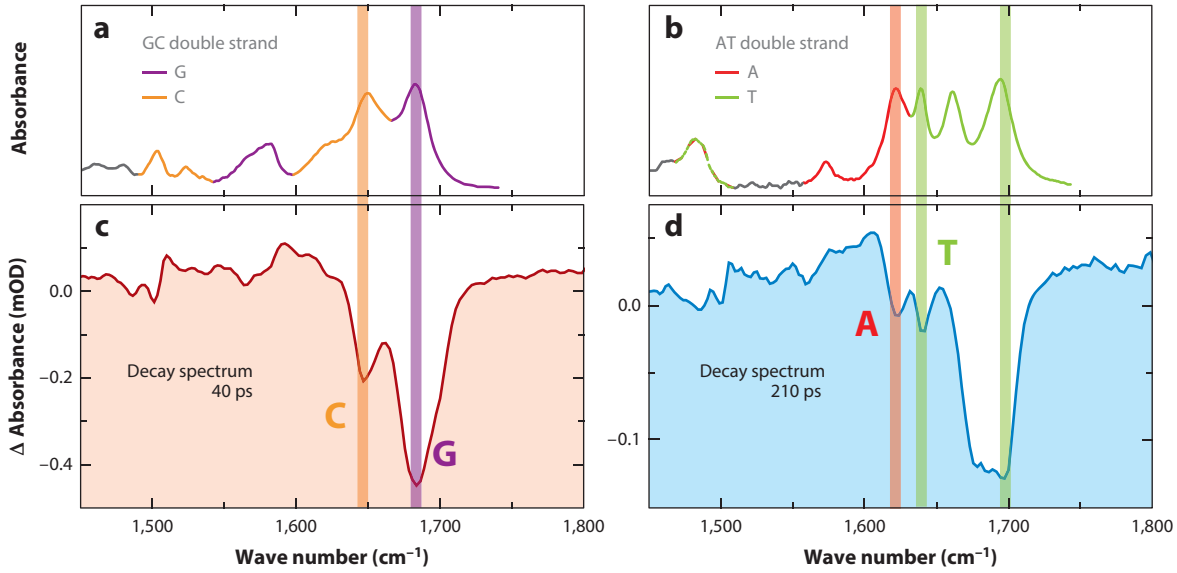

Figure 4

(*a*,*b*) Absorption spectra of a $(dGdC)_4 \cdot (dGdC)_4$ and a $(dA)_{18} \cdot (dT)_{18}$ duplex with the assignment of the IR absorption bands to the respective bases. (*c*,*d*) Decay-associated spectra for the long-living components of natural calf thymus DNA, showing the concerted recovery of paired bases. Figure adapted from Reference 74.

Very recently, Bucher et al. (74) published time-resolved data from UV pump/IR probe experiments on double-stranded DNA (see **Figure 4**). Transients on a 5-ps timescale showed signatures of vibrational cooling and pointed to a partial rapid decay of the excited state to the ground state. The remaining absorption differences (approximately 50%) displayed signatures that allow the assignment to specific bases. A signal decay in the 40-ps range (**Figure 4*c***) is related to the recovery of the ground state absorption of guanine and cytosine (**Figure 4*a***), whereas the transient spectrum of the longer time constant of approximately 210 ps (**Figure 4*d***) is dominated by contributions from adenine and thymine. Surprisingly, the data revealed that paired bases connected by Watson-Crick hydrogen bonds recovered with the same time constant. Additional information was obtained from short sequences designed to demonstrate the differences occurring upon pairing. Whereas the charge transfer states in the single strands had long and very different decay times (G^+A^- decays with 490 ps, C^+U^- with 65 ps), the combination of the two single strands to a duplex yielded only one, short, time constant of 40 ps for the recovery of guanine and cytosine. Paired bases are again synchronized.

Different models have been discussed to explain the differences in the excited state decay or ground state recovery of single- and double-stranded DNA. Domcke and colleagues (41–43) used the direct coupling of the excitation of one base to interchain charge transfer and ultrarapid motion along the proton transfer coordinate to explain the ultrarapid decay seen in single paired bases (without stacking). Kohler and colleagues (61, 62, 75) presented another model (see above) in which the excitation and charge transfer occur within one strand and the decay is modulated only by the pairing interactions. A third possibility involves an excited state (excitonic or charge separated) that induces (double) proton transfer between paired bases (76, 77). Although each model can explain individual experimental observations, additional experiments and theoretical studies are required to decide on the molecular structure of the long-living states and decay mechanisms.

This knowledge would considerably promote the understanding of the excited states preceding lesion formation in the DNA duplex.

3. UV-INDUCED PHOTOLESIONS

3.1. Pyrimidine Pyrimidone Adducts: The (6-4) Lesion

In a (6-4) lesion, the two pyrimidine bases adjacent on the DNA strand are linked by a single bond between the carbon atoms at the 6 and 4 position of the two rings (see **Figure 3**). The quantum yields for these lesion are of the order of 10^{-3} (13, 54). The highest yields were reported for (6-4) lesions involving a cytosine thymine base sequence (44). In the following, we focus on (6-4) formation between two thymine bases. This (6-4) lesion contains a pyrimidinone moiety with an absorption band peaking at 325 nm (**Figure 1*a***) (78).

This band is well separated from the one of the thymine base (see **Figure 1*a***) and can serve as an indicator for lesion formation in a time-resolved experiment. A laser flash photolysis experiment on $(dT)_{20}$ dissolved in water showed that, upon 266-nm excitation, the band characteristic for the pyrimidinone moiety rises within a few milliseconds. On this timescale, the ground state bleach remains constant (78). The characteristic time for the rise is orders of magnitude longer than the respective decay for the triplet [10 ns (79)] or the singlet [~500 fs (17)] excitation of thymine. This implies that the (6-4) lesion is formed via a ground state intermediate, which is spectroscopically silent in the visible to near-UV range. The observation is in agreement with suggestions (35, 59, 78) that this intermediate bears an oxetane ring (see **Figure 5*a***). The formation of this ring would involve the carbonyl function at the 4 position of one thymine and the C=C double bond of a second thymine. Because of its smaller extent of π-conjugation in comparison to the thymine moiety, this oxetane is not expected to absorb in the visible or near UV. Such an addition between an excited carbonyl compound and an alkene is a well-known reaction in organic photochemistry and is referred to as the Paternò-Büchi reaction (80). Depending on the compounds involved, the reaction can yield oxetanes with long lifetimes, which can be isolated, or short-lived oxetanes, which undergo rapid ring opening. The millisecond rise observed in Reference 78 describes this ring opening. In a thymidylyl-(3′,5′)-thymidine (TpT) model compound in which one carbonyl oxygen at the 4 position was replaced by a sulfur atom, a photoproduct containing a four-membered ring (thietane moiety) was isolated and characterized (81). This gives further (indirect) support that the (6-4) lesion forms via an unstable four-membered ring.

3.2. Dewar Valence Isomer

The (6-4) photolesion has a modified electronic system with a pyrimidinone moiety, and its long-wavelength band appears in the UV-A range at approximately 325 nm, well separated from the absorption spectra of pyrimidine and purine bases (see **Figure 1*a***). The strongly increased solar irradiance in this spectral range, combined with a comparatively high yield for transformation into the Dewar photoproduct of 5–8% in dimeric samples (53), may lead to the rapid conversion of the (6-4) lesion in bright sunlight (13, 82). The photoreaction from the (6-4) lesion to a Dewar photoproduct shows interesting features (see **Figure 5*b***). Time-resolved experiments have been performed on a T(6-4)T model compound, derived from a T-T dinucleotide with a bioisosteric formacetal linker forming the backbone instead of the natural phosphodiester. Excitation of the T(6-4)T lesion at approximately 320 nm populates an excited electronic state with $^1\pi\pi^*$ character (53). The reaction dynamics are observed in absorption and fluorescence spectroscopy. Spectroscopy in the visible (**Figure 5*c***) shows absorption transients with different time constants.

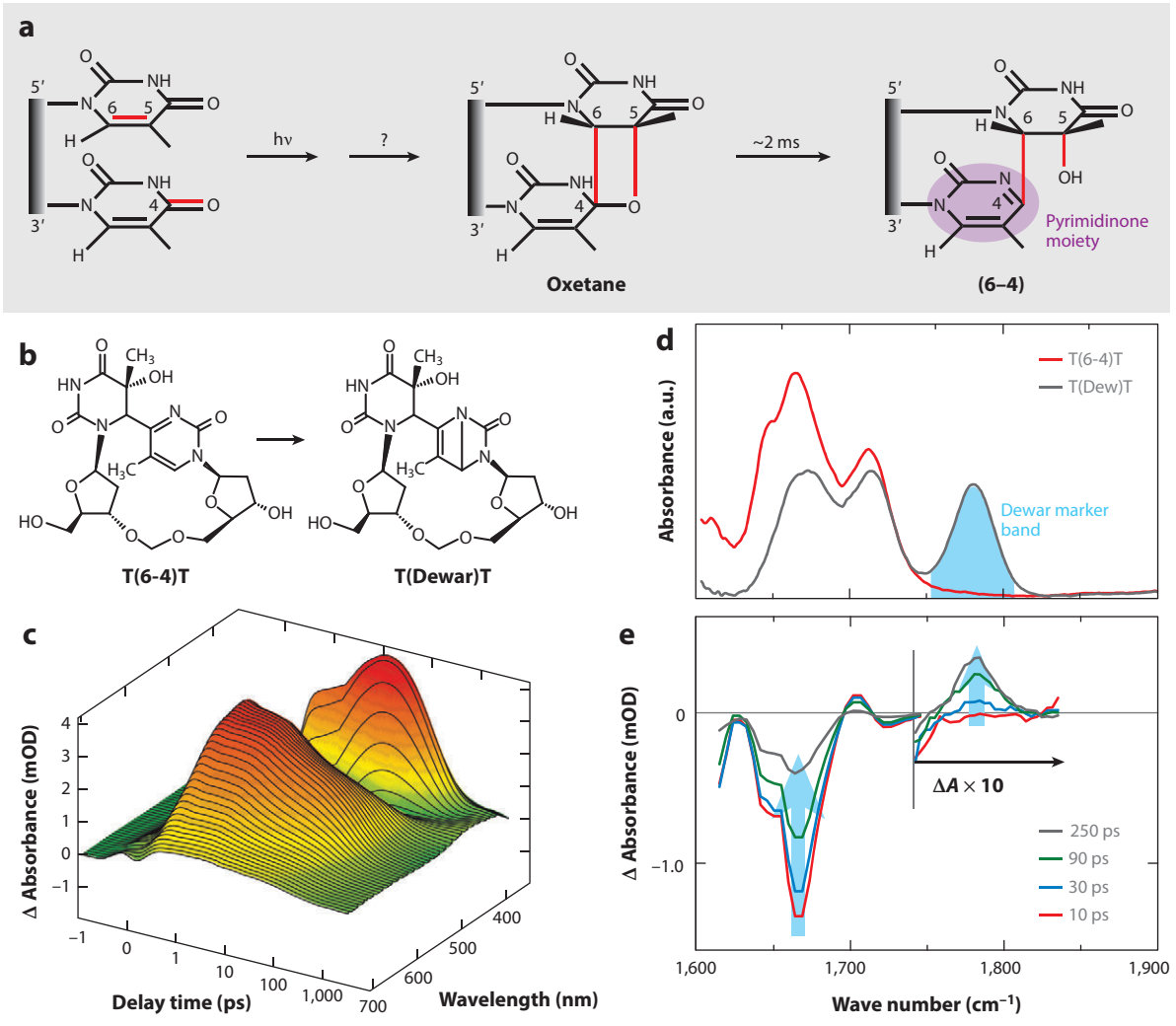

Figure 5

(*a*) Formation of the (6-4) lesion between two thymine bases. Bonds broken and formed during the reaction are highlighted in red. An intermediate oxetane has been suggested. The pyrimidinone moiety (lower heterocyclic ring, highlighted in *purple shading*) of the (6-4) lesion allowed the monitoring of the final step in the reaction. (*b*) Structures of T(6-4)T and T(Dewar)T. (*c*) Absorption changes recorded in the UV/visible range after illumination of T(6-4)T by pulses at 325 nm. (*d*) Stationary IR absorption data for T(6-4)T before UV illumination (*gray*) and after illumination (*red*), highlighting the marker band for the Dewar form at 1,780 cm^{-1}. (*e*) Time-resolved IR experiment showing the decay of the excited electronic state (~1,670 cm^{-1}) and the formation of the T(Dewar)T band at 1,780 cm^{-1} on a timescale of 100 ps.

A first 2.5-ps process is related to motions on the excited state potential surface away from the Franck-Condon region. Further time constants at room temperature are 130 ps and 1.2 ns. The UV experiments yield time constants but not direct molecular insight; time-resolved IR measurements (**Figure 5*e***) show that the marker band at 1,780 cm^{-1} (see **Figure 5*d***), characteristic for the Dewar form, appears with the 130-ps process. At later times, absorption transients are observed, which point to the formation of a triplet state with a yield of 2–10% (82). Similar kinetics (a long-lived

excited electronic $^1\pi\pi^*$ state followed by triplet formation) have been observed for 1-methyl-2(1*H*)-pyrimidinone, mimicking the chromophore part of T(6-4)T (83). However, the formation of a Dewar-like photoproduct could not be observed in 1-methyl-2(1*H*)-pyrimidinone (83). This is in agreement with illumination experiments on a T(6-4)T model compound in which no Dewar photoproduct was found after the cleavage of the formacetal linker (53). Quantum chemical modeling was used to deduce the excited state potential surfaces from the originally excited Franck-Condon region of T(6-4)T to the region leading to Dewar photoproduct formation (84). The calculations show that the relevant conical intersection depends strongly on details of the forces between the two heterocyclic systems and that the removal of the backbone prevents Dewar photoproduct formation. In addition, one may expect that geometric changes upon the incorporation of T(6-4)T into a double strand may considerably influence the Dewar photoproduct formation yield.

3.3. Spore Photoproduct

A different type of DNA lesion induced upon UV-C radiation is found in bacterial spores (44). In spores, DNA is organized in a way that keeps the genetic information functional even under extremely harmful conditions over long periods of time (85). Dormant spores are an order of magnitude more resistant to UV radiation than are growing cells (86). Spore UV resistance has been attributed to two main factors: (*a*) the different photochemistry of spore DNA and (*b*) the effcient repair mechanisms in the early minutes of spore germination and outgrowth. Whereas CPD and (6-4) photolesions are the major lesions formed under UV-C irradiation in bacterial vegetative cells, only minor amounts of these lesions are found in spore DNA. Instead, the photolesion that is almost exclusively formed in spore DNA is 5-(a-thyminyl)-5,6-dihydrothymine, arising from two adjacent thymine residues in one strand. The lesion contains a bond between the methyl group of the 3′-end thymine and the C5 atom of the 5′-end thymine residue (see **Figure 3**) (87, 88). Additionally, there is evidence that only the 5R isomer is formed because of the constraints imposed by the double helix (89, 90). As found for *Bacillus subtilis* spores, the spore photoproduct also remains the major lesion under combined UV-B and UV-A irradiation. Detailed reviews of the unique photoresistivity and photoreactivity of spore DNA are given in References 54 and 85.

In spores, DNA is tightly packed and bound to specific proteins (e.g., SASP) that are synthesized in the developing spore and then degraded after the completion of spore germination (85). As a result, the DNA molecule is arranged in a crystalline-like structure, causing major changes in its photochemical reactivity. Spore photoproducts formed under UV-C irradiation are efficiently repaired by a special spore photoproduct lyase, keeping the genetic information intact (60).

Although much research has been done on the nature of these lesions and the influence of various factors, such as humidity, temperature, binding to certain proteins, and the use of different model systems, there is still a lack of knowledge on the exact reaction mechanism (54, 85). To this point, several reaction mechanisms have been proposed (4, 54, 60, 88, 91, 92).

Varghese (88) suggested that the formation includes two independently UV-generated thymine-derived radicals: 5-a-thyminyl and 5,6-dihydrothymin-5-yl. Yet, how these radical species are generated was not explained. Additionally, a concerted reaction pathway including the methyl group of one thymine and the C5=C6 double bond of the other thymine has been discussed (4).

More recent data support a consecutive formation mechanism (see **Figure 6**) (92). As such, the lesion is formed via a triplet state of an excited thymine base and the formation of a pair of radicals at the C5=C6 double bond. The radical at the C5 position attacks the C5 methyl moiety of the adjacent 3′ thymine base. The latter reaction results in a radical pair forming the methylene bridge between the bases. Additionally, it has been suggested that the triplet-induced CPD lesion

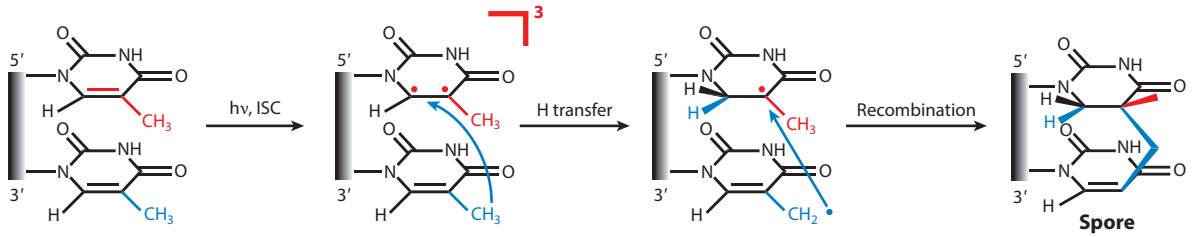

Figure 6

Proposed reaction mechanism for the formation of the spore photoproduct between adjacent thymine bases. Upon UV excitation and intersystem crossing (ISC), a radical pair is formed. Abstraction of a hydrogen atom from the 3′ thymine methyl group results in the formation of a 5-a-thyminyl and 5,6-dihydrothymin-5-yl radical. Recombination of the two radicals yields the spore photoproduct.

and the spore photoproduct share the same triplet precursor (93). Yet, time-resolved experiments are needed to proof the above hypothesis on the early events in the reaction pathway. However, the experiments are hampered by the overall small quantum yields and the required reaction conditions. Therefore, along with an improvement of the signal-to-noise levels, model systems with higher quantum yields are necessary to address the reaction in further detail.

3.4. Cyclobutane Pyrimidine Dimer

CPD lesions are the photoproducts that cause the most DNA damage under UV-B and UV-A irradiation in cellular DNA, as well as in DNA model systems (94). The lesion is formed via a cycloaddition between the C5=C6 double bonds of two pyrimidine bases, resulting in a four-membered cyclobutane ring that links the two bases together (see **Figure 3**) (11, 12). CPD formation is documented for all four possible doublets of pyrimidines and for 5-methylcytosin. Considering the possible stereoisomers, only the *cis-syn* and, to a lesser extent, the *trans-syn* conformations are found in di- and polynucleotides owing to steric constraints (59, 95). In B-DNA duplex conformations, only the *cis-syn* isomer is found (44).

The observed quantum yields for CPD formation in pyrimidine steps are of the order of 0.01 or smaller and are strongly dependent on the pyrimidine bases involved (4, 44, 96). Detailed studies based on chromatographic quantification methods on isolated and cellular DNA demonstrated that the TT CPD is generally the major product, followed by TC, CT, and CC (44, 59, 97). The frequency of CPD at a TT step is usually one order of magnitude higher than that at a CC step (44).

Owing to their relatively high abundance, CPD lesions between thymine bases became the focus of scientific research. The reaction occurs via a [2 + 2] cycloaddition, which is photochemically allowed [orbital symmetry rules by Woodward & Hoffman (98)] but thermally forbidden. The latter is in line with calculations for ground state geometries that show a high activation barrier between the educt and product state (99, 100). Accordingly, the reaction is expected to occur on the excited state surface via excited singlet or triplet states.

Ever since the pioneering work of Beukers et al. in the 1960s, the underlying reaction mechanism of CPD formation has been under debate (4, 12, 47, 78, 79, 101–103). Early experiments relied on the determination of quantum yields under specific experimental conditions and yielded support for both the singlet and the triplet pathway (4, 104). **Figure 7** presents an overview of reaction pathways leading to the CPD via singlet and triplet states. It is well known that triplet photosensitization can lead to CPD formation between isolated pyrimidine nucleotides as well as

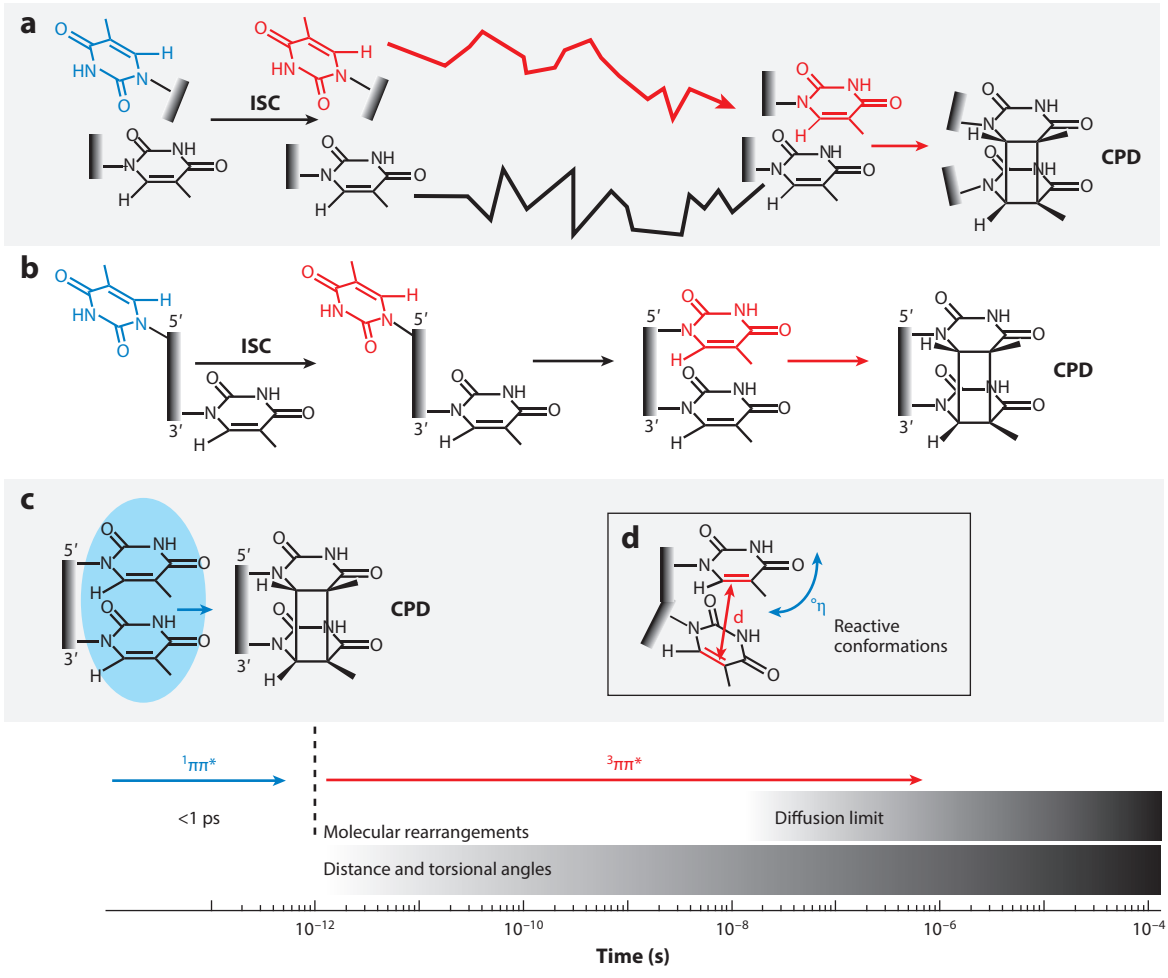

Figure 7

Schemes for cyclobutane pyrimidine dimer (CPD) formation. (*a*) The singlet excited state (*blue*) formed after the UV excitation of a single thymine base is depopulated on a timescale of 1 ps, whereas time constants for diffusion-limited processes and molecular rearrangements span timescales of several tens of picoseconds to milliseconds. Therefore, reactions between monomeric thymine bases or thymine nucleotides in solution are expected to involve a triplet state intermediate (*red*) obtained after intersystem crossing (ISC). (*b*) In thymine aggregates or DNA strands, however, thymine bases are in close proximity, and a diffusional process is not required. DNA is a highly dynamic molecule and motions such as the stacking and unstacking of bases, base-pair breathing and opening, torsional oscillations, and helix bending will frequently bring a given bipyrimidine doublet into and out of favorable geometries for dimerization. Therefore, long-lived triplet states of thymine can acquire reactive conformations during their lifetimes and contribute to the overall CPD quantum yield. (*c*) Thymine bases that are in a reactive conformation at the instant of UV absorption can directly react to form a thymine dimer within the lifetime of the singlet excited state. In this case, the reaction is controlled by the ground state geometry, and the equilibrium distribution of the relative orientation of thymine bases determines the amount of CPD lesions formed. (*d*) The reactivity of a conformation depends on distance (d) and the torsional angle (η) of the two double bonds. Figure adapted with permission from Reference 79. Copyright 2014 American Chemical Society.

in DNA strands (4, 105–107). Additionally, the lowest excited triplet state of the four different DNA bases is the one of thymine (87, 108). Therefore, it has been proposed that energy transfer from higher- to lower-lying triplet states is strongly favored, and triplet states might tend to be localized on thymine bases (107, 109, 110). This would make them prone to be hotspots for mutations. Yet, the importance of a triplet channel for CPD formation after the direct UV irradiation of DNA has been questioned by early experiments on thymine bases or nucleotides in frozen matrices and aggregates. The latter were interpreted in terms of a singlet reaction channel that would allow for dimer formation in an ultrafast photoreaction for properly oriented bases (111–114).

The first transient absorption experiments on all-thymine oligonucleotides that addressed CPD formation on the femtosecond to nanosecond timescale were performed with probing in the UV. However, because of the lack of CPD marker bands in the UV (see **Figure 1*a***) or the lack of temporal resolution, these experiments could not provide a conclusive picture (61, 78). Therefore, the reaction was readdressed using UV pump/IR probe spectroscopy (47). The study was based on IR marker bands between 1,300 cm^{-1} and 1,500 cm^{-1}, characteristic for the formation of the *cis-syn* CPD lesion (see **Figure 8**). For $(dT)_{18}$, these marker bands were observable as early as ~1 ps after UV excitation. These measurements provided the first unequivocal evidence that CPD lesions

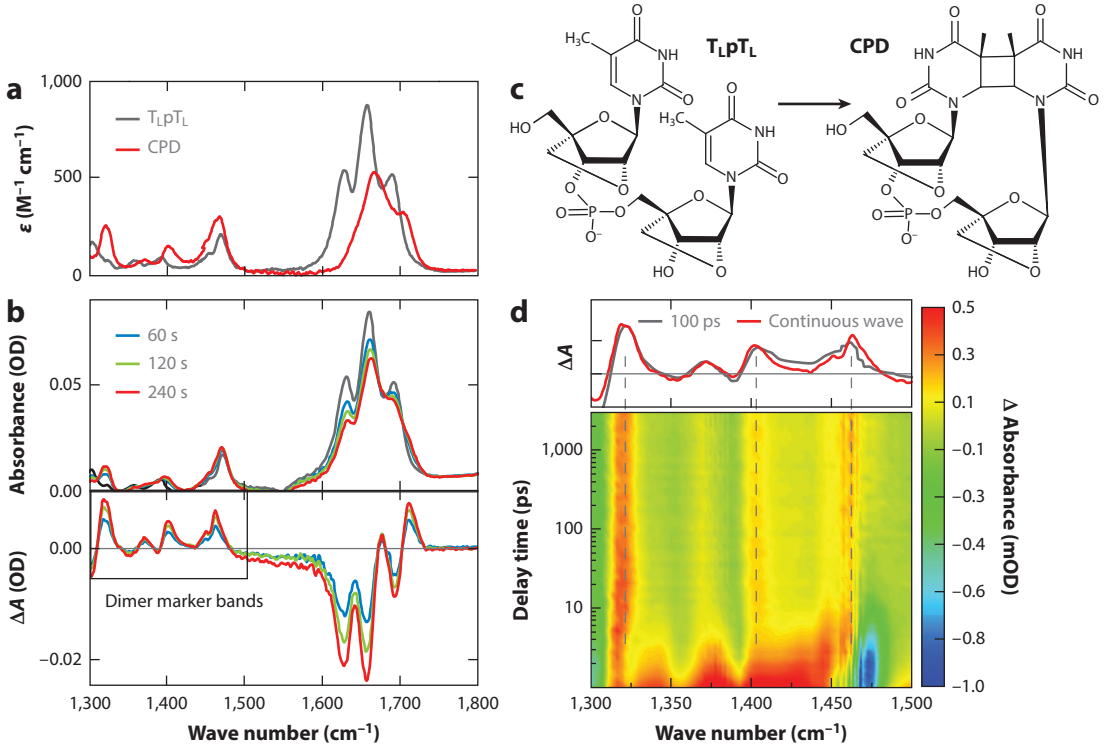

Figure 8

Cyclobutane pyrimidine dimer (CPD) formation in the locked dinucleotide T_LpT_L. (*a*,*b*) Identification of CPD marker bands via steady-state irradiation experiments using 266-nm illumination and comparison with stationary IR spectra of the educt and product molecule. (*c*) Structures of T_LpT_L and the corresponding CPD lesions. In T_LpT_L, the sugar moiety is locked into a 3-endo conformation. This results in an increased amount of base stacking and a six-fold increase in Φ_{CPD} compared to the dinucleotide TpT. (*d*) Time-resolved transient data showing the instant presence of the CPD marker bands within 1 ps after UV excitation at 266 nm.

are predominantly formed in an ultrafast photoreaction. The ultrafast nature of the reaction was interpreted as concerted CPD formation from the initially excited singlet state with $\pi\pi^*$ character, and it was suggested that the low quantum yield Φ_{CPD} for CPD formation [e.g., ~5% for $(dT)_{20}$] (115) resulted from the rareness of reactive conformations in the thermal ensemble (47). The result was further validated by a follow-up study on a series of all-thymine DNA model systems, including the locked dinucleotide T_LpT_L (**Figure 8*c***). In T_LpT_L, a methylene clamp forces the sugar into a C3-endo puckering conformation, resulting in a six-fold increase of the CPD yield compared to the dinucleotide TpT (102, 116). Measurements using circular dichroism in the UV range showed that this increase is accompanied by a significantly higher amount of base-stacking interactions. The identification of IR marker bands (see **Figure 8*a*,*b***) in time-resolved UV pump/IR probe experiments confirmed that the predominant amount of CPD formation occurs within 1 ps (**Figure 8*d***) from the excited singlet state. This finding is in line with quantum chemical studies on stacked thymine bases and TpT dinucleotides (40, 117–119).

The notion that triplet states represent only a minor reaction channel for CPD lesion formation in single-stranded DNA is supported by a comparison of the wavelength-dependent quantum yields for CPD and triplet state formation (35). Whereas the quantum yield for CPD formation is shown to be essentially constant over the main absorption band between 250 and 300 nm, the yield of triplet state formation increases by approximately one order of magnitude between 280 nm and 250 nm. Taking these results into account, investigators estimated that the overall contribution of triplet states to CPD formation in single-stranded DNA is expected to be below 10% (35). Recently, Pilles et al. (79) directly addressed the fate of triplet states in all-thymine single-stranded DNA. Monitoring the characteristic IR signature of the thymine triplet state in $(dT)_{18}$, the authors showed that triplet states predominantly decay to the electronic ground state via an intermediate on a 10-ns timescale. The intermediate species was tentatively assigned to a biradical, as suggested by experimental and theoretical studies (79, 99, 120, 121). The observed decay of thymine triplet states is in line with findings of low efficiencies for CPD formation from triplet states of monomeric thymine in aqueous solutions (122) and the low propensity of thymine triplets to form CPDs in sensitization experiments (107). It also explains why triplet states were not observed in nanosecond laser-flash experiments on dT_{20} by Marguet & Markovitsi (78).

As the formation of CPD lesions via the singlet state is significantly faster than base motions that would bring adjacent bases into a favorable position (see **Figure 7*c***), the reaction probability mainly depends on the arrangement of the bases at the instant of UV absorption (47). The rareness of the reactive conformations causes the low dimerization yields. In 2007, Johnson & Wiest (123) used molecular dynamics simulations on a 50-ns timescale to study the populations of reactive conformers that exist at any given time in $(dT)_{18}$ single-stranded DNA. Trajectory analysis showed that only a small percentage of the conformations fulfill the distance and dihedral requirements for thymine dimerization. In a related study, Law et al. (124) used the distance (d) and torsion angle (η) between the C5–C6 double bonds (**Figure 7*d***) to describe reactive thymine-thymine steps in TpT. Based on the sampling of the conformational space of TpT in various solvents and the comparison with experimentally obtained quantum yields, the authors defined reactive conformations for $d < 3.63$ Å and $\eta < 48.2°$. The two-parameter model is also supported by a study on the electronic structure during the formation of a CPD using semiempirical and first-principles approaches (125).

Another set of studies applied a single-parameter model to explain the observed quantum yields in several DNA structures, including synthetic DNA hairpins possessing TT steps (126, 127). The authors suggested that the primary constraint on dimerization is the distance d between the two double bonds, and they found that $d < 3.52$ Å leads to quantum yields that match the observed trends within a factor of three. Constraints on the dihedral angle between the two double bonds

are not seen as equally important (126). Yet the single-parameter model has its limitations and is not expected to be applicable to hairpins or duplexes in which the TT steps are flanking purine bases (127).

Currently, there is no evidence that the direct induction of CPD lesions by irradiation with UV-B or UV-C light might occur via a different channel in double-stranded DNA. Instead, the steric models were rather successful in predicting CPD quantum yields and can, for example, explain the observation of damage hotspots in core DNA (128). Yet the influence of flanking bases on the quantum yields of CPD formation is still not fully understood, and sequence-dependent thymine dimer formation is an area of intense research activity (129–135). Pan et al. (132) suggested that a combination of ground state conformation, ground state electron donor-acceptor interactions, and excited state exciplex formation is responsible for the reduced CPD yields observed for bipyrimidine steps with flanking purine bases. The investigation of these interactions and the disentanglement of steric from electronic effects is one of the main challenges for future studies on CPD formation (132, 135, 136). The latter is also important in light of a study by Holman et al. (134) suggesting that a DNA self-repair mechanism might occur via electron transfer from neighboring purine bases. Further research is also needed to elucidate the finding that methylation of cytosine (mC) enhances CPD formation (8, 13, 119, 137, 138). In a recent combined theoretical and experimental study on mC-containing trinucleotides, Esposito et al. (139) concluded that the presence of a methyl group at the 5 position of cytosine strengthens the interaction with the flanking thymine base and disfavors stacking with an adjacent guanine. Thus, reactive conformers leading to CPD are favored.

Although it has been shown that CPD lesions occur with the highest abundance in UV-B and UV-C irradiation, it is not yet fully understood how CPD lesions are formed via UV-A irradiation. In the UV-A, CPDs are formed with quantum yields that are at least two orders of magnitude smaller than those in the UV-B and UV-C range. Thereby, CPDs could arise via the direct absorption of UV-A photons owing to the weak absorption of DNA in the UV-A range or by photosensitization via the excitation of endogenous chromophores with subsequent intersystem crossing to the triplet state (13). Yet, studies comparing UV-A-induced CPDs for isolated and cellular DNA yielded similar results (140), suggesting that endogenous chromophores and photosensitization play only a minor role. Instead, there is support for a direct excitation pathway with UV-A irradiation (115). Quantum chemical calculations by Banyasz et al. (35) on TpT suggest that singlet excited states forming after the absorption of UV-A photons exhibit significant charge transfer character. It has been proposed that interconversion between charge transfer and singlet $\pi\pi^*$states is possible through vibronic coupling. Yet the probability that singlet $\pi\pi^*$ states are repopulated this way is not high and, together with the conformational constraints that define a reactive conformation, could explain the two orders of magnitude lower quantum yields found for UV-A irradiation in contrast to those for UV-B and UV-C.

4. OUTLOOK

Nature uses different strategies to ascertain the integrity of genetic information under UV irradiation. Time-resolved methods now allow us to obtain better insight into the dynamic processes induced by the absorption of UV photons in DNA and to learn about the processes leading to photolesions. Important progress has recently been made by the combination of different ultrafast spectroscopic techniques, especially in the use of time-resolved IR spectroscopy. It has been shown that single DNA bases dissipate the excitation very efficiently, on a subpicosecond timescale. However, stranded DNA shows longer-lived excited states. Charge transfer facilitated by the stacking of DNA bases plays an important role in single-stranded DNA. For natural

double-stranded DNA, the situation is more complex, and further investigations are required for a sufficient understanding of the nature of the long-lived excited states and their role in DNA integrity. Recent progress in time-resolved techniques allows a ready description of the formation of CPD and Dewar photolesions from the excited singlet states. The ground state conformation and backbone geometry determine the efficiency of lesion formation. The roles of flanking bases and of UV-A in direct lesion formation still remain unresolved. Additionally, direct investigations of the formation of the less frequent photolesions are still missing. Further improvements in time-resolved techniques combined with new types of model systems and sophisticated theoretical modeling could yield new insights, not only into lesion formation, but also into lesion repair.

SUMMARY POINTS

1. UV pump/IR probe spectroscopy allows one to follow photophysical and photochemical processes in real time. It has established itself as an invaluable tool to derive and identify reaction intermediates and photoproducts.
2. Long-lived excitation in single-stranded DNA is related to intrastrand charge transfer and charge delocalization. Their role in DNA damage formation or repair is under debate.
3. In double-stranded DNA, the pairing of nucleobases is reflected in a concerted decay of the long-lived excitation of paired bases. Base pairing, not base stacking, controls these states.
4. The CPD lesion is predominantly formed from the excited electronic singlet state $^1\pi\pi^*$ within 1 ps. As a direct consequence of the short formation time, the ground state conformation at the instance of excitation determines the reaction probability.
5. Triplet states have been identified for thymines. In all-thymine single strands, they decay on a 10-ns timescale and turn out to be only a minor channel for CPD formation.
6. The formation of the Dewar valence isomer from the (6-4) moiety occurs on a 100-ps timescale. Under bright solar irradiation, the (6-4) photolesion is efficiently converted into a Dewar valence isomer lesion.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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