Interaction of the Antitumoral Drug Pazelliptine with Polynucleotides: A Subpicosecond Transient Absorption Study

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The flat heterocyclic antitumoral drug pazelliptine (PZE) strongly interacts with nucleic acids. The transient absorption spectra of PZE/polynucleotide complexes have been investigated with subpicosecond time resolution on a large spectral range, 400−850 nm. The relaxation dynamics are found to vary widely depending upon the type of polynucleotide in which PZE has been inserted. For fully protonated PZE and for PZE complexed to poly(dA−dT)−poly(dA−dT), a similar fast spectral relaxation was detected and assigned to a deprotonation of the excited singlet state of the drug. In the case of the PZE/poly(dG−dC)−poly(dG−dC) complex, the evolution of the transient absorption spectra suggests that there are at least two different drug binding sites. The kinetics of the differential absorption reveals the activation of a radiationless transition $S_1 \rightarrow S_0$ upon PZE association to poly(dG−dC)−poly(dG−dC). The possible mechanism of this fast molecular process is discussed in connection with previously reported results on heterocycle−nucleotide interactions.

Introduction

Pazelliptine (PZE), a 9-azaellipticine derivative bearing a 3-(diethylamino)propylamino side chain, exhibits important antitumor activities.[1−3] In vivo studies have revealed that the drug induces DNA strand breaks (endonuclease).[4−6] However, it is not clear whether these DNA damages result from direct interaction with the nucleotides or if the participation of topoisomerase I is also required.[4−9] The efficient interaction of PZE with B-DNA ($K \sim 10^6$ M^{-1}) is of an intercalative nature as suggested by fluorescence energy transfer and viscosimetric measurements.[10,11] This is a prerequisite but not a sufficient condition for its biological activity, which is supposed to be based on redox reactions.[4] It appears that beside the nuclease activity, PZE also possesses photosensitizing properties: we have recently reported that the optical irradiation of PZE produces cleavages of single and double strand DNA with a preference for guanine sites.[12] This is a valuable property for a DNA-targeted drug since it allows one to use light as a trigger of the nuclease activity which may be clinically useful for increasing lethal effects against abnormal cells. Alternatively, this property permits one to extend the use of PZE as a probe for DNA structure and conformation. The study of the reaction pathway leading to the photonuclease has been undertaken; however, it is not yet clearly established.[12]

Strong fluorescence quenching has been detected upon PZE binding to calf thymus DNA (CT-DNA), which is a common feature of some intercalated heterocycles.[10,13−15] It has been shown also that fluorescence quenching is very efficient (90%) when PZE is complexed to poly(dG−dC)−poly(dG−dC) while, for the PZE/poly(dA−dT)−poly(dA−dT) complex, fluorescence is enhanced.[10] Nanosecond laser flash photolysis studies have revealed that the PZE triplet yield is also reduced in the presence of poly(dG−dC)−poly(dG−dC).[12] These results prove that the interaction between PZE and G−C base pairs activates a new radiationless transition, resulting in a significant shortening of the excited singlet state (S_1) lifetime. A closer examination of the fast photophysics of PZE should provide a new insight into this process, which may be at the origin of the photonuclease activity.

In the present work, we have investigated the interaction between PZE and polynucleotides by subpicosecond transient absorption spectroscopy. The evolution of the differential absorption spectra of the free and complexed drug was analyzed on a large spectral domain (400−850 nm). The effects identified in both kinetics and spectra are discussed in connection with their possible molecular causes.

Materials and Methods

The antitumoral drug PZE is a generous gift of Drs. Bisagni and Rivalle (Institute Curie, Orsay, France), being prepared as described in the literature.[16] The synthetic polynucleotides and calf thymus DNA were purchased from Pharmacia and used as received without purification. Drug and DNA concentrations were determined spectrophotometrically with a Cary 210 (Varian) apparatus, using the molar absorption coefficients at wavelengths corresponding to their absorption maxima: $\epsilon_{350\ nm} = 5000\ M^{-1}\ cm^{-1}$ for PZE,[17] $\epsilon_{260\ nm} = 6600\ M^{-1}\ cm^{-1}$ for poly(dA−dT)−poly(dA−dT),[18] $\epsilon_{254\ nm} = 8400\ M^{-1}\ cm^{-1}$ for poly(dG−dC)−poly(dG−dC),[19] and $\epsilon_{260\ nm} = 6600\ M^{-1}\ cm^{-1}$ for DNA.[20] For all solutions, the drug concentration was $\sim 7 \times 10^{-4}$ M; the absorption spectra did not reveal any traces of drug self-association.

For the pH influence study, free PZE was dissolved in 50 mM acetate buffer at pH 4.0, in 50 mM KH_2PO_4 phosphate buffer at pH 5.6, or in 50 mM KH_2PO_4/K_2HPO_4 phosphate buffer at pH 7.0, respectively. In every case, 100 mM NaCl was added. The PZE polynucleotide complexes were prepared in the buffer at pH 7.0. The mixing ratio (*P*/*D*) corresponding

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to the nucleotide base concentration to drug concentration was equal to 10 for all complexes. Under these experimental conditions, the concentration of free PZE in the samples was always negligible.[10]

Transient absorption measurements have been carried out using a pump−probe system based on a Nd:YAG-pumped dye laser,[11] which delivers short pulses (0.8 ps duration) at a repetition rate of 10 Hz and an energy of 400 μJ. The 624 nm laser output was directed to a 2 mm long adenosine 5′-diphosphate (ADP) crystal. The resulting UV radiation was extracted by a dichroic filter and used as the excitation beam at a pulse energy of 20 μJ. The remaining laser beam was focused into water in order to generate a continuum light for the probe beam; the continuum was then split into a sample and a reference beam. A properly oriented half-wavelength plate placed in the pumping beam turns its polarization vector to the magic angle with respect to that of the probing beam. The sensitivity of the measurement was improved by focusing the pumping and the probing beams into the sample to a diameter of 0.3 mm. The sample and reference spectral intensities were simultaneously monitored with an OMA Spec 4000 System (EG&G, Princeton Applied Research) equipped with a CCD detector. Since PZE in its ground state has a negligibly small absorbance in the probed spectral interval (400−800 nm), the resulting differential absorption spectra simply corresponds to the absorption of the formed transient species. The probing beam was optically delayed with respect to the pumping beam. In a given experiment, transient spectra were determined for a set of up to 20 chosen delay times (from −4 to +700 ps) which were automatically scanned for, at least, 30 times. For each delay, the spectra were accumulated over 50 laser shots every scan. The transient spectra were corrected for the wavelength dependence of the delay times. The corresponding absorbance was obtained by the subtraction of the apparent transient absorption measured in the absence of sample pumping from that measured with pumping. The base line, including the fluorescence contribution, was measured for a negative delay of the probe beam and subtracted from the transient spectra. Calculated decays were fitted to the experimental curves by iterative integration of the rate equations by varying the physical parameters involved.

Results and Discussion

Photophysics of Free PZE in Aqueous Solutions. PZE is a molecule with complex acid−base properties. Three different protonation states have been reported[21] corresponding to the following p*K* values: 5.5, 6.5, and 9.3. In highly alkaline solutions the PZE molecule is neutral (**IV**, Figure 1), while in acidic solutions (pH below 5.4) it is a triply charged cation (**I**). The most acidic position is N2, followed by N9 and N(R3). These complex acid−base properties could play an important role in the photophysics of PZE, as well as in the interaction between PZE and nucleotides. Moreover, it has been already pointed out that the proton concentration near the DNA surface corresponds to an effective pH of 4.5.[22] For this reason we have investigated the transient spectra of free PZE in aqueous solutions at three different pH values: 4.0, 5.6, and 7.0, respectively.

The steady-state absorption and fluorescence properties of PZE in different protonation states have already been reported.[21] The position of the first absorption maximum is sensitive to the solvent polarity and varies between 350 nm (in water) to 375 nm (in dioxane). The second absorption maximum is located at about 300 nm. Its amplitude is very sensitive to the

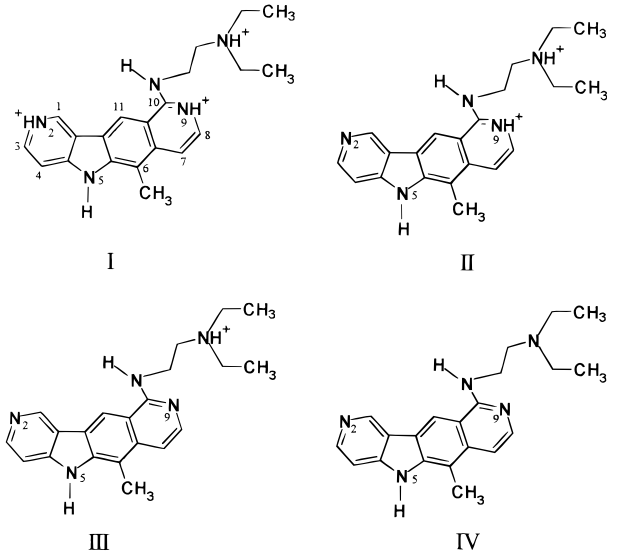

Figure 1. Molecular structure of PZE in its different protonation states numbered from **I** (fully protonated form) to **IV** (fully deprotonated form).

protonation state of the molecule. Its emission maximum is at about 445 nm, except for **I** for which it is located at 405 nm. Furthermore, for this fully protonated PZE, time-resolved fluorescence measurements have revealed a fast shift of the emission band (200 ps) which was proposed to be due to a deprotonation at N2, the most acid position of PZE.[21]

The subpicosecond photophysical study of free PZE was initiated at pH 7.0. Under these conditions, the prevailing molecular form is **III**. Its ground-state absorption and emission spectra are given in Figure 2a and the transient absorption spectra in Figure 2b. The transient absorbance develops out within the laser pulse duration, as illustrated in Figure 3. The excitation wavelength corresponds to the $S_0 \rightarrow S_2$ transition (Figure 2a). The internal conversion $S_2 \rightarrow S_1$ was too fast to be detected in our transient spectra. Thus, the transient species appearing in Figure 2b is very likely to be the excited singlet state S_1. We note that the maximum at 700 nm coincides with the absorption maximum of the hydrated electron; however, it belongs to the excited PZE as far as it was also detected for PZE in dioxane. Moreover, the examination of the absorption spectrum in Figure 2a suggests that the maximum at 700 nm corresponds to the transition $S_1 \rightarrow S_3$. In the spectral domain 400−525 nm, the transient spectra could be distorted by the stimulated emission $S_1 \rightarrow S_0$.

During the time interval probed, the transient spectra decay slowly without any detectable change in their shape. We conclude that the S1 state of **III** is stable in this time domain, except for a slow decay. The decay time was estimated as 1 ns, in good agreement with the excited singlet state lifetime obtained from time-resolved fluorescence measurements.[21]

A different situation occurs in the case of **I**. The transient absorption spectra of PZE in aqueous solution at pH 4.0 are presented in Figure 4a for the spectral domain 400−600 nm. We note that the prompt spectrum is not identical with that of PZE at pH 7.0 (Figure 2b): a local minimum appears at about 425 nm; on the other hand, in the spectral domain 650−850 nm, the transient absorption spectra of the two molecular forms are similar. However, the striking feature of the transient spectra of **I** is the spectral evolution detected on the blue side: an anomalous increase of the optical density at wavelengths below 500 nm is observed during the first 200 ps after the optical excitation (Figure 4a). The wavelength dependence of the

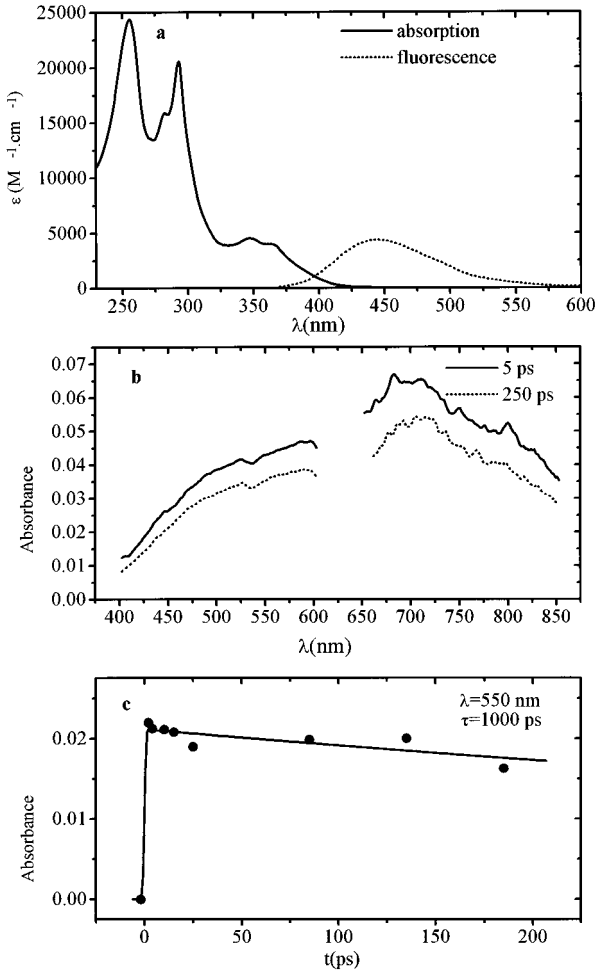

Figure 2. (a) Absorption and emission (arbitrary units) spectra of PZE in aqueous solution at pH 7.0. (b) Transient differential absorption spectra of a 0.7 mM solution of PZE in 50 mM phosphate buffer and 100 mM NaCl, pH 7.0. (c) Decay of the differential absorbance of the same PZE solution.

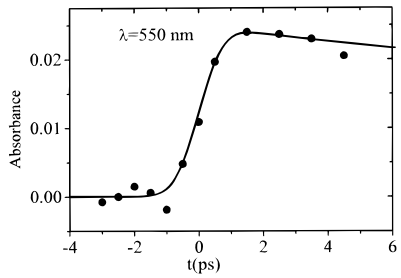

Figure 3. Rise of the differential absorbance of PZE in aqueous solution at pH 7 upon 312 nm laser pulse excitation.

differential absorption kinetics illustrated in Figure 4b is consistent with this evolution: the decay at 550 nm was fitted by a monoexponential of 1 ns lifetime, while the fitted decay at 430 nm includes an exponential with negative amplitude superimposed on the decreasing component 1 ns lifetime. A more suggestive and precise representation of this spectral evolution is provided by the kinetics of the ratio *R* between the optical density at 450 nm and that at 550 nm, respectively (Figure 4c). The experimental points were fitted assuming a two state molecular system with an internal unimolecular rate of 5×10^9 s^{-1} constant (200 ps lifetime).

One notes a coincidence between the position of the PZE emission band (Figure 2a) and the spectral interval in which the spectral relaxation occurs. However, the detected effect

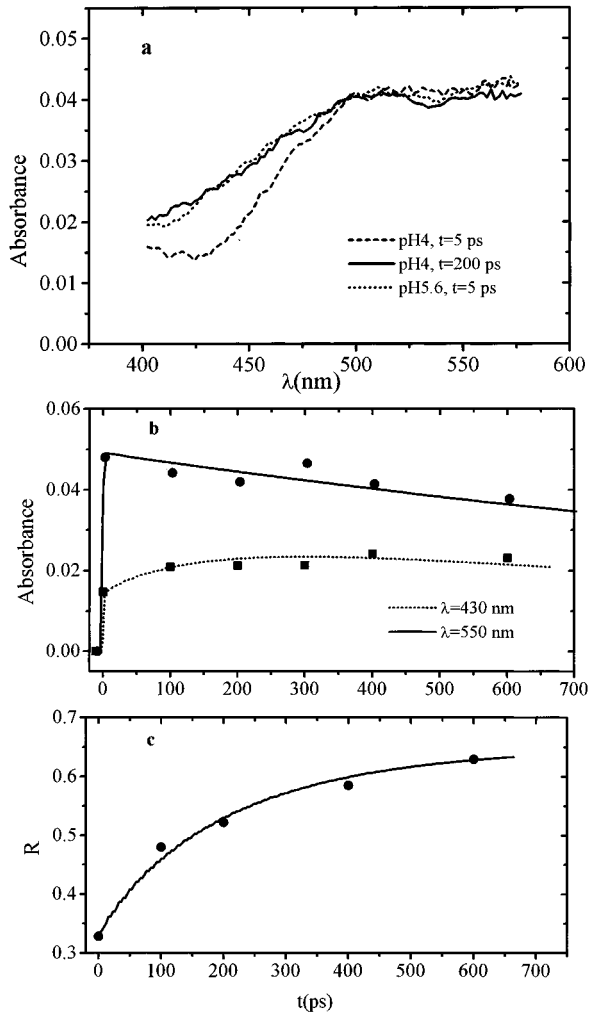

Figure 4. Spectral relaxation of the differential absorption of a 0.7 mM solution of PZE in 50 mM acetate buffer and 100 mM NaCl, pH 4.0: (a) transient spectra (all spectra have been scaled for a common value of the differential absorbance at 500 nm); (b) wavelength dependence of the differential absorption decay; (c) kinetics of the ratio *R* between the differential absorbancies at 430 and 550 nm, respectively.

could not be due to a decrease of the contribution of stimulated emission to the transient spectra. Indeed, the time-resolved fluorescence measurements did not reveal any fast decay of the fluorescence intensity. Moreover, the transient absorption belonging to pure $S_1 \rightarrow S_N$ transitions (wavelengths greater than 500 nm) does not include any fast component.

On the basis of the coincidence between the rate constants, we conclude that the present spectral relaxation is the counterpart of the emission band relaxation already reported.[21] Consequently, we have tested the deprotonation at the N2 position as a possible cause of the spectral evolution presented in Figure 4a. Since the molecular form of PZE prevailing at pH 5.6 is **II**, we have compared the prompt transient spectrum at this pH to the relaxed spectrum of PZE at pH 4.0. The two spectra, presented in Figure 4a, are closely related. One can conclude that the present results are consistent with the hypothesis of a deprotonation occurring at the N2 position upon optical excitation of PZE. On the basis of the rate constant of this process, one can evaluate[22] an excited-state pK_a value of -1 to be compared to the ground-state value of 5.5. Variations of the pK_a value by six units or more upon $S_0 \rightarrow S_1$ transition are usual for aromatic molecules.[23,24]

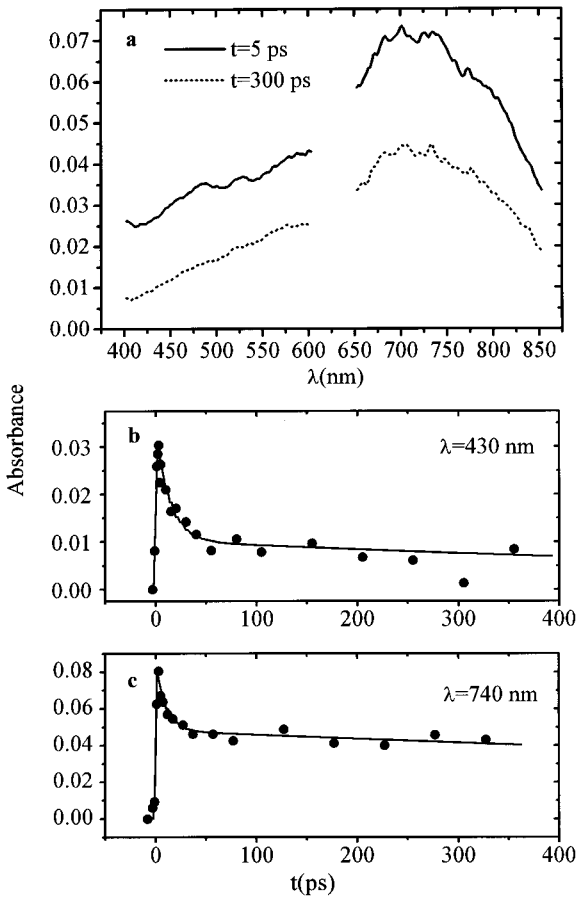

Figure 5. Differential absorption of a 0.7 mM PZE and 7 mM poly(dG−dC)−poly(dG−dC) solution in 50 mM phosphate buffer and 100 mM NaCl, pH 7.0: (a) transient spectra; (b) decay of the differential absorbance at 430 nm; (c) decay of the differential absorbance at 740 nm.

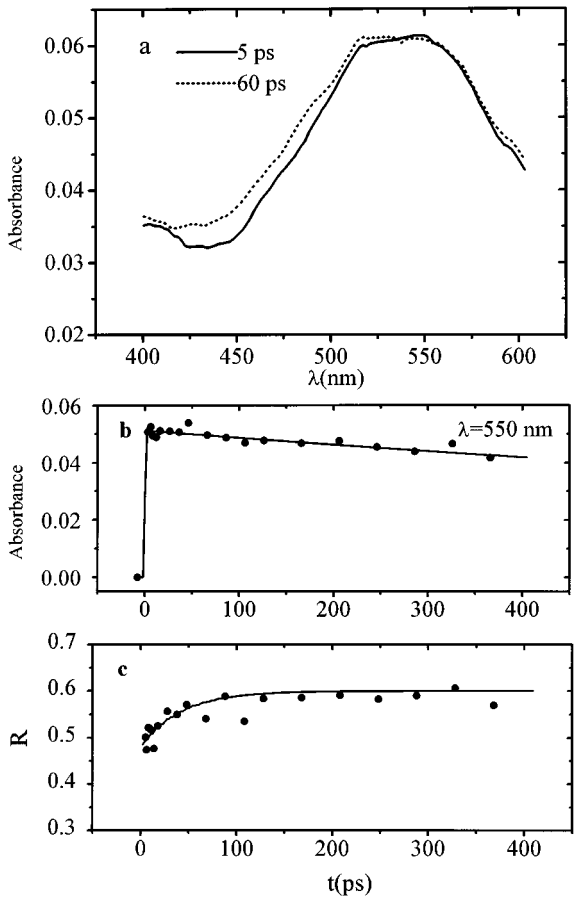

Figure 6. Differential absorption of a 0.7 mM PZE and 7 mM poly(dA−dT)−poly(dA−dT) solution in 50 mM phosphate buffer and 100 mM NaCl, pH 7.0: (a) transient spectra; (b) decay of the differential absorbance at 550 nm; (c) kinetics of the ratio *R* between the differential absorbancies at 440 and 550 nm, respectively.

Photophysics of PZE−Polynucleotide Complexes. The interaction between PZE and polynucleotides has already been investigated by steady-state absorption and fluorescence measurements.[10] It was found that the addition of polynucleotides to a PZE aqueous solution induces a red shift and a hypochromism on both the 350 and 300 nm absorption bands of PZE, which is a characteristic of intercalated drugs.[13,24,25] The fluorescence of the drug is enhanced in the presence of poly(dA−dT)−poly(dA−dT) and strongly quenched in the presence of poly(dG−dC)−poly(dG−dC) and DNA. These properties of the PZE complexed to polynucleotides, as well as the polarization of its fluorescence upon complexation, are consistent with a strong interaction. The efficient singlet−singlet energy transfer from the nucleic acid bases to PZE[10] and the increase of the viscosity of polynucleotide solutions in the presence of PZE[11] prove that at least a fraction of the drug intercalates between the nucleic acid base pairs.

The transient absorption measurements in the picosecond time range reveal important differences between PZE/poly(dG−dC)−poly(dG−dC) and PZE/poly(dA−dT)−poly(dA−dT) complexes. For instance, the prompt differential absorption spectra in the 400−600 nm spectral interval (Figures 5a and 6a, respectively) are very different. They are also different from the prompt differential absorption spectrum of free PZE in aqueous solution (Figures 2b and 4a). Significant redistribution of the electronic density in the PZE molecule upon complexation could be responsible for this situation. However, in the range 400−500 nm, there are some similarities between the prompt spectrum of the PZE/poly(dA−dT)−poly(dA−dT) complex (Figure 6a) and that of fully protonated free PZE (**I**) (Figure 4a) which presents the same minimum at 430 nm. Adding the observation that for both these species the fluorescence maximum is shifted to 405 nm,[10,21] one may infer that a change in the protonation state of PZE bound to poly(dA−dT)−poly(dA−dT) does occur, as already proposed.[10] Theoretically, the protonation of the polynucleotide-bound PZE is possible since the calculated H^+ concentration near the DNA surface was found to correspond to an effective pH of about 4.5.[26] However, in the case of the PZE/poly(dG−dC)−poly(dG−dC) complex, the prompt differential absorption spectrum does not show any evidence for such a protonation change.

In agreement with the previously reported quenching of PZE fluorescence in the presence of poly(dG−dC)−poly(dG−dC), the kinetics of differential absorption of PZE/poly(dG−dC)−poly(dG−dC) complex reveals a significant fast component of 15 ± 2 ps (Figure 5b,c). The kinetics is biexponential, the longer lifetime being evaluated as 1000 ± 250 ps. The preexponential factor of the fast component is wavelength dependent and decreases from 0.70 at 400 nm to 0.45 at 600 nm; between 600 and 850 nm no variation of the preexponential factors has been detected. Accordingly, a spectral evolution was identified on the blue side of the differential absorption spectrum: the ratio between the optical density at 400 nm and that at 600 nm decreases during the fast decay. The biexponential structure of the differential absorption decay, as well as the actual spectral evolution, suggests that at least two excited

molecular species are involved in the present transient spectra. One may naturally suppose that there are at least two binding sites in the PZE/poly(dG−dC)−poly(dG−dC) complex associated with different lifetimes of the excited PZE and slightly different differential absorption spectra.

A common feature for a number of polycyclic planar molecules is the quenching of their fluorescence upon intercalation between base pairs in polynucleotides. Such a quenching has been reported from some acridine dyes,[27] viologens,[28] anthryls,[13] or phenotiazinium dyes.[24] Often, this effect was assigned to an electron transfer from the nucleic acid bases to the excited polycycle, which has been only effectively observed in a few cases.[25,28] In fact, there are few molecular complexes of this kind investigated by transient absorption measurements with equivalent time resolution and on a large spectral domain allowing the identification of the radicals presumed to be formed. Among these few examples one notes the complexes of phenotiazinium dyes, methylene blue and thionine, formed with mono- and polynucleotides. Initially, the fast component detected in the decay of differential absorption of complexed methylene blue has been attributed to an electron[29] or a hydrogen atom[30] transfer from guanine to the excited dye. However, the systematic investigation of the transient differential absorption between 400 and 850 nm has shown no traces of semireduced methylene blue, the radical which would be formed by the proposed electron transfer.[15] Moreover, it was found that the deactivation of the excited singlet state of the dye and the ground-state recovery were simultaneous in the limit of a time resolution of 0.5 ps. On the other hand, a correlation between the rate constant of the deactivation process and the ΔG°[31] values for the electron transfer in the dye/nucleotide base system has been identified. It was then concluded that, in the case of methylene blue−nucleotide complexes, the fast deactivation of the S_1 state of the dye is due to a nonradiative $S_0 \leftarrow S_1$ transition via a low-lying and very short-lived charge transfer state.[32] A similar mechanism was proposed for thionine−nucleotide complexes.[33]

Both the reduced and oxidized radicals of PZE have absorption bands in the 400−500 nm spectral domain with extinction coefficients greater than 3000 mol^{-1} L cm^{-1} .[34,35] Given the sensitivity of our experimental system, these bands would appear in the transient spectra of the PZE/poly(dG−dC)−poly(dG−dC) complex if an electron transfer were involved in the fast decay of the PZE excited singlet state. Thus, the spectral evolution presented in Figure 5a is in disagreement with the electron transfer hypothesis. Therefore, it is likely that a mechanism similar to that proposed for the phenothiazinium dyes−nucleotide complexes is active in the present case also.

The differential absorption of the PZE/poly(dA−dT)−poly(dA−dT) complex decays slowly (Figure 6b) over the whole investigated spectral range (400−850 nm) with a lifetime of 2000 ± 250 ps. However, the transient spectra exhibit an additional rapid evolution in the spectral domain 425−500 nm. The spectral evolution is confirmed by the kinetics of the ratio between the optical density at 440 nm and that at 550 nm (Figure 6c). The time constant of the spectral relaxation was found to be 40 ± 10 ps. The spectral evolution of PZE complexed to poly(dA−dT)−poly(dA−dT) is similar to that detected in the case of **I** (Figure 4a) and supports the hypothesis of a more acid environment of complexed PZE.

The results obtained on the complexes of PZE with synthetic polynucleotides provide a basis for the analysis of the complexes with natural polynucleotides where the base pairs G−C and A−T are simultaneously present. We have investigated the

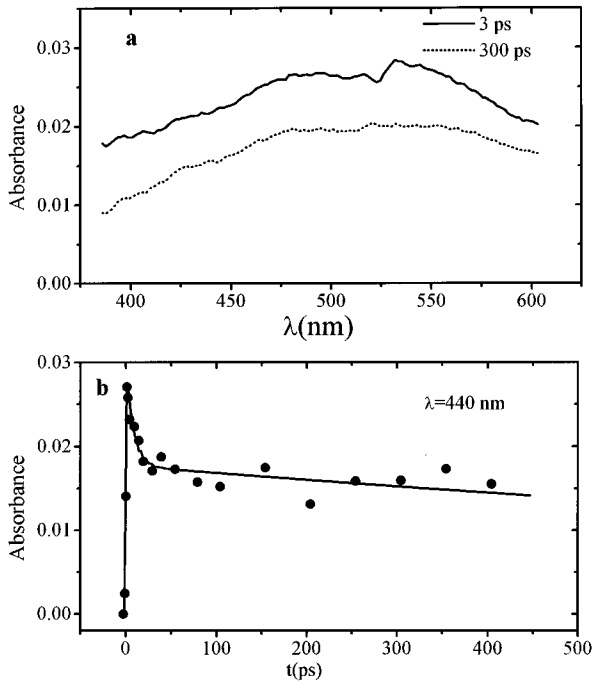

Figure 7. Differential absorption of a 0.7 mM PZE and 7 mM CT-DNA solution in 50 mM phosphate buffer and 100 mM NaCl, pH 7.0: (a) transient spectra; (b) decay of the differential absorbance at 440 nm.

complex formed by PZE with the DNA extracted from calf thymus, a natural polynucleotide containing almost equal amounts of G−C and A−T base pairs.[36] The aspect of the differential absorption spectra presented in Figure 7a is somehow intermediate between that of PZE/poly(dG−dC)−poly(dG−dC) and that of PZE/poly(dA−dT)−poly(dA−dT). The decay of the differential absorbance (Figure 7b) was fitted by a sum of two exponentials as follows: $\tau_1 = 10 \pm 2$ ps, $a_1 = 0.38 \pm 0.05$ and $\tau_2 = 1000 \pm 200$ ps, $a_2 = 0.62 \pm 0.05$. The smaller relative amplitude of the fast component and the intermediate aspect of the transient spectra may be explained by assuming that in DNA the drug is intercalated not only between two G−C base pairs but also between A−T and G−C or two A−T base pairs. However, from the weights of the fast component in the decays presented in Figures 5c and 7b, we conclude that the properties prevailing in the PZE/DNA complexes are those of the drug intercalated between two G−C base pairs. This conclusion is in satisfactory agreement with the results obtained by steady-state fluorescence measurements[10] and by nanosecond transient absorption spectroscopy.[12]

Conclusions

The interaction between PZE and polynucleotides has been thoroughly investigated by transient absorption spectroscopy in the picosecond time domain. The transient absorption band between 400 and 600 nm was found to be very sensitive to the type of PZE−polynucleotide complex. For **I** and for PZE complexed to poly(dA−dT)−poly(dA−dT), a similar fast spectral relaxation was detected and assigned to a deprotonation of the drug in its excited singlet state. This result also supports the hypothesis of a modified protonation state of complexed PZE. The evolution of the differential absorption spectra of PZE/poly(dG−dC)−poly(dG−dC) suggests that, in this complex, there are at least two different binding sites of the drug. Furthermore, the kinetics of the differential absorption reveals the activation of a radiationless transition $S_0 \leftarrow S_1$ upon PZE

association to poly(dG−dC)−poly(dG−dC). We have also found that the interaction between PZE and DNA mainly corresponds to the drug intercalated between two G−C base pairs. Under these circumstances, the rapid deactivation of the excited state should decrease the yield of the photooxidation process responsible for the DNA induced damages.

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