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DNA Repair by Photolyase: A Novel Substrate with Low Background Absorption around 265 nm for Transient Absorption Studies in the UV†

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ABSTRACT: CPD photolyase enzymatically repairs the major UV-induced lesion in DNA, the cyclobutane pyrimidine dimer (CPD), by photoreversion of the damage reaction. An enzyme-bound reduced flavin ($FADH^-$) cofactor functions as photosensitizer. Upon excitation, it transiently transfers an electron to the CPD, triggering scission of the interpyrimidine bonds. After repair completion, the electron returns to the flavin to restore its functional reduced form. A major difficulty for time-resolved spectroscopic monitoring of the enzymatic repair reaction is that absorption changes around 265 nm accompanying pyrimidine restoration are obscured by the strong background absorption of the nondimerized bases in DNA. Here we present a novel substrate for CPD photolyase that absorbs only weakly around 265 nm: a modified thymidine 10-mer with a central CPD and all bases, except the one at the 3′ end, replaced by 5,6-dihydrothymine which virtually does not absorb around 265 nm. Repair of this substrate by photolyases from *Anacystis nidulans* and from *Escherichia coli* was compared with repair of two conventional substrates: a 10-mer of unmodified thymidines containing a central CPD and an acetone-sensitized thymidine 18-mer that contained in average six randomly distributed CPDs per strand. In all cases, the novel substrate was repaired with an efficiency very similar to that of the conventional substrates (quantum yields in the order of 0.5 upon excitation of $FADH^-$). Flash-induced transient absorption changes at 267 nm could be recorded on a millisecond time scale with a single subsaturating flash and yielded very similar signals for all three substrates. Because of its low background absorption around 265 nm and the defined structure, the novel substrate is a promising tool for fast and ultrafast transient absorption studies on pyrimidine dimer splitting by CPD photolyase.

DNA photolyases are light-dependent enzymes that revert UV-induced damage in DNA (for reviews, see refs (*1*−*3*)). The most abundant UV lesion is a linkage of two neighboring pyrimidines by two covalent bonds, the *cis-syn* cyclobutane pyrimidine dimer (CPD).[1] This lesion is specifically repaired by CPD photolyase. It comes in two types (according to the light harvesting cofactor: reduced folate or 5-deazaflavin) and is found widely in living organisms (with the remarkable exception of placental mammals).

Photolyase binds specifically to DNA containing a CPD. The CPD is flipped out of the double helix and enters into the binding pocket of the enzyme. A widely accepted mechanism of CPD repair involves ultrafast (∼150 ps) electron transfer from the photoexcited singlet state of the FAD cofactor of photolyase in its fully reduced form ($FADH^-$) to the CPD. After cleavage of the CPD, transfer of an electron back to the flavin completes the enzymatic cycle. This enzymatic photorepair is highly efficient; according to the most recent comprehensive review, its quantum yield could approach unity for direct excitation of $FADH^-$ ((*1*); but see Discussion).

For transient absorption studies of photorepair *in vitro*, it is convenient to use CPD-containing oligonucleotides as substrates. For proper binding to photolyase, the CPD-containing strand should be at least five bases long (*4*−*7*). A straightforward and common method to prepare such a synthetic substrate for photolyase consists of exposing acetone-containing solutions of oligo(dT) strands to UV light above 300 nm that is nearly exclusively absorbed by the solvent (*8*). Using sufficiently high UV doses, one can form several CPDs per strand. However, such substrates are heterogeneous because the sites of CPD formation within the strand are arbitrarily distributed. On the other hand, the simplest *homogeneous* substrate for photolyase is a single CPD flanked by several unmodified nucleotides. It has a strong absorption around 265 nm, making the detection of the small repair-induced absorbance changes in this spectral region a challenging task. The problem is worsened by the fact that, if repair is studied under conditions where each photolyase has a substrate bound, an excess of strands is required. This may be one

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[1]Abbreviations: CPD, *cis-syn* cyclobutane pyrimidine dimer; FWHM, full width at half-maximum; DMAD, *p*-(dimethylamino)-benzenediazonium tetrafluoroborate; DHT, 5,6-dihydrothymine; MTHF, 5,10-methenyltetrahydrofolate; AM, actinometer; PL, photolyase; PR, photorepair.

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of the reasons that, with one notable exception (*7*), all published transient absorption studies on enzymatic photorepair (*9*−*12*) were limited to the spectral region >370 nm, despite the fact that the only conclusive spectral evidence for photorepair is the reestablishment of the absorption of the normal bases around 265 nm.

In order to overcome these difficulties, we devised a novel substrate (5′-$(DHT)_4$-T<>T-$(DHT)_3$-T-3′) that combines homogeneity with low background absorption around 265 nm. It is composed of a central CPD (T<>T) surrounded by eight thymidines, four on each side, seven of which have been replaced by 5,6-dihydrothymidine that virtually does not absorb around 265 nm (the molar decadic extinction coefficient of 5,6-dihydrothymine is below 10 M^{-1} cm^{-1} (T. Ito and S. Nishimoto, personal communication)). The terminal base at the 3′end is an intact thymine, providing for an easy means of spectroscopic concentration determination based on the characteristic 265 nm absorption peak.

In this contribution, we present evidence that this novel substrate is repaired by both folate and deazaflavin type photolyases with an efficiency comparable to that for two reference substrates (acetone-sensitized dT_{18} and T<>T-CPD flanked by four nonmodified thymidines on each side), both under continuous and laser flash illumination. The novel substrate is thus a promising candidate for further *in vitro* repair studies by transient absorption spectroscopy in the UV.

In this context, we also determined absolute quantum yields of photorepair and consistently obtained repair quantum yields between 0.45 and 0.57 for direct excitation of $FADH^-$, i.e., lower than those cited in ref *1*. We will discuss possible origins of this discrepancy.

MATERIALS AND METHODS

Proteins. *Anacystis nidulans* photolyase was overproduced in *Escherichia coli* (*13*), purified as described by Eker et al. (*14*), and was devoid of the antenna cofactor (hereafter called apophotolyase). Holophotolyase from *E. coli* (both flavin and antenna cofactor 5,10-methenyltetrahydrofolate (MTHF) present) was overexpressed and purified as described previously (*15*). *E. coli* E109A mutant photolyase that lacks the antenna cofactor (*16*) was a gift from E. Schleicher (Freiburg). The purified enzymes contained a mixture of two redox states of the flavin adenine dinucleotide (FAD) cofactor: the neutral radical FADH° (major form) and the fully oxidized FAD_{ox} (minor form). Photolyases were stored at −80 °C in the presence of 2-mercaptoethanol and 20% glycerol. Immediately before measurements, the photolyase storage buffer was exchanged against repair buffer (100 mM NaCl, 10 mM phosphate buffer, pH 7.0) with polyacrylamide chromatography microcolumns at 4 °C.

CPD-Containing Oligonucleotide Substrates. Acetone-sensitized dT_{18} ("asT18") was prepared as described in ref *8* and UV-treated as described in ref *17*, resulting in ∼6 CPD per strand; 5′-$(DHT)_4$-CPD-$(DHT)_3$-T-3′ ("DHT10") and 5′-T_4-CPD-T_4-3′ ("T10") were custom-synthesized, reverse-phase HPLC purified, and quality controlled by MALDI-TOF mass spectrometry by Eurogentec (Liège, Belgium) using HPLC-purified *cis-syn* thymine dimer phosphoramidite (catalog no. 11-1330 from Glen Research, VA; purity 98.1%, as communicated by Glen) and, for DHT10, 5,6-dihydro-dT-CE phosphoramidite (catalog no. 10-1530 from Glen Research; 5*S*:5*R* stereoisomer ratio 90:10, as communicated by Glen). Deprotection of the phosphoramidite building blocks was performed by ammonium hydroxide at room temperature in the dark in order to avoid degradation. More details provided by Glen Research and Eurogentec (including MALDI spectra of the DHT10 and T10 substrates) are presented in the Supporting Information. For earlier reports on introduction of *cis-syn* thymine dimers into oligonucleotides and synthesis of DNA fragments containing DHT, see refs *18* and *19*. DMAD (*p*-(dimethylamino)benzenediazonium tetrafluoroborate) was used as purchased from Wako Chemicals (Neuss, Germany).

Photoreduction. For anaerobic photoreduction of FADH° to catalytically active $FADH^-$, samples were prepared under nitrogen atmosphere in a glovebox and there transferred to an anaerobic quartz cell. Photoreduction was carried out in the presence of 1 mM L-cysteine (Fluka) with continuous light from a LQX1800 xenon lamp (Linos) and monitored by the decrease of the 580 nm absorption peak of FADH°. Our photolyase samples contain a certain fraction of oxidized flavin which is still tightly bound to the protein, as judged both by the typical vibrational structure of the 450 nm absorption peak and by the fact that this oxidized FAD cannot be removed by washing on a 15 kDa microcolumn. This oxidized flavin can be reduced by illumination with blue light in the presence of an electron donor such as cysteine. Our observation indicated, however, that oxidized photolyase thus reduced had a tendency of quickly reoxidizing, probably due to some subtle modification in the flavin binding environment that made possible the first oxidation. In order to exclude these unstably reduced photolyases from the assay, photoreduction of the photolyase sample was performed with an OG515 cutoff filter that let pass only light of wavelengths that were exclusively absorbed by the flavin radical FADH°. The concentration of photolyases containing fully reduced flavin was estimated from the bleaching at 580 nm during photoreduction assuming an extinction coefficient $\varepsilon_{580} = 5\ mM^{-1}\ cm^{-1}$ (*20*) for FADH° ($FADH^-$ does not absorb at 580 nm).

After completion of photorepair, a control was performed to check whether during the illumination procedure reduced flavin had been released from the enzyme. The sample cell was opened and shaken in order to facilitate oxygen exposure. No traces of formation of 450 nm absorption as indicative for oxidized flavin (expected to be formed rapidly from reduced free flavins in the solution (*21*)) were detected.

Experimental Procedures of Photorepair Quantum Yield Determination. Samples (typically 3 μM reduced photolyase, substrate with a total of 10 μM CPD, 1 mM cysteine in repair buffer) of 250 μL total volume were placed in a 10 × 2 × 8 mm (length × width × height) anaerobic quartz cell with four clear windows (Starna, Germany), allowing for experiments using 10 and 2 mm optical paths (2 mm was used when the absorption over 10 mm exceeded 2 units). Illumination was along the 2 mm optical path with continuous light from a LQX1800 xenon lamp (Linos) passed through an optical fiber and the following combination of filters: 3 mm colored glass B390 (Hoya), interference filter IF380 (Pomfret), and 2 mm neutral density filter NG11 (Schott) resulting in UV light centered at 384 nm with 10 nm fwhm spectral width. A DG535 function generator (Stanford Research) coupled to the mechanical shutter (integrated in the lamp) controlled the duration of the light pulses (170 ms for DMAD, 2−50 s depending on the photolyase).

Alternatively, series of 25 flashes of 5 ns duration and ∼3 mJ/cm^2 energy at 355 nm were provided by a tripled Brilliant B Nd:YAG laser (Quantel, France) at 2 Hz. Laser flash energy was

measured with an energy meter (SOLO with a QE 25 measuring head from Gentec EO) before and after illumination of photolyase and again before and after illumination of the actinometer solution in order to monitor long-term stability of the laser energy.

After each illumination, samples were homogenized by gentle shaking, and absorption spectra were recorded over 10 mm (for asT18 and DHT10) or 2 mm (for T10) with an Uvikon XS spectrophotometer (Secomam). These illumination/recording cycles were repeated up to ten times on the photolyase sample; then the absorption cell was washed, rinsed, and dried, and the procedure was repeated with the same cell, this time containing 250 μL of ~2 μM DMAD solution in 50 mM H_2SO_4.

The continuous light photon flux determination by the DMAD actinometer was cross-checked using a ferrioxalate actinometer according to the reported procedure (*22*) using 4.2 mM ferrioxalate and 1.8 mM phenanthroline, assuring at our low light doses a sufficiently large excess of phenanthroline ([phenanthroline]:[Fe(II)] > 50:1) to avoid potential errors from slow color development (*23*). Pure potassium ferrioxalate was prepared from 150 mL of 1.5 M potassium oxalate (Sigma) and 50 mL of 1.5 M ferric chloride (Sigma) and three times recrystallized in hot water following the reported procedure (*22*).

Data Evaluation for Photorepair Quantum Yield Determination. For a unimolecular photochemical reaction, the first-order rate constant k is determined by the incoming photon flux I (photons per second per centimeter squared), the absorption cross section σ (centimeters squared) of the reacting molecule, and the quantum yield φ of the reaction:

$$k = I\sigma\varphi \quad (1)$$

where we assume that the solution is sufficiently dilute that variations of I over the optical path can be neglected. In photochemical actinometry, one circumvents the difficulties of photometrically determining the photon flux by studying the effect of that flux on the reaction of a well-studied actinometer (AM) with known σ_{AM} and φ_{AM} under exactly the same illumination conditions as for the reaction of interest, photorepair in our case. This procedure allows estimating the quantum yield φ_{PR} for photorepair by writing eq 1 for both actinometer (AM) and photolyase (PL) and then equaling the flux I for both:

$$\varphi_{PR} = (k_{PR}/k_{AM})(\sigma_{AM}/\sigma_{PL})\varphi_{AM} \quad (2)$$

Further, σ_{AM}/σ_{PL} can be replaced by $\varepsilon_{AM}/\varepsilon_{PL}$. Finally, we need to express the reaction rate constants k via the measured effects, in our case, change of absorbance A during illumination. For the case of an actinometer that is consumed by the reaction, we have $[AM] = [AM]_0 \exp(-k_{AM}t)$ yielding a linear dependence of ln[AM] on illumination duration t:

$$\ln[AM] = -k_{AM}t + \ln([AM]_0)$$

As the detected absorbance A_{AM} is proportional to [AM] (reaction products do not absorb at the detection wavelength 378 nm), we can obtain k_{AM} directly from the slope of a plot of $\ln(A_{AM})$ versus t.

For photorepair, under conditions of substrate excess and limiting light intensity, i.e., the photochemical repair step is the kinetically limiting step (this was the case in our experiments, as verified by the independence of CPD repair from substrate concentration; not shown), the product (TT resulting from CPD repair) concentration change is given by $d[TT]/dt = [PL]k_{PR}$. Integrating over an illumination period Δt and using $\Delta A_{PR} = \Delta[TT]\Delta\varepsilon_{PR}l_{Det}$, one obtains

$$\Delta A_{PR} = [PL]k_{PR}\Delta\varepsilon_{PR}l_{Det}\Delta t \quad (3)$$

where [PL] is the concentration of active (i.e., reduced) photolyase, $\Delta\varepsilon_{PR}$ is the differential molar absorption coefficient at 265 nm due to CPD repair, and l_{Det} is the optical path length of the detection light. Substituting k_{PR} from eq 3 into eq 2, we obtain

$$\varphi_{PR} = \Delta A_{PR}\varepsilon_{AM}\varphi_{AM}/(\Delta t k_{AM}\Delta\varepsilon_{PR}[PL]l_{Det}\varepsilon_{PL})$$

We define $[PL]l_{Det}\varepsilon_{PL} = A_{PL}{}^{X}$, which is the absorbance of the sample's active photolyases at the excitation wavelength over the optical path length used for the detection of photorepair. Hence

$$\varphi_{PR} = \Delta A_{PR}\varepsilon_{AM}\varphi_{AM}/(\Delta t k_{AM}\Delta\varepsilon_{PR}A_{PL}{}^{X}) \quad (4)$$

Of the values entering eq 4, $\Delta A_{PR}/\Delta t$ and k_{AM} are determined experimentally from illumination of the photolyase and DMAD solutions. Based on the measured absorption spectrum of DMAD and the published value (*24*) of $\varepsilon_{378} = 36.1\ mM^{-1}\ cm^{-1}$, we calculate an averaged $\varepsilon_{AM} = 34.7\ mM^{-1}\ cm^{-1}$ over the emission band of our lamp/filter combination, and $\varphi_{AM} = 0.48$ is obtained for 10 °C by interpolation of published values (*25*) for 5 °C (0.46) and 25 °C (0.53).

The differential extinction coefficient for CPD repair $\Delta\varepsilon_{PR}$ is slightly dependent on the neighboring bases and thus on substrate type. From measurements of hypochromism increasing with strand length (*26*), it was found that the extinction coefficient of thymine at the edge of and inside an oligo(dT) strand is respectively 0.93 and 0.88 times that of an isolated T (ε_T), suggesting $\Delta\varepsilon_{PR} = 1.86\varepsilon_T$ for an isolated CPD (in DHT10) and $\Delta\varepsilon_{PR} = 1.66\varepsilon_T$ for an embedded CPD (in T10, the bases next to the CPD being transformed from "edge" to "inside" upon repair). These ratios were found to be in agreement with observed absorbance changes due to substrate addition and complete repair (not shown). Based on an extinction coefficient of $\varepsilon_T{}^{265} = 9.9\ mM^{-1}\ cm^{-1}$ for a thymidine monomer in solution (*27*), we thus obtain 18.4 and 16.4 $mM^{-1}\ cm^{-1}$ for CPD repair in DHT10 and T10, respectively. As in dT_{18} the CPDs are introduced randomly upon UV illumination, the asT18 strand is heterogeneous and carries CPDs in various environments ranging from CPDs on both sides (rarely, corresponding to the situation in DHT10) to pieces of intact T (increasingly often with ongoing repair, corresponding to the situation in T10). In view of the average initial 6:6 partition of the CPDs and intact Ts, the most probable situation is a CPD between two isolated Ts that change to "edge" upon repair, corresponding to a net change of $\Delta\varepsilon_{PR} = 1.62\varepsilon_T = 16\ mM^{-1}\ cm^{-1}$. Table 1 summarizes the absorption properties of the three substrates studied in this work.

The last parameter in eq 4, $A_{PL}{}^{X}$, seems straightforward to be taken from the absorption spectra of the samples (none of the substrates absorbs at the excitation wavelengths used here). However, the quantification of reduced flavin (only form active in CPD repair in photolyase) based on the UV absorbance as apparent from the absorption spectrum rests on the assumptions that (a) all flavins are reduced (no radical, no oxidized), (b) all flavins are bound (no free flavin), and (c) there is no light scattering. Meanwhile, on one hand excess reductant/prolonged photoreduction can result in increased light scattering, and on the other hand insufficient reduction leaves part of the proteins unreduced. Thus, it is difficult to meet simultaneously assumptions a and c. In order to make sure that only photons absorbed

Table 1: Properties of the DNA Photolyase Substrates Used in This Study

substrate			absorption properties at 265 nm		
short-hand	composition	no. and position of CPDs	ε^a (mM^{-1} cm^{-1})	$\Delta\varepsilon_{PR}$ (mM^{-1} cm^{-1})	ratio $\Delta\varepsilon_{PR}/\varepsilon$
DHT10	5′-$(DHT)_4$-T <> T-$(DHT)_3$-T-3′	one central	9.9	18.4	1.9
T10	5′-T_4-T <> T-T_4-3′	one central	71.7	16.4	0.23
asT18	acetone-sensitized dT_{18}	six[b] random	8.9−9.9[c]	16−18[c]	1.6−2.0[c]

[a]Of the whole strand per CPD contained in the strand. [b]On average. [c]Span for a strand with six CPDs.

by photochemically active reduced proteins are taken into account for our quantum yield determination, we estimated the $A_{PL}{}^X$ value indirectly: The absorbance of $FADH^-$ formed during photoreduction was calculated from the amount of FADH° absorbance disappearing at 580 nm using reference spectra for the enzyme with the cofactor in its different redox forms (*20*). Thus, the absorbance of $FADH^-$ at 355 nm was taken to be 1.12 times and at 384 nm 0.88 times that of the decrease of the 580 nm peak (integration over the spectral output of the light source (370−400 nm) gives a mean factor of 0.86 for the latter). For the MTHF antenna cofactor of photolyase from *E. coli*, a spectrum was obtained by subtracting properly weighted portions of FADH° and FAD_{ox} spectra from the spectrum of the holoenzyme. Thus, the absorbance of MTHF around 384 nm (after integration over the spectrum of the lamp) was found to be 5.5 times that of FADH° at the 580 nm peak.

Transient Absorption Spectroscopy at 267 nm. Continuous monitoring light from a T9F26 light emitting diode (Seoul semiconductor) at 265±7 nm (at half-height) was passed through a narrow-band (2 nm fwhm) 266 nm interference filter (LL01-266 from Semrock), the 10 mm optical path of the cell, and through a broad-band 265 nm interference filter (265FS25 from LOT Oriel) onto the cathode of a solar-blind R6834 photomultiplier tube (Hamamatsu). The spectral composition of the light from the emitting diode that passed through this filter combination was quantified with a HR4000 fiber spectrometer (Ocean Optics) and found to be 267 ± 1 nm (at half-height). The output of the photomultiplier was fed into the 11A33 plug-in of a DSA 602A digital oscilloscope (Tektronix). A capacitor of 100 nF was set parallel to the input of 1 MΩ impedance. Single repair flashes (355 nm, 5 ns, < 7 mJ) from a frequency-tripled Brilliant B Nd:YAG laser (Quantel) were fired perpendicular to the monitoring light beam via a weakly diverging lens and a $\lambda/4$-plate that circularized the polarization, onto the 8 × 10 mm surface of the cell. A small fraction of these flashes was monitored simultaneously by a FND100Q photodiode (EG&G) coupled to the 11A52 plug-in of the oscilloscope. Before and after signal recording, the sample was protected from monitoring light by a mechanical shutter.

In principle, the monitoring light at 267 nm might induce formation of CPDs and (6−4) photoproducts between neighboring nondimerized thymines in the T10 and the T18 substrates and also in already repaired DHT10. Based on the fluence rate of ~100 $\mu W/cm^2$ for the monitoring light and assuming quantum yields for CPD and (6−4) photoproduct formation of 0.013−0.028 and 0.001−0.004, respectively (*28*), the probability of formation of any of these products in any of our substrates within the 2 s detection period is below 0.002 per strand. In line with this prediction, we observed no detectable bleaching at 267 nm in control experiments without excitation flash (not shown).

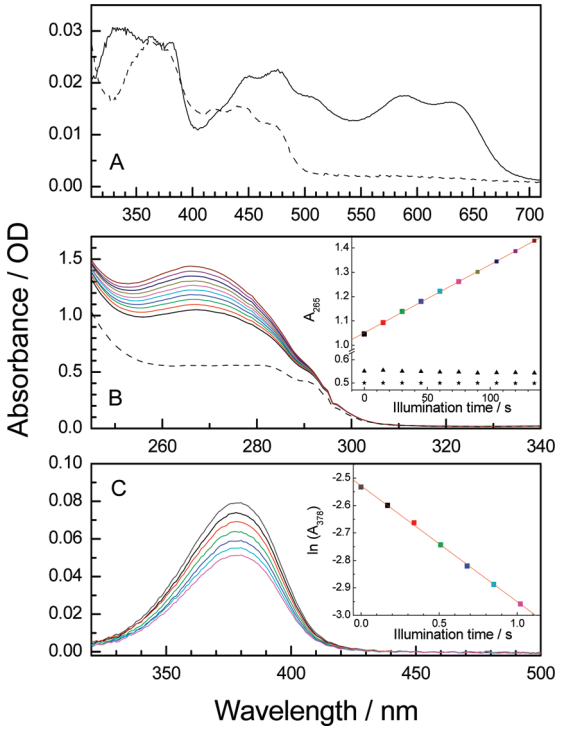

FIGURE 1: Spectroscopic determination of photorepair quantum yield for apophotolyase from *A. nidulans* and the DHT10 substrate using actinometric photon flux determination under identical geometry. (A) Absorption spectra measured before (solid) and after (dashed) photoreduction by orange light. (B) UV absorption spectra measured before (dashed) and after (black solid) addition of ~50 μM DHT10 and after successive 15 s periods of illumination with 384 nm light. Inset: squares, peak (265 nm) absorbance as a function of total illumination time; triangles, same for photolyase without substrate; stars, same for substrate without photolyase. (C) UV absorption spectra of 2.2 μM DMAD in 50 mM H_2SO_4 upon successive 170 ms periods of illumination at otherwise identical conditions as in (B). Inset: Semilogarithmic plot of the peak (378 nm) absorbance vs total illumination time. All spectra were measured over a 10 mm optical path length. Colored squares in the insets correspond to spectra of the same color in the corresponding main panels.

General. Samples were kept at 10 °C during all experiments and on ice in between. All experiments and procedures in the presence of substrate were carried out in darkness or under dim yellow light (λ > 520 nm) to avoid photoexcitation by ambient light ($FADH^-$, MTHF, and DMAD do not absorb at wavelengths above 520 nm).

RESULTS

Determination of Photorepair Quantum Yield. In order to characterize our novel, weakly UV absorbing substrate (5′-$(DHT)_4$-T <> T-$(DHT)_3$-T-3′, "DHT10"), we compared the efficiency with which it is repaired by photolyases to that of two

Table 2: Quantum Yields of Photorepair by Photolyases from *A. nidulans* (without Antenna) and *E. coli* (without and with Antenna) for Three Different Substrates

		continuous illumination at 384 ± 5 nm		
substrate	*A. nidulans* (apo)[a] laser flashes at 355 nm	*A. nidulans* (apo)[a]	*E. coli* (apo)[b]	*E. coli* (holo)[a]
DHT10	0.57 ± 0.10	0.45 ± 0.05	0.48 ± 0.01	0.35 ± 0.01
T10	0.57 ± 0.03	0.45 ± 0.02	0.50 ± 0.01	0.40 ± 0.03
asT18[c]	0.54 ± 0.04	0.46 ± 0.03	0.55 ± 0.02	0.42 ± 0.02

[a]Errors are standard deviations from ≥three independent measurements. [b]Errors are uncertainties in the fitted slopes to data as in insets of Figure 1b,c. [c]Calculated with $\Delta\varepsilon_{PR} = 16.6\ mM^{-1}\ cm^{-1}$.

reference substrates: T <> T-CPD flanked by four unmodified thymidines on each side ("T10") and acetone-sensitized dT_{18} containing in average six UV-induced CPDs ("asT18"). Figure 1 shows original data used to determine according to eq 4 the photorepair quantum yield of *A. nidulans* apophotolyase (devoid of the antenna cofactor) for the novel DHT10 substrate. The absorption spectra before and after photoreduction of $FADH^0$ (panel A) allow the estimation of the absorption $A_{PL}{}^X$ of the active (i.e., $FADH^-$ containing) photolyases at the wavelength of excitation in the photorepair experiment (see Materials and Methods). UV/vis absorption spectra measured between successive 384 nm photorepair illumination periods (panel B) yield the absorption increase due to thymine restoration per illumination time interval, $\Delta A_{PR}/\Delta t$. Control measurements with photoreduced photolyase alone (no substrate present) yielded a very minor bleaching of the protein absorption (triangles in the inset of panel B), that was taken into account for the evaluation of the repair quantum yield. The bleaching of the 380 nm absorption band of the DMAD actinometer upon illumination in the same geometry and by the same light source as for the photorepair experiments (panel C) allows to determine the light reaction rate k_{AM} of the actinometer that is a measure of the photon flux (see Materials and Methods). From these data, together with the constants ε_{AM}, φ_{AM}, and $\Delta\varepsilon_{PR}$ as given in Materials and Methods, eq 4 yields a repair quantum yield of 0.45 ± 0.05 (standard deviation from three independent experiments).

Repair quantum yields were similarly determined for all nine combinations of photolyases and substrates available to us (see Table 2). For *A. nidulans* apophotolyase, we also determined the repair quantum yields for excitation by laser flashes at 355 nm (Table 2). Examination of all data in Table 2 indicates that there is no systematic variation in repair yield between the three substrates; i.e., our novel, weakly UV-absorbing substrate DHT10 is repaired as well as conventional substrates.

Comparison between the three different photolyases examined under continuous illumination reveals a significantly lower repair quantum yield for the holoenzyme from *E. coli* compared to its apo form (lacking the MTHF antenna cofactor). The lower quantum yield can be explained by an additional process, i.e., excitation energy transfer from the antenna cofactor to the reduced flavin. We estimate the efficiency of this transfer (φ_{EET}) according to $\varphi_H = (86.5\%\ \varphi_{EET} + 13.5\%)\varphi_A$, where 86.5% and 13.5% are the fractions of total light quanta absorbed by the antenna and the flavin directly (according to their respective extinction coefficients at 384 nm), respectively. Averaging over the data for all three substrates, we obtain $\varphi_{EET} \approx 0.73$, which compares reasonably well with literature values of 0.62 (*13*) and 0.92 (*29*).

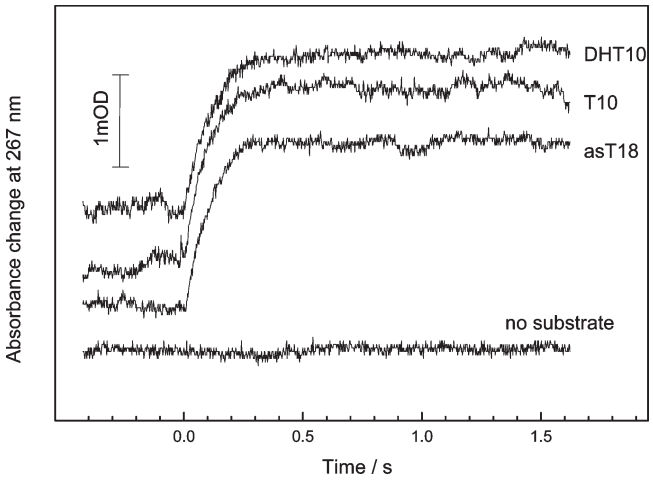

FIGURE 2: Absorption changes at 267 nm of ~3 μM photoreduced photolyase from *A. nidulans* alone (bottom) or mixed with various substrates upon excitation at time 0 by a single laser flash at 355 nm of ~2 mJ/cm^2 energy density (traces are offset for clarity). The CPD concentrations were 40 μM for DHT10 and asT18 and 13 μM for T10.

A slight difference in repair yields under continuous illumination is observed between apophotolyases from *E. coli* (0.51 in average) and *A. nidulans* (0.45) (Table 2). The origin of this difference is not clear. Pulsed laser excitation of *A. nidulans* photolyase with subsaturating flashes at 355 nm appeared to be more efficient (0.56 average repair yield) than continuous illumination at 384 nm (0.45). This difference might indicate that we underestimated the absorption of $FADH^-$ at 355 nm relative to that at 384 nm. It should be mentioned, however, that the laser data scattered much more than those using continuous light, most likely because of fluctuations of the laser flash energy.

Transient Absorption Monitoring of Enzymatic Photorepair on a Millisecond Time Scale. The novel DHT10 substrate was designed for fast and ultrafast transient absorption studies of the photorepair mechanism. We are presently constructing a setup with 300 ps time resolution for absorption changes at 266 nm induced by 355 nm excitation flashes (based on the principles of a recent setup for the visible (*30*)). It hence appeared useful to check for the amplitude of the flash-induced absorption increase (due to the restoration of two intact thymines per CPD repaired) on a slower, more easily accessible time scale. Using a light emitting diode as monitoring light source and a solar blind photomultiplier that was cabled for slow response (RC = 100 ms), we were able to resolve the absorption increase at 267 nm induced by a single weak (~2 mJ/cm^2) excitation flash given to *A. nidulans* apophotolyase in the presence of either of the three substrates described above (Figure 2). The three substrates yielded comparable long-lived absorption increases, with an instrument-limited rise time of ~100 ms. The observed amplitude of 1.5−2 mOD corresponds to successful photorepair in 1 out of ~30 reduced photolyase−substrate complexes, as expected for the low excitation energy used. As a control, photolyase without substrate did not show any significant absorption change at this time resolution (bottom trace in Figure 2).

Due to the lower background absorption of the DHT10 substrate compared to conventional T10 (compare Table 1), a higher concentration of the former (40 μM compared to 13 μM for the latter) could be used without degrading the signal-to-noise ratio. This larger excess is critical to enable accumulating an accordingly larger number of shots per sample in future

experiments on fast time scales that will require signal averaging to obtain a good signal-to-noise ratio.

DISCUSSION

The aim of the present study was to introduce a homogeneous substrate for photolyase that has low background absorption around 265 nm, the wavelength where thymine formation upon photorepair would ideally be detected. In the novel DHT10 substrate, seven of the eight thymidines in the T10 substrate are replaced by dihydrothymidines that virtually do not absorb around 265 nm. Thus, the background absorption of DHT10 is eight times lower than that of T10. In all of our assays (photorepair by continuous light at 384 ± 5 nm, photorepair by laser flashes at 355 nm, flash-induced transient absorption changes at 267 nm on a millisecond time scale) and for three different photolyases (apophotolyases from *A. nidulans* and from *E. coli* and holophotolyase from *E. coli*), DHT10 was repaired as well as T10 or asT18 substrate that contains several randomly distributed CPDs. Hence DHT10 is a promising substrate for future fast and ultrafast transient absorption studies in the 265 nm band, where a large number of signals will have to be averaged to obtain a sufficiently high signal-to-noise ratio. Under sample and excitation conditions of the top trace in Figure 2, for example, about 400 shots can be given until all of the DHT10 substrate initially present is repaired. The improvement compared to conventional T10 is by a factor of 8 based on equal background absorptions of the two samples at the start of the experiments (compared to the pentameric substrate used in ref *7*, the improvement is by a factor of 3). Taking into account the increase of 265 nm absorption due to photorepair during the course of the experiments, one might also choose to compare samples that have identical background absorption when all substrate is repaired. In this case, the improvement would still be by a factor of 3.3 (three intact thymidines absorbing in repaired DHT10 compared to ten in repaired T10).

As compared to unmodified thymine, 5,6-dihydrothymine lacks aromaticity, and the methyl group at C5 is moved out of the plane. As observed for several other enzymes acting on DNA (*31*), these differences could in principle affect the binding of photolyase to the DHT10 substrate compared to substrates containing unmodified thymines flanking the CPD. For photolyase, it is established, however, that binding of the substrate induces flipping of the CPD out of the strand and that flanking bases are involved in substrate binding essentially via their backbone phosphates (*4*−*6*). It was hence not unexpected that the DHT10 substrate was repaired as well as T10, also regardless of the presence of the two C5 stereoisomers of 5,6-dihydrothymine (see Materials and Methods).

Surprisingly, the absolute quantum yields determined in this study (0.45−0.57 for apophotolyases from *A. nidulans* and from *E. coli* and 0.35−0.42 for holophotolyase from *E. coli*) are well below the values of 0.8−1 and 0.70−0.75, respectively, that appear to be accepted in the literature (*1*). We are aware that absolute quantum yield determinations are prone to many errors from different sources. In our method, systematic errors may result from erroneous extinction coefficients for CPD repair ($\Delta\varepsilon_{PR}$), FADH°, $FADH^-$, MTHF, and DMAD, and from an erroneous quantum yield of the DMAD actinometer. $\Delta\varepsilon_{PR}$ is rather well-known (estimated errors less than ±5% for DHT10 and T10 and less than ±10% for asT18). Our DMAD actinometer has been verified against the ferrioxalate actinometer. We determined the photon flux of our illumination setup under identical conditions with both actinometers and obtained 12.6 ± 0.6 and 13.5 ± 0.4 nE s^{-1} cm^{-2} for DMAD and ferrioxalate, respectively. With respect to the extinction coefficients of photolyase cofactors, note that only the ratios $\varepsilon_{355}(FADH^-)/\varepsilon_{580}(FADH°) = 1.12$, $\varepsilon_{384\pm5}(FADH^-)/\varepsilon_{580}(FADH°) = 0.86$, and $\varepsilon_{384\pm5}(MTHF)/\varepsilon_{580}(FADH°) = 5.5$ (see Materials and Methods) enter into our calculation of the quantum yields. The former two ratios deviate by less than 15% from previous studies (*32*, *33*) that obtained higher repair quantum yields than ours. Taking together all of these potential errors, our data for apophotolyases from *E. coli* and from *A. nidulans* appear hardly consistent with a repair quantum yield exceeding 0.7.

Let us stress that quantum yields of 0.45−0.57 for antenna-free photolyases are not in contradiction with the ~90% quenching of $FADH^-$ fluorescence by electron transfer to added substrate (excited state lifetime reduction from ~1.7 to ~0.2 ns) observed earlier (*17*, *34*, *35*) but would rather indicate that not all of the transferred electrons result in CPD repair because of competition with electron back-transfer prior to bond scission.

Interestingly, two well documented *in vitro* studies on apophotolyase from *E. coli* reported yields of ~0.65 (*33*) and ~0.7 (*36*), which is just within the error margin of our results. For apophotolyase from *A. nidulans*, however, the (to our knowledge) only well-documented study yielded a repair quantum yield of unity (*32*), well outside our error margin. For holophotolyase from *E. coli*, uncertainties with respect to the extinction coefficient and the stoichiometry of the MTHF antenna pigment provide additional error sources. It is of note, however, that a quantum yield ratio of ~0.75 between holo- and apophotolyase from *E. coli* observed in the present study is in line with the expected loss during energy transfer from the MTHF antenna pigment to $FADH^-$ in the holoenzyme (*29*, *34*).

In conclusion, we cannot yet explain why, despite considerable efforts to exclude or limit systematic errors in our experiments, we obtain lower absolute repair quantum yields than most of the previous studies on comparable enzyme−substrate combinations. This unresolved question does, however, not affect the main conclusion of our study: that the novel weakly UV-absorbing substrate DHT10 is repaired as well as conventional substrates and is hence a promising tool for future fast and ultrafast transient absorption studies on the repair mechanism of photolyase.

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SUPPORTING INFORMATION AVAILABLE

Conditions for deprotection of the phosphoramidite building blocks, analytical data for the *cis-syn* thymine dimer phosphoramidite, and MALDI-TOF mass spectra of the T10 and DHT10 substrates. This material is available free of charge via the Internet at http://pubs.acs.org.

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