Photoprotected Genes

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Photoactivation of a Psoralen-Blocked Luciferase Gene by Blue Light**

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The unleashing of photocleavable ("photoprotected") compounds has been used extensively for several decades now. The approach turned out to be particularly valuable for triggering small-molecule activity inside living cells, including neurotransmitters, cyclic nucleotides, and calcium cations.[1] More recently, the concept has been expanded to the control of large biopolymers, such as proteins and nucleic acids.[2] However, as it took decades to optimize photoprotection of small molecules, the search for simple and convenient methods that work on proteins and nucleic acids is yet ongoing. The activation of psoralen cross-linked nucleic acids by a light-trigged electron transfer is a novel photoprotection strategy that has the potential to overcome some of the weaknesses of currently used approaches.

Two strategies for photoprotecting nucleic acids are usually applied. Photolabile protecting groups are either attached to the nucleobase or to the backbone phosphate groups.[1,2] In the first strategy, the protecting group is installed at the nucleotide level and then incorporated site-specifically into single-stranded DNA or RNA by means of solid-phase synthesis.[3] This allows for the exact control of the modification in the resulting oligomer; however, the later incorporation into plasmids or large RNA transcripts can be cumbersome. In the second strategy, the photocleavable protecting groups are introduced stochastically into biochemically generated nucleic acids such as plasmids and RNA.[4] However, random introduction allows little control over the modification site. In general, the effect of a single protection group is insufficient to entirely block the function of a nucleic acid,[5] and thus multiple modifications are required to reduce the residual activity.[2–5] This is particularly severe for the stochastic modification of plasmids and mRNAs.[4] Since light-driven deprotection is never a clean reaction, one always has to carefully optimize the degree or the site of modification to balance the residual activity of the photoprotected compound against the feasibility for photoactivation. In particular for randomly photoprotected nucleic acids, the activity of the unlashed nucleic acid is often only five to ten times that of the photoprotected precursor.[4]

Psoralen cross-links[6] may offer a simple and efficient solution to this problem. Even though psoralen is a small molecule (258 Da), a single cross-link somewhere inside a plasmid, gene, or RNA transcript reliably shuts down the function of the nucleic acid to virtually zero residual activity. Thus, psoralen cross-links provide a strong means for the manipulation of gene function. Such strong inhibitory effects result from the formation of interstrand cross-links at 5′-d(TA) or 5′-r(UA) motifs by two subsequent [2+2] photocyclizations between the psoralen and the two pyrimidine bases (Figure 1).[6] Owing to the covalent linkage, no cellular machinery can unwind the two strands to read out the genetic information. Furthermore, cellular cross-link repair is slow and error-prone and involves several repair pathways.[7] For this, psoralen has been used to shut down genes, to stimulate homologous recombination, or to study DNA repair.[8,9] Techniques have been developed to incorporate psoralen cross-links site-specifically into plasmids[8] and even chromosomes[9] to manipulate their function. Recently, we demon-

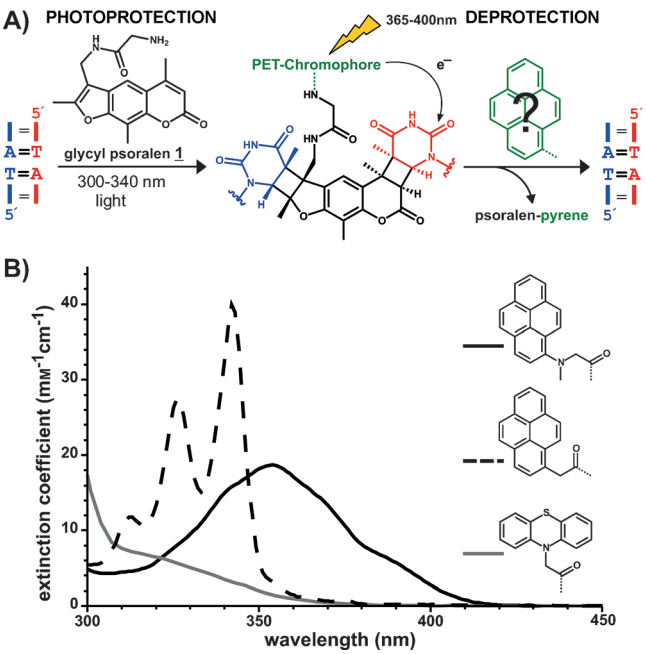

Figure 1. Psoralen photoprotection strategy. A) Psoralen interstrand cross-links completely block gene function by covalent linkage of the pairing strands. Photoelectron transfer (PET) from an external PET chromophore could be applied to photoactivate cross-linked genes if a suitable chromophore is found. B) UV spectra of pyrene, the previously used phenothiazine, and the 1-aminopyrene PET-chromophore explored in this study.

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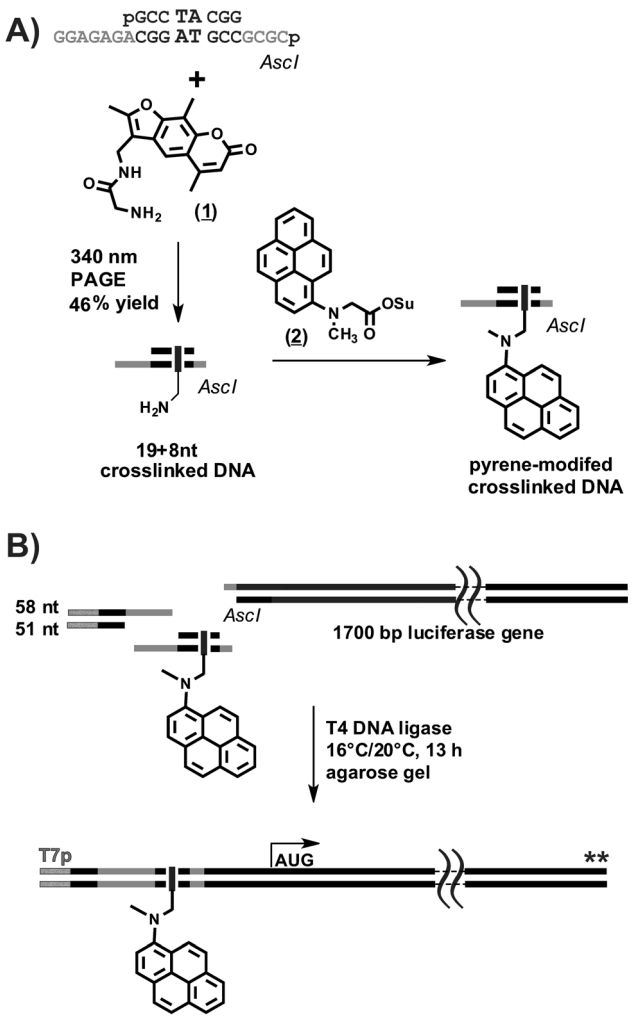
strated that psoralen cross-links can be reversed in a photolyase-like fashion, paving the way for their potential application in photoprotection.[10]

The photolyase-like repair of the psoralen-cross-link relies on the photoreductive transfer of a single electron from an external donor to the cross-link. In our initial study we had chosen phenothiazine as donor owing to its strong photoreductive power.[10] However, phenothiazine is a poor light collector ($\lambda_{max} = 315$ nm with $\varepsilon \approx 5\ mL\,mol^{-1}\,cm^{-1}$), and long irradiation times (hours) of harmful light (330 nm) were required to achieve cross-link removal. Such conditions are incompatible with cellular systems and thus hamper the application in biology. Considering the electron injection energetics, we estimate the minimal light energy required to be ≥ 3.0 eV (ca. 2 eV[11] for reduction of the cross-link; ≥ 0.5 eV for oxidation of the donor; ≥ 0.5 eV additional driving force to overcome the reorganizational energy). To provide ≥ 3.0 eV using light, absorption of photons with $\lambda \leq 410$ nm would be sufficient. Thus, the system can be clearly further improved by the choice of a better suited electron donor.

Screening the literature on electron transfer studies in DNA, we found the class of pyrene donors particularly promising owing to their strong absorbance at 345 nm ($\varepsilon \approx 35\ mL\,mol^{-1}\,cm^{-1}$) and strong photoreductive power ($E^* \approx 2.5$ eV).[12] Several red-shifted pyrenes have been reported, including some that are directly conjugated to uridine.[13] However, their photoreduction potentials seem to be too low for efficient cross-link repair.[14] Adding an amino group to an aromatic ring usually results in the red-shift of the absorbance and the lowering of the oxidation potential (Figure 1B). In accordance, 1-aminopyrene absorbs broadly between 330 nm and 400 nm with $\varepsilon \approx 3$–$19\ mL\,mol^{-1}\,cm^{-1}$ at a relatively low oxidation potential of 0.6 eV.[12] Thus, 1-aminopyrene fits exactly to the requirements defined above. Fortunately, Majima and co-workers recently described the synthesis and properties of an *N,N*-dialkyl 1-aminopyrene that can be easily incorporated into nucleic acids by peptide bond formation.[15] Similar to their description, we synthesized the *N*-hydroxysuccinimide ester **2** of this derivative for the incorporation into a cross-linked gene (Scheme 1A).

To test the applicability of psoralen cross-links in a photoprotection approach, we generated a complete functional luciferase gene (1.8 kb) that carries a single psoralen cross-link at one specific site between the promoter and the translation start site (Scheme 1B). To have full knowledge and control of the cross-link site, we decided to assemble the gene by a three-component ligation strategy from a T7 promotor (51 bp), a well-defined cross-link site (27nt), and the luciferase gene (1700 bp). The latter was obtained from a plasmid by restriction digest. The defined cross-link site was generated by irradiating a stoichiometric mixture of an 8nt oligomer 5′-d(pGCCTACGG) and the complementary 19nt oligomer (5′-d(pCGCG CCGTAGGCAGAGAGG) in presence of *N*-glycyl-amino-methyltrisoralen (**1**) at 340 nm (Scheme 1A). The product was purified by 20% polyacrylamide gel electrophoresis and was obtained in 46% yield after isolation and precipitation. The integrity of the cross-linked species was confirmed by MALDI mass analysis (Supporting Information, Figure S2). This 19 + 8nt cross-linked DNA was then further modified at the cross-link amino group by incubation with aminopyrene succinimide ester **2**. The integrity of the single modified species was confirmed by MALDI-MS (Supporting Information, Figure S4). The 19 + 8nt cross-linked DNA piece with or without the pyrene modification was then ligated between the T7 promotor and the luciferase gene. As a positive control, a full gene lacking the cross-link was assembled by the same strategy. All of the assembled genes were purified on agarose gel, stained with GelRed (Biotium), and cut out under green light (505 nm).



Scheme 1. Assembly of a psoralen-photoprotected luciferase gene. A) The psoralen-cross-link was inserted site-specifically into a duplex formed by a 19nt with an 8nt oligomer. The cross-linked species was further modified with the aminopyrene donor by peptide bond formation directly at the cross-link site. B) The sticky ends at the cross-linked piece were used to attach a T7 promotor and the full luciferase gene by action of the T4 DNA ligase.

In contrast to our previous study,[10] several improvements regarding the synthesis and modification of psoralen cross-linked nucleic acids have been achieved. The direct modification of aminomethyl trisoralen cross-links at the amino group under peptide bond formation turned out to be very inefficient, which is due to steric hindrance. However, incorporating an additional glycyl residue to the aminomethyl

trisoralen moiety (compound **1**) allowed for the highly efficient modification (full conversion within 1 h, see the Supporting Information) of the cross-linked DNA with *N*-hydroxy-succinimide esters. Second, during UV-dependent cross-linking, the 19 + 8nt construct was prone to the formation of additional psoralen monoadducts that were inseparable during PAGE purification. Using our previous procedures, about 50% of the cross-linked DNA carried an additional psoralen monoadduct, probably at the *AscI* restriction site. However, adding uracil (6 mM) to the cross-linking buffer (Supporting Information) and applying an additional irradiation step gave the 19 + 8nt cross-linked DNA that is free from any additional adducts.

Initially, we studied the light-driven opening of the 19 + 8nt cross-linked DNA piece alone. For this the starting material was diluted to a final concentration of about 2 μM into phosphate buffer (10 mM, 100 mM NaCl, pH 7.0). Irradiation was performed in a fluorescence spectrometer equipped with an excitation monochromator (± 20 nm) and a 75 W xenon lamp. The sample (50 μL) was irradiated at 365 nm or 400 nm inside a PCR tube. At various time points, samples (5.0 μL) were taken and the conversion was analyzed by 18% PAGE (7 M urea). To determine kinetics, a dilution series of 100%, 50%, 25%, and 12.5% of both the 19nt product and the 19 + 8nt starting material were loaded onto the gel. After SybrGold staining and LASER scanning, kinetics were determined by densitometry.

Both UV light (365 nm) and blue light (400 nm) promote the conversion of the cross-linked starting material into the desired 19nt product with very similar features (Figure 2). First, conversion is initially very fast but slows down rapidly and finally stops after about 70% conversion. Second, only about 50% of the expected amount of 19nt product was generated. Instead, a side product was formed with similar kinetics as the product. Already in our previous study, we observed the formation of psoralen monoadducts as reaction intermediates.[10] Opening of such monoadducts is driven by a second electron transfer or by direct light absorption. MALDI mass analysis of the reaction mixture after 60 seconds irradiation with UV light (Supporting Information, Figure S8) clearly shows the formation of both the desired product (5983 Da) and its psoralen monoadduct (6568 Da). Interestingly, we also found that substantial amounts of the starting material (9058 Da) had lost the aminopyrene donor (minus 271 Da). We then tested the photostability of the donor and found, in accordance with previous reports[15] that the 1-aminopyrene derivative is unstable and fully decomposes upon irradiation (Supporting Information, Figure S14). Thus, the limited photostability of the electron donor explains the premature stop of the photoactivation we observe on the gel. Despite those two drawbacks, the desired, modification-free 19nt template is formed in substantial amounts (20–30%) upon irradiation. Furthermore, the efficiency of photoactivation is remarkable: under UV light conditions the steady-state (20% product) is obtained within 1 min; under blue light conditions the steady state (23% product) is obtained after 5 min (Figure 2). From the initial slopes, we can estimate the life-times for the opening of the cross-linked species before donor decomposition interferes. This is 1.6 min with UV light and 7.1 min with blue light. To estimate the quantum yield, we compared these data with the photodecay of commercially available dimethoxynitrobenzyl-photoprotected cAMP under the same conditions (Supporting Information). As the quantum yield and extinction coefficients are known for the latter, we can directly estimate the product $\varepsilon\Phi$ for our photoactivation reaction to be about 1100 $L\,mol^{-1}\,cm^{-1}$ at 365 nm and about 250 $L\,mol^{-1}\,cm^{-1}$ at 400 nm. Taking the excitation coefficient of 15500 $L\,mol^{-1}\,cm^{-1}$ for our donor at 365 nm into account, we obtain a quantum yield $\Phi \approx 7\%$ for the initial cross-link reversal. To our knowledge, this is an extraordinarily high quantum yield for a chemical reaction driven by a photoelectron transfer.

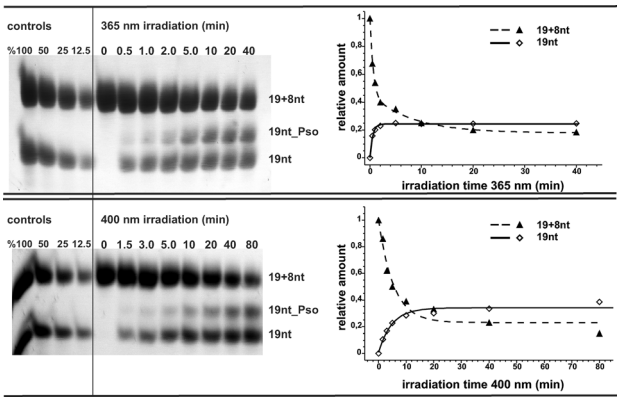


Figure 2. PAGE analysis of the light-dependent release of the desired 19nt product upon irradiation of 19 + 8nt cross-linked DNA modified with the aminopyrene donor by UV light (365 nm) or blue light (400 nm). Relative amounts of starting material and product were estimated densitometrically by comparing the band stain with the controls.

To test the applicability of psoralen photoprotection in a biochemical assay, we then studied the photoactiviation of the assembled luciferase gene containing the cross-linked piece downstream the T7 promotor (Scheme 1B). For this, the assembled genes were diluted into phosphate buffer (1 mM, 10 mM NaCl, pH 7.0) to a final concentration of about 40 nM. Irradiation was carried out under identical settings in PCR tubes inside a fluorescence spectrometer. Samples (4.0 μL) were taken out at various time points to template in vitro transcription/translation mixtures (PURExpress, New England Biolabs, see Supporting Information). After 2.5 h incubation (37 °C), the activity of the translated luciferase protein was measured.

The strength of the psoralen photoprotection now becomes apparent (Figure 3). Introducing a single cross-link reduces the luciferase activity by a factor of ≥ 1000. The residual signal is virtually zero after background subtraction. Notably, very short irradiation with UV light (10 s) already substantially recovers luciferase activity (30%). According to the kinetics for the reversal of the cross-link, the maximum signal (68% of the positive control) is obtained after 1–2 min irradiation. Owing to the low starting signal, the calculation of photoreactivation factors is error-prone; however, factors of ≥ 500 are accessible. To confirm the necessity of the pyrene donor, we also ligated the 19 + 8nt cross-linked DNA without the pyrene modification into the luciferase gene. However,

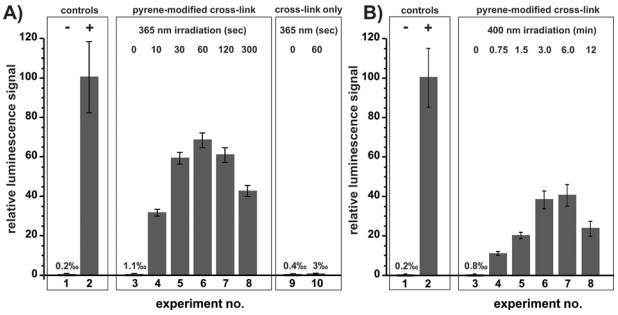


Figure 3. In vitro transcription/translation assay. A) Experiment 1,2: positive and negative controls (water versus cross-link-free gene); 3–8: luciferase signal with increasing irradiation time at 365 ± 20 nm, templated by the psoralen-cross-linked and pyrene-modified gene; 9,10: control for the necessity of pyrene modification: the cross-linked gene without the pyrene modification was irradiated; B) 3–8: luciferase signal with increasing irradiation time at 400 ± 20 nm, templated by the psoralen cross-linked and pyrene-modified gene.

this gene was silent and cannot be substantially activated by UV light (Figure 3A, experiment no. 9,10). As expected, photoactivation of the gene also succeeds with blue light. However, five times longer irradiation times are required, which arised from the five times lower excitation coefficient of the donor at 400 nm ($2.8\,mL\,mol^{-1}\,cm^{-1}$) versus 365 nm ($15.5\,mL\,mol^{-1}\,cm^{-1}$). At short irradiation times (0.75 to 3 min), the luciferase signal depends linearly on the irradiation time. However, in accordance to the kinetics of the 19 + 8nt cross-linked DNA, the maximum yield is reached after 3–6 min. To simulate photoactivation under cellular-like conditions, we repeated the irradiation experiment at 365 nm (60 s) with the same amount of template but directly inside the fully assembled transcription/translation mixture prior to incubation (Supporting Information, Figure S10). The activity was light-activated up to 45% of the positive control, thus demonstrating its applicability in presence of proteins, salt, magnesium, and reducing agents.

The application of psoralen cross-links to light-activate gene function is a novel concept. Herein we provide a proof of principle and demonstrate its feasibility on the whole gene level. To make the system more suitable for practical applications, we successfully established an aminopyrene derivative as an electron donor. Even though full reconstitution of gene function was not achieved, an extraordinarily efficient photoreactivation enables for the fast and substantial activation of psoralen-photoprotected genes in a very simple setting (fluorescence spectrometer) with either UV light (365 nm) or blue light (400 nm). Owing to the complete suppression of residual gene activity, notably high photoactivation factors were obtained in a luciferase assay. We expect our strategy beneficial for situations in which a high photoactivation or a virtually complete suppression of any residual gene function is required. In contrast to classical protection with photolabile protecting groups,[1,2] psoralen photoprotection separates the protecting moiety from the light collector. This will enable us to consider the sequence specific photoactivation of a particular gene in a mixture of several cross-linked genes by addressing the donor site-specifically with the help of a PNA[16] or a polyamide[17] probe. In general, we anticipate that psoralen-dependent photoprotection is readily transferrable and applicable to virtually any conceivable DNA and RNA construct.

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