Two wavelength femtosecond laser induced DNA–protein crosslinking

Ch. Russmann[1], J. Stollhof, C. Weiss, R. Beigang and M. Beato[1,*]

Fachbereich Physik, Universität Kaiserslautern, Erwin-Schrödinger-Straße 46, D-67663 Kaiserslautern, Germany and [1]Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Straße 2, D-35037 Marburg, Germany

ABSTRACT

Nucleic acid–protein interactions are essential for storage, reproduction and expression of genetic information. Biochemical methods, such as dimethyl sulfate genomic footprinting, have been developed to study stable protein–DNA interactions *in vivo* and chemical crosslinking has been used for less stable interactions, but the chemical agents are slow, damage cells and perturb native equilibria. To avoid these perturbations, UV laser crosslinking offers an alternative, although the energies required for significant cross-linking cause extensive DNA damage. We find that a combination of femtosecond laser pulses at two different wavelengths, in the UV and the visible range, increases the crosslinking efficiency while minimizing DNA damage. This technique also allowed us to directly measure the singlet S_1 lifetime of native DNA ($\tau_{S1} = 3.2 \pm 0.2$ ps), which is mainly determined by the lifetime of thymine [$\tau_{S1} = 2.8 \pm 0.4$ ps for $(dT)_{16}$], the photochemically most reactive base. Our results suggest that two wavelength femtosecond laser pulses are well suited for the identification of transcription factors interacting with defined sequences and for studying the kinetics of protein–nucleic acid interactions in intact cells.

INTRODUCTION

Nucleic acid–protein interactions are essential for all biological processes involving storage, reproduction or expression of genetic information. To study these interactions in intact cells gentle, non-invasive methods are required. Chemical agents have been used for genomic footprinting (1) or for DNA–protein crosslinking (2–4) but they damage cells and perturb their native equilibria. Crosslinking by conventional UV light (5) has also been employed for less stable interactions, but is relatively inefficient and generates heat which can also perturb the interactions to be studied. Furthermore, both crosslinking methods are slow, requiring times in the range of minutes, and therefore are not appropriate for the study of rapid kinetics. UV-laser crosslinking offers an alternative that has been used in the recent years with different laser systems and various biological models (6–12). However, except for the DNA double strand binding transcription factor RAP1 models (6) and the progesterone receptor (8), little is known about the potential of UV lasers for the study of specific interactions between eukaryotic transcription factors or regulatory proteins and their target sequences in DNA.

The practical use of UV lasers is limited not only by the efficiency of the crosslinking reaction, which strongly depends on the laser parameters (8), but also by the availability of adequate methods to determine DNA–protein contact points (13) and to identify the crosslinked proteins (6,14,15). Immunoprecipitation combined with DNA hybridization or primer extension are the classical methods (14,15), but it should also be possible to identify the crosslinked proteins using state of the art mass spectrometry methods (16,17). However, the major problem in using UV-laser crosslinking is that conditions which lead to high crosslinking efficiency also result in high DNA damage. Because the analysis of the crosslinking events relies on primer extension of the DNA by PCR techniques requiring the integrity of the DNA double helix flanking the crosslink, we defined a new parameter called the 'effective crosslink yield' (8). This operational parameter is the fraction of extendible DNA multiplied by the crosslink yield. Here we report the first study of femtosecond (fs) UV laser DNA crosslinking to a eukaryotic transcription factor, Nuclear Factor 1 (NF1), using combinations of UV and visible pulses as a promising new strategy to reach high crosslinking efficiency combined with low DNA damage, thus raising the effective crosslinking yield. In addition, the use of two independent fs pulses at different wavelengths allows us to investigate the dynamics of laser-induced crosslinking.

UV crosslinking for a DNA base can be represented as a two-step process (10) in a simplified energy level diagram (Fig. 1). A first UV photon excites the DNA base from the singlet S_0 ground state to the first excited S_1 manifold. From the excited S_1 level the molecule can absorb a second photon which promotes the DNA base to high lying singlet S_N levels above the ionization limit (I.L.). The generated cation radical and the electron may recombine (18,19), either by 'germinate recombination' of a just formed charged pair or by 'volume recombination' of cations and electrons from different molecules. The volume recombination of the cation radical then initiates crosslinking with hydrogen-bonded amino acid and the formation of other photoproducts. There is, however, a finite probability for an intersystem crossing (I.C.) to the triplet T_1 manifold which has a long lifetime compared to the singlet S_1 level. Absorption of a second photon then leads to a transition to high lying T_N levels above the ionization limit. The ions produced via the triplet channel can also initiate crosslinking to the contacting proteins. Processes leading to DNA damage can be initiated at any of the four excited states S_1, S_N, T_1 and T_N, but only S_N and T_N lead

*To whom correspondence should be addressed. Tel: +49 6421 28 6286; Fax: +49 6421 28 5398; Email: beato@imt.uni-marburg.de
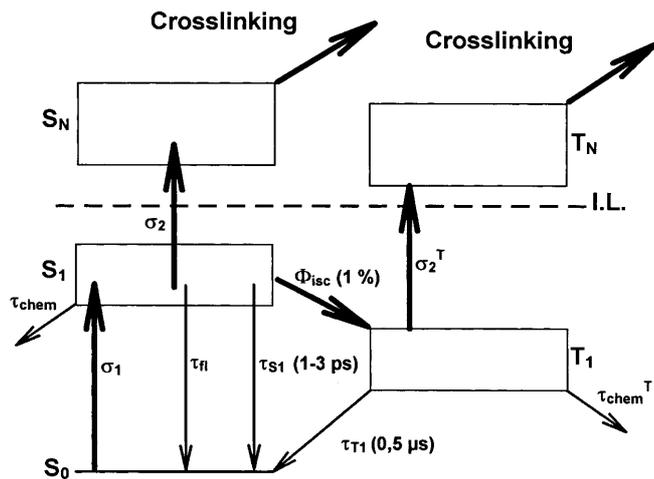
Figure 1. Simplified energy level diagram of a typical DNA base. The pathways leading to crosslinking via the singlet S_1 and the triplet T_1 states are indicated. After excitation of the singlet S_1 state the bases are promoted either directly with a second photon to high lying S_N states above the ionization threshold (dashed line) or, after intersystem crossing to the T_1 level, to states T_N also above the ionization limit. I.L., ionization limit; ϕ_{isc}, probability for intersystem crossing; τ_{S1}, S_1 lifetime; τ_{T1}, T_1 lifetime; τ_{chem}, lifetime for chemical processes; τ_{fl}, fluorescent lifetime; σ_i, absorption cross section for absorption to level i.

to crosslinking (20). Thus, to reduce DNA damage the population times in the non-productive states S_1 and T_1 should be minimized.

MATERIALS AND METHODS

Analysis of crosslinked DNA–protein complexes

Recombinant pig NF1 (CTF-2; 21) was expressed as a His-tagged protein in Sf9 cells infected with a baculovirus NF1 expression vector, and purified by Ni-chelate chromatography. NF1 (10 ng in TGA buffer containing 10 mM Tris–HCl pH 7.5, 10% glycerol, 1 mM EDTA and 90 mM NaCl) and an oligonucleotide (22) (5′-AAT TCC TTT TTT <u>TGG ATT GAA GCC A</u>AT CGG ATA ATG AGG-3′, 20 fmol, 20 000 c.p.m. Cerenkov counting), which encompasses an NF1 binding site (underlined), were incubated for 20 min in 25 μl TGA buffer containing 1.5 μg bovine serum albumin and 1 μg poly(dI–dC) and irradiated in microcentrifuge tubes with the unfocussed laser beam. The irradiated samples were heated to 100°C with 1/3 vol of SDS sample buffer (200 mM Tris–HCl pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol, 1% 2-mercaptoethanol, 1 M urea) (6) for 5 min and loaded onto an 8% SDS–polyacrylamide gel (SDS–PAGE). The gel was dried and analyzed with a PhosphorImager™ (Molecular Dynamics Inc.). The fraction of NF1 crosslinked to the DNA probe was multiplied by the fraction of NF1 complexed with the DNA (binding efficiency), as determined in the band shift assay. This new parameter is the crosslink yield (8).

Analysis of the integrity of DNA

A plasmid containing the complete MMTV promoter was irradiated, cleaved with the restriction enzyme *Hae*III (Boehringer Mannheim), and analysed by 30 cycles of primer extension using the following [32]P-5′-end-labeled primer (8): 5′-GACGAG-CGGAGACGGGATGGCGAACAG-3′. Reaction products were
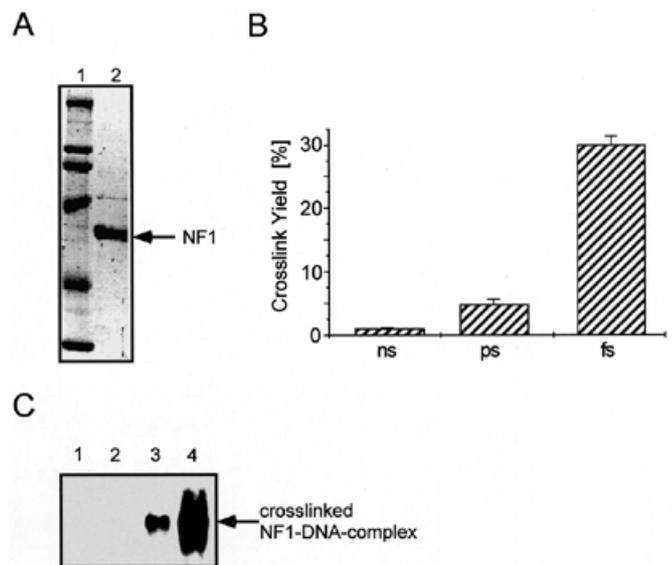

Figure 2. Determination of crosslink yield. (**A**) SDS–PAGE of recombinant NF1. After electrophoresis, the gel was stained with Coomassie blue. Lane 1, size markers (molecular weight in kDa); lane 2, recombinant NF1. (**B**) Comparison of the crosslinking efficiency of NF1 with 5 ns, 100 ps and 200 fs UV pulses (8). The best absolute crosslink yields determined after irradiation with equal amounts of applied energy (3 J/probe) are shown. In the case of the 5 ns and 100 ps pulses, the light intensity was comparable ($I = 10^{12}/Wm^2$), whereas in the case of 200 fs the light intensity was three orders of magnitude lower. (**C**) PhosphorImager image of an SDS–PAGE analysis of crosslinked NF1–DNA complexes. The labeled NF1 oligonucleotide was incubated with NF1, irradiated with the laser system described in Figure 3, and analyzed by SDS–PAGE (8). Lane 1, unirradiated control; lane 2, irradiated with blue laser pulses [1 nJ/pulse, 1 J]; lane 3, irradiated with UV laser pulses [0.25 nJ/pulse, 0.25 J]; lane 4, irradiated with UV [0.25 nJ/pulse, 0.25 J] and 300 fs-delayed blue laser pulses [1 nJ/pulse, 1 J]. Error bars are indicated.

visualised on a 6% sequencing gel followed by analysis of the dried gel on the PhosphorImager. The amount of full length extension products obtained from irradiated DNA was divided by the amount of full length extension products obtained from unirradiated DNA. This number is an operational information qualitatively reflecting the amount of intact DNA (extendable DNA) and does not represent a stringent measurement of DNA damage. The fraction of extendable DNA was multiplied by the crosslink yield and called 'effective crosslink yield'.

Laser set-up

The cw mode-locked Ti:sapphire laser used generates 150 fs long pulses at a repetition rate of 82 MHz with an average power of up to 2 W in a wavelength range from 720 to 850 nm. The infrared pulses were frequency doubled and tripled in two β-bariumborate crystals, and pulses at the second harmonic frequency 2ω (λ = 400 nmcrosslinking) and the third harmonic frequency 3ω (λ = 266 nm) were used simultaneously. The average output power at the third harmonic frequency was as high as 150 mW, corresponding to a pulse energy of ~2 nJ per pulse and a peak power of 10 kW. The pulse length was increased to ~200 fs due to the dispersion of the non-linear crystals. Using a variable delay line with a temporal resolution of 10 fs, the probe was irradiated with UV and blue with a well-defined time delay.
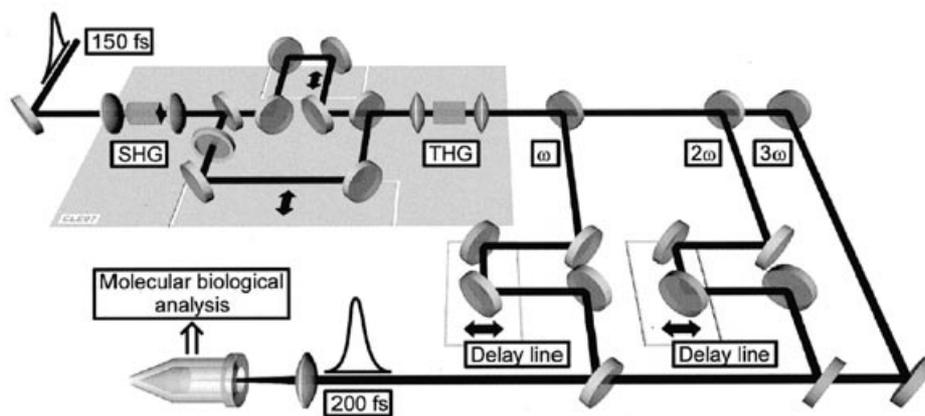
Figure 3. Schematic diagram of the experimental set-up used for the time resolved measurements. The cw mode-locked Ti:sapphire laser used generates 150 fs long pulses at a repetition rate of 82 MHz with an average power of up to 2 W in a wavelength range from 720 to 850 nm. The infrared pulses were frequency doubled and tripled in two β-bariumborate crystals, and pulses at the second harmonic frequency 2ω ($\lambda = 400$ nm) and the third harmonic frequency 3ω ($\lambda = 266$ nm) were used simultaneously. The average output power at the third harmonic frequency was as high as 150 mW, corresponding to a pulse energy of ~2 nJ per pulse and a peak power of 10 kW. The pulse length was increased to ~200 fs due to the dispersion of the non-linear crystals. Using a variable delay line with a temporal resolution of 10 fs the probe was irradiated with UV and blue with a well-defined time delay.

Rate equations

For the calculations we have used a rate equation model taking into account all relevant levels of the DNA (23).

RESULTS AND DISCUSSION

Comparison of crosslinking efficiency with UV pulses of different length

As a model system to explore the possibilities of UV laser-mediated crosslinking to specific regulatory sequences, we have studied the binding of recombinant NF1 (Fig. 2A) to the MMTV promoter (24). NF1 binds to a palindromic site on the MMTV promoter located downstream of the hormone responsive region, and synergizes with hormone receptors for transactivation of the promoter (24). For our studies, we used an oligonucleotide containing this palindromic NF1 binding site (Material and Methods). A comparison of the optimal NF1 crosslinking efficiency achieved using monochromatic laser pulses at 266 nm and 5 ns, 100 ps and 200 fs pulse length, respectively, is shown in Figure 2B. The strong increase in crosslinking yield for the fs pulses cannot be simply explained in terms of higher peak intensities, which was kept orders of magnitude below the intensities used with ns and ps pulses. Obviously, the short pulse length is responsible for the increase in crosslinking efficiency, as only in the case of 200 fs pulses the pulse length was shorter than the lifetime in the intermediate S_1 level of the DNA. In addition the high repetition rate leads to accumulative population in the triplet levels of the DNA from where crosslinking can also be initiated.

As expected, the enhancement in crosslinking is accompanied by a higher DNA damage, which reduces the increase in effective crosslink yield (see also Fig. 4B). Similar results have been obtained with the progesterone receptor and an oligonucleotide with an hormone responsive element (8).

Combination of UV and blue pulses

In order to reduce the UV-induced DNA damage we have applied UV pulses for the excitation to the singlet S_1 and blue pulses for the ionization of the excited DNA base molecules. This strategy

has the advantage that the intensity of the UV pulses in the first step can be kept low, thus reducing DNA damage caused by the UV photons. A high crosslinking efficiency can still be attained by applying the second pulse at a wavelength which is too long to excite DNA bases from the ground state, and therefore alone does not damage DNA, but provides enough additional energy to cross the ionization threshold.

The experimental set-up is shown in Figure 3. The UV pulses ($\lambda = 266$ nm) were used to excite the singlet S_1 levels of the DNA bases. The probe was irradiated with blue ($\lambda = 400$ nm) pulses with a well-defined time delay. The crosslinking yield was determined by electrophoresis on SDS–polyacrylamide gels (Fig. 2C) as described (8). The maximum amount of applied UV energy was limited to 200 mJ, explaining the lower crosslinking efficiency (2.6%) reached with UV pulses alone (dashed line in Fig. 4A) in comparison to the results shown in Figure 2B. Using blue pulses alone there is no crosslinking (Fig. 2C, lane 2), as this would require a non-resonant 2-photon excitation. The crosslinking yield after the two wavelength pulses is shown as a function of the temporal delay between the UV and the blue pulses. There is an increase in crosslinking when two pulses arrive almost simultaneously at the probe with a maximum at a delay of 300 fs between the UV and the blue pulses (see also Fig. 2C, lane 4). This time corresponds to the maximum population of the singlet S_1 level. The increase in crosslinking yield is mainly caused by a two-step excitation via the singlet S_1 state. The experimental decay of the increased crosslinking efficiency is caused by the short lifetime of the intermediate S_1 level and corresponds to an S_1 lifetime of 3.2 ± 0.2 ps. For comparison, we have also measured the lifetimes of single-stranded DNA by crosslinking the single-stranded binding protein (SSB) to homo-oligomers containing only thymine $(dT)_{16}$ or guanine $(dG)_{16}$ bases (data to be published elsewhere). The corresponding lifetimes were $\tau_{S1}[(dT)_{16}] = 2.8 \pm 0.4$ ps and $\tau_{S1}[(dG)_{16}] = 1.0 \pm 0.2$ ps. These values differ slightly from the recently determined singlet lifetime of thymine 1.2 ps $\pm$ 0.2 ps in concentrated aqueous solution (3.2 mM) under high intensity excitation (19) and from similar determinations performed with adenine and calf thymus DNA (25). Our direct experimental measurement of this parameter for

A

B
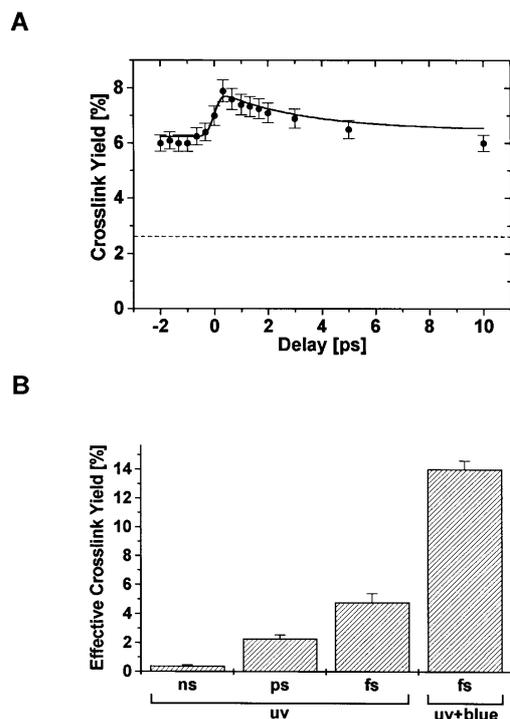
Figure 4. Two-wavelength crosslinking of NF1. (**A**) An oligonucleotide encompassing the NF1 binding site was used for two-wavelength crosslinking with a UV and a blue photon. The samples were irradiated for 10 s with an average output power of 20 mW in the UV and 100 mW in the blue spectral region. The crosslinking efficiency is shown as a function of the temporal delay of the blue pulses with respect to the UV pulse. The solid line represents the result of our rate equation model (Materials and Methods). The dashed line indicates the crosslinking yield obtained using only UV pulses of 20 mW. (**B**) The figure shows the best effective crosslink yields of NF1 determined after irradiation with UV laser pulses [266 nm] of 5 ns [30 mJ/pulse, 2 J], 100 ps [3 mJ/pulse, 1.5 J] or 200 fs [82 MHz, 1 nJ/pulse, 1 J] alone and with a combination of 200 fs UV laser pulses [266 nm, 0.25 nJ/pulse, 0.25 J] and blue laser pulses [400 nm, 1 nJ/pulse, 1 J]. Error bars are indicated.

Conclusions

A comparison of the effective crosslink yield obtained with UV pulses alone and with an optimized combination of UV and blue pulses shows that the latter technique leads to a significant improvement (Fig. 4B). Thus, our new method of two wavelength fs laser crosslinking characterized by its high efficiency and low DNA damage should be useful for establishing libraries of DNA binding sites for transcription factors (26). It opens the possibility for crosslinking nucleic acids and proteins within living cells in order to stabilise transient interactions for further analysis by genomic footprinting and other techniques.

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bases within a native DNA molecule under physiological conditions indicate that the lifetime in native DNA is mainly determined by the lifetime of the pyrimidines which are photochemically more reactive than the purines (20).

There is also an increase in crosslinking when the two pulses are separated in time by more than the lifetime of the intermediate S_1 level. This is caused by the high repetition rate of the mode-locked laser. The time between two consecutive pulses (13 ns) is small compared to the lifetime of the triplet T_1 state (0.5 μs; 18) which is always populated by internal conversion processes. The blue photon then leads to crosslinking from the T_1 level explaining the relatively high values of crosslinking found within the time period tested (Fig. 4A).

The solid line in Figure 4A represents a calculation using a rate equation model which takes into account all relevant levels for crosslinking (Materials and Methods) and is in excellent agreement with the experimental values. Thus, we are able to qualitatively and quantitatively describe the crosslink process and to determine the singlet lifetime in native DNA. With this technology it will also be possible to describe other processes leading to DNA damage in native DNA and to measure additional photophysical parameters of DNA.