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Dynamic probing of nucleic acid-protein interactions by biphotonic laser chemistry♣

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In this study, we describe a new approach for a millisecond dynamic study with high spatial resolution of non-equilibrium protein–DNA interactions in solution. The approach is based on mapping the time-resolved UV laser–induced biphotonic DNA oxidative lesions during DNA recognition and binding of a specific protein. Our laser "photofootprinting" approach was applied for studying the interaction of the human necrosis factor NF– B p50 homodimer bound to a 37 base pair DNA. Evidence is provided for the occurrence of a two-step process: rapid (30 ms) formation of the pre-equilibrium complex and slow (1 s) protein rearrangement and DNA conformation accommodation.

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1. Introduction

Protein-DNA complexes play a crucial role in coordinating events involved in gene expression, DNA replication and repair. High-intensity UV laser (λ ~ 260 nm) DNA-protein "footprinting" is a direct and powerful approach to investigate transient protein-DNA interactions. Exposure of DNA to high-intensity UV laser pulses results in the induction of specific oxidative photolesions [1, 2]. These arise via energy and charge transport mediated resonance biphotonic ionization of the nucleobases (Fig. 1) and strongly depend on both the DNA sequence and its local secondary structure [3]. Oxidatively modified nucleotides are selectively cleaved by alkali or enzymatic treatment, enabling their mapping at a nucleotide level [4]. The interaction of a protein with DNA alters the local DNA structure, giving rise to strong changes in the photoreactivity of the nucleobases (the yields of the different lesions). This could be used for studying the protein-DNA interactions at a nucleotide level, i.e. to carry out a UV laser footprinting [5-7]. This photochemical approach enables "visualizing" protein binding to its DNA target sequence, through the sensitivity of the laser induced DNA photochemistry to the resulting structural deformations. This approach exhibits a tremendous advantage over existing traditional methods for studying protein-DNA interactions: it could be used to study the kinetics of rapid protein-DNA interactions at a nucleobase resolution level. In other words, it should be able to detect the "dance" of a protein upon interaction with its recognition DNA sequence.

This technique has been recently demonstrated in studying stationary (equilibrium) complexes of the transcription factor NF-κB with its DNA target sequence [7, 8]. Basic events at the heart of every inflammatory response are underpinned by the dynamic relationships between genomic *cis*-regulatory (DNA sequence) determinants and interacting factors, such as NF-κB. Since the number of functional κB sites and the number of NF-κB molecules are of the same order, then how does NF-κB interact and exchange with this large number of target sites? What is the diffusion involved (2D, 3D, mixed), and how does NF-κB discriminate between highly sequence-specific (functional) and less specific binding sites? Why is binding to highly specific sequences not inhibited by the nucleosome?

Now, by using an original and unique combination of biphotonic laser chemistry and quenched-flow fast mixing techniques, we were able to directly address these questions, achieving a millisecond time- and a nucleotide space-resolution simultaneously. Our dynamic footprinting study showed that the formation of the equilibrium NF-κB– DNA complex is accomplished by a sequential two-

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step process: fast (30 ms) pre-equilibrium binding and slow (1000 ms) subsequent rearrangements involving different protein and DNA domains.

2. Experimental

Biochemicals. Synthetic 37 - mer oligonucleotides: containing the high affinity pseudosymmetrical MHC H-2 site (GGGGATTCCCC), were purchased from MWG (Germany) and purified on a 20% polyacrylamide gel, as previously described [4]. Typically 10 pmol from either the top or the bottom strand was 5'-end labelled by [γ-^{32}P]ATP by T4 polynucleotide kinase and annealing with the complementary strand by briefly heating and slow cooling down to room temperature [5]. Under the conditions used, 100% of duplexes were obtained. The protocol of Adams and Workman [9] was used to produce and purify the recombinant human NF-kB p50.

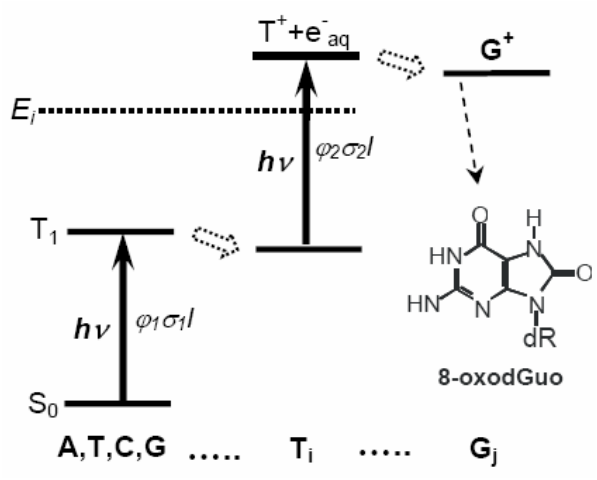

Fig. 1. Energy migration and charge transport mediated by two-photon ionization of DNA (E_i is the ionization potential). An arbitrary nucleotide (A,T,C,G) is excited to the T_1 state through S_1 by one-photon absorption. Then, a neighbouring (T_j) becomes T_1 excited by energy migration, and is ionized upon absorption of a second photon. Finally, the radical cation is trapped by a neighbouring guanine (G_j) giving rise to G^+. The latter is rapidly transformed to 8-oxodGuo which is a cleavage substrate to the Fpg glycosylase. Note that energy-migration and hole transport phenomena are highly sensitive to the DNA sequence, as well as to its secondary and higher order structures [3]. The probability for G^+ formation depends on the availability of protein inducing conformational changes in DNA in the interaction site.

Dynamic UV laser footprinting. Binding reactions were performed in 25 μL volumes in a buffer: 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 5 % glycerol, 0.1 mg/ml BSA, 0.01 % NP40, 30 mM NaCl that contained 0.2–0.5 nM of the labelled 37 bp DNA. The concentration of the protein necessary to achieve the formation of 100% complexes (~1–3 nM) was adjusted by control EMSA experiments, as described [8]. Equal volumes (12.5 μl) of DNA and protein solutions, at a concentration allowing the formation of 100% complexes, were rapidly mixed (10 ms) by a modified model SEM-300/S stop-flow (BioLogic, Grenoble, France). At variable time delays (10 ms – 2.5 s), mixes were exposed to a single UV laser pulse ($\lambda \sim 266$ nm, $\tau_p \sim 4 - 5$ ns, $E_p \sim 0.1$ J/cm^2) provided by the fourth harmonic generation of a Q-switched, model Surelite II Nd:YAG laser (Continuum, Santa Clara, CA). The energy of individual pulses was measured using a calibrated pyroelectrical energy meter (Ophir Optronics, Evry, France) and an 8% reflection fused silica beam splitter (Fig. 2).

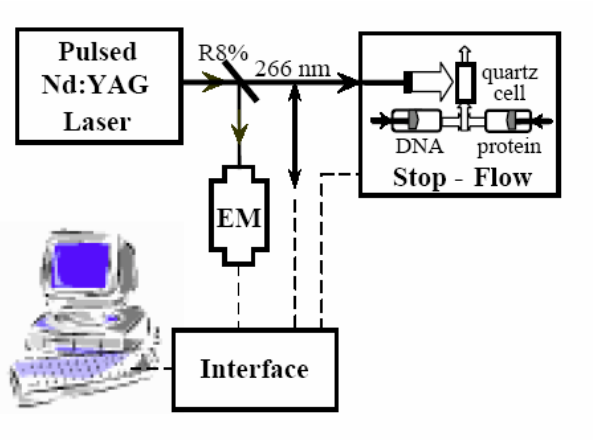

Fig. 2. Experimental set-up for high-resolution UV laser induced dynamic DNA – protein footprinting.

Each irradiated sample was recovered from the irradiation quartz chamber; the DNA was extracted by phenol-chlorophorm and ethanol precipitation, treated by Fpg glycosylase for cleaving DNA at 8-oxoG, and analyzed by 15% acrylamide-urea sequencing gel electrophoresis [5-7]. Digital images were quantified by rectangle integration, using InageQuant 4.1 (Molecular Dynamics, USA) software. DNA cleavage rates at guanines within the NF-κB binding site ($G_{\pm 2}$ - $G_{\pm 5}$) were normalized to the guanines outside the binding site, and plotted as a function of the time delay between the mixing and the laser pulse irradiation. The zero-time response corresponded to naked DNA (absence of protein).

3. Results and discussion

The Rel/NF-κB protein family regulates several vital processes in mammalian cells, including inflammation and immune responses, cell adhesion, cancer, and apoptosis [10–13]. The large role of these transcription factors in the cellular life cycle explains the high interest in their function. When bound to DNA the Rel/NF-κB proteins are found either as homo- or hetero-dimers. The crystal structures of NF-κB p50 and NF-κB p52 homo-dimers

bound to DNA have been solved previously [14–16]. A detailed picture of the structure and dynamics of these complexes in solution is, however, still missing. We first analyzed the equilibrium binding of NF-κB p50 to its recognition sequence by using the recently developed UV laser footprinting technique. The binding of a transcription factor induces a local change in the DNA structure at the binding site, which affects the spectrum of the lesions induced upon laser irradiation.

Because the DNA base photo modifications can be mapped at the nucleotide level, this allows the visualization of local changes in the DNA conformation at a nucleotide resolution, *i.e.* to carry out UV laser footprinting.

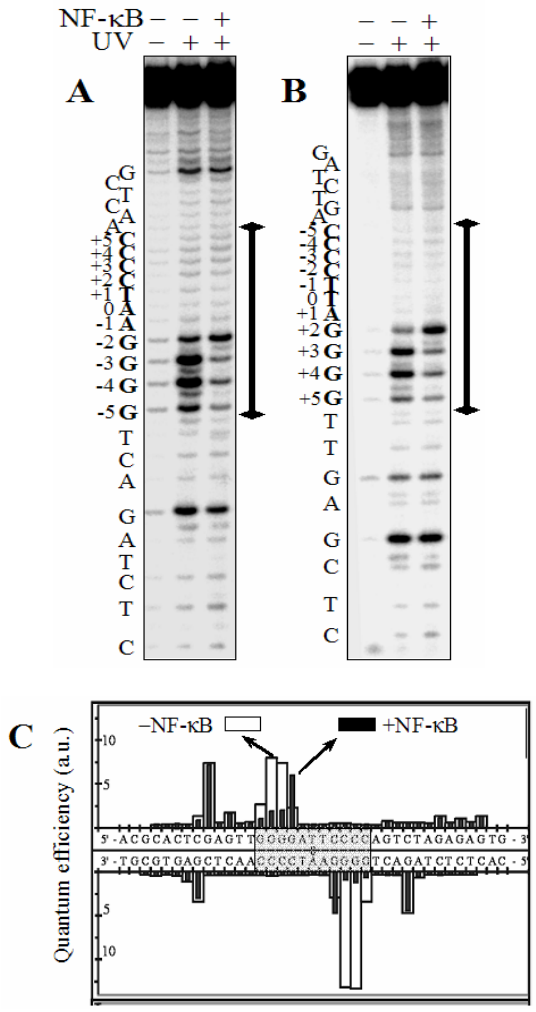

*Fig. 3. UV laser footprinting of NF-κB p50 homodimer-DNA complexes. A 37-mer double-stranded oligonucleotide containing the binding sequence of NF-κB was allowed to interact with NF-κB p50. The complex and the naked DNA control were exposed to a single high intensity UV laser pulse (E_p ~ 0.1 J/cm^2) and the footprinting analysis was carried out as described. Gel images of the top strand - **A**, bottom strand - **B**. **C** - quantified data.*

Initially, we studied, by UV laser footprinting, the solution structure at equilibrium of the NF-κB p50 homodimer complexed with a 37-bp DNA duplex that contained the major histocompatibility complex H2 NF-κB p50 binding site GGGGATTCCCC. The complex was exposed to a single high-intensity 266 nm laser pulse, and the resulting 8-oxodG lesions were mapped with Fpg glycosylase induced cleavage. This allowed the visualization of the changes in the nucleobase photo reactivity upon binding of the transcription factor. The results showed a clear footprint within the DNA binding sequence (Fig. 3). The quantitative analysis of the laser photolysis-mediated one-electron oxidation nucleobase lesions revealed interesting structural alterations concerning the NF-κB DNA binding site within the complex (Fig. 3 C). Indeed, the quantum efficiency for 8-oxoGua formation decreases considerably for the two central residues $G_{\pm3}$ and $G_{\pm4}$, and to a smaller extent for the residues $G_{\pm5}$, but decreases for the residues $G_{\pm2}$. This particular pattern might be considered as a specific "signature" of the NF-κB dimer bound to its target DNA sequence.

To carry out dynamic photofootprinting experiments, we synchronized the laser to the stopped-flow device using a custom designed microprocessor controlled interface, which allowed very rapid mixing and time-delayed photochemical probing of the substrate (Fig. 2). Since the volume of the stopped-flow quartz chamber is very small (20 μl), the dead time of the mixing device does not exceed few milliseconds. This enabled us to study the kinetics of the protein DNA interactions for a few milliseconds after mixing (Fig. 4).

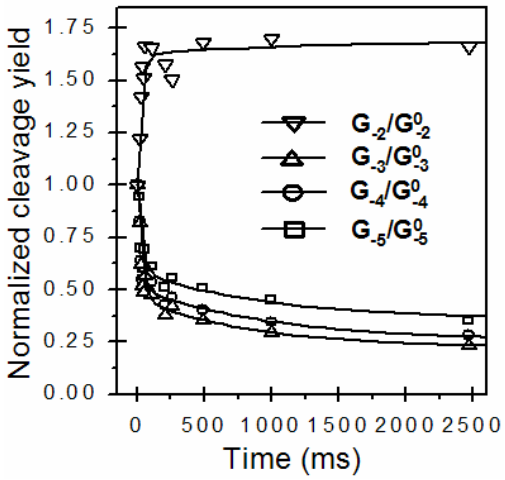

Fig. 4. Biphotonic UV laser dynamic photo footprinting of the NF-κB – DNA complex. Uniquely 5'-end labelled 37 bp DNA (the bottom strand), containing the NF-κB binding site, was submitted to a single UV laser pulse (E=0.1 J/cm2) either naked or in a complex with NF-κB at the times after fast (10 ms) mixing as indicated. DNA was extracted, treated with the Fpg protein and analyzed by sequencing gel electrophoresis. Normalized to the naked DNA cleavage yield at guanines as a function of the delay time of the laser pulse after fast mixing (dead

time 10 ms) of NF-κB with DNA. Data were fitted by a bi- exponential (decay for G_{-3}–G_{-5}, or grow G_{-2}) function: $Y= A_1exp(\pm t/t_1) + A_2exp(\pm t/t_2) + y_0$, with best fitting parameters: G_{-3}–G_{-5}: A_1≈65-70%, t_1≈25-30 ms, A_2≈35-30%, t_2≈1s ; G_{-2}: A_1≈95%, t_1≈25 ms, t_2≈1 s.

NF-κB first binds with a diffusion controlled rate (~30 ms) followed by slow (1s) local DNA rearrangements. Interestingly, this process of slow rearrangement is absent for $G_{\pm 2}$, located close to the centre of the recognition sequence, that might be due to the different protein domains involved in respective interactions.

This demonstrates directly the great difference in the times required for proper adaptation and folding of different domains within the complex, and represents an experimental basis for further molecular dynamics modelisations. Finally, this peculiarity of the DNA recognition can provide some rationale on the nucleosome "transparency" for the specific NF-κB binding [7, 8]. However, further experiments within nucleosomal templates aimed at shedding more light on this important question are under way.

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