Single-Molecule Conformational Dynamics of Fluctuating Noncovalent DNA−Protein Interactions in DNA Damage Recognition

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Single-molecule spectroscopy is a powerful methodology[1−4] that provides new information unobtainable by conventional ensemble-averaged experiments due to intrinsic sample heterogeneities.[5−7] Here we report a single-molecule spectroscopy study that reveals dynamics of fluctuating molecular noncovalent interactions within single DNA-protein complexes. These data will facilitate a molecule-level understanding for the dynamics and mechanisms of the damage-recognition process in DNA repair.[8]

Xeroderma Pigmentosum group-A protein (XPA) is involved in recognizing DNA lesions.[8,9] Our experiments used recombinant, full-length *Xenopus* XPA[9] and a double-stranded (ds) 55-mer oligonucleotide with fluorescein covalently attached at the 5′-end. Fluorescein is a *bona fide* lesion for DNA repair,[9] and fluorescence quenching is observed upon XPA binding.[9] Single DNA-XPA complexes at nM concentration[10] were imbedded in 20-μm thick, 0.5% agarose gel, sandwiched between two glass coverslips,[4,10] and located with use of an inverted fluorescence confocal microscope[4] by raster-scanning the sample with focused diffraction-limited lasers at 442 or 488 nm.[4,11]

We observed fluorescence intensity fluctuations in single DNA-XPA complexes at two different time scales. Figure 1A shows a typical autocorrelation function, $C(t) = \langle \Delta I(t)\ \Delta I(0)\rangle / \langle \Delta I(0)^2 \rangle$, derived from a single-complex trajectory, $I(t)$. $C(t)$ shows a decay that can be fit to a double exponential with $A_f = 0.3$, $k_f = 45 \pm 3\ s^{-1}$, $A_s = 0.5$, and $k_s = 5 \pm 2\ s^{-1}$, where $C(t) = A_f \exp(-k_f t) + A_s \exp(-k_s t)$ for $t > 0$. To determine whether the fast (k_f) and slow (k_s) fluctuations are spontaneous or photoinduced,[11] we recorded single-molecule fluorescence intensity trajectories with excitation rates of 1×10^5 to 7×10^6 ct/s, and found that there was no measurable excitation intensity dependence of the autocorrelation decay rates. Since single DNA-XPA complexes were freely rotating at a much faster rate ($>100\ s^{-1}$)[2,12] than the observed fluctuation rates, the intensity fluctuations cannot be attributed to rotational and translational motions.[12] Possible DNA hydrodynamic bending motions for this oligonucleotide are most likely at the microsecond time scale.[2] Therefore, k_f and k_s rates are attributed to conformational fluctuations of two quasi-independent nuclear coordinates (or sets of collective nuclear coordinates) changing at different rates. Otherwise, there should be a single-exponential decay rate of $k_f + k_s$ if the nuclear coordinates are coupled, according to the fluctuation−dissipation theorem. Emission spectral trajectories

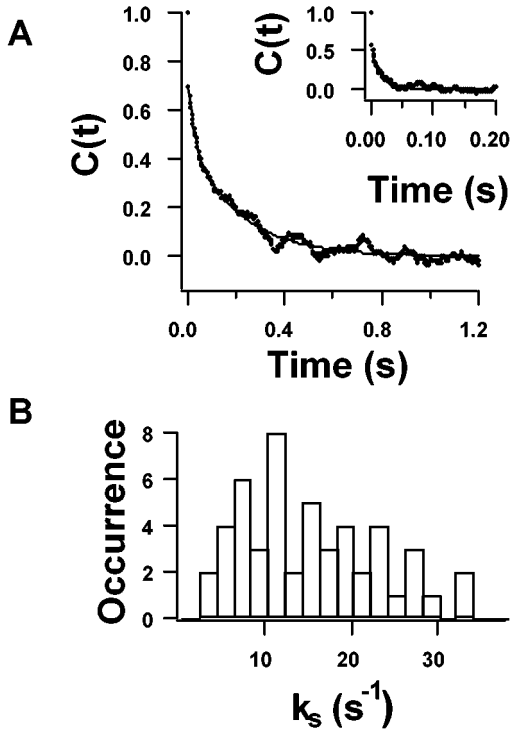

Figure 1. (A) The autocorrelation function, $C(t)$, calculated from a fluorescence intensity trajectory of a single DNA-XPA complex. Non-single exponential decay is evident (the noise in the $C(t)$ is mainly due to the finite length of the single-molecule trajectories[4,11]). The solid line is a biexponential fit with fast and slow decay rates of 45 ± 3 and $5 \pm 2\ s^{-1}$, respectively. Inset: The autocorrelation function calculated from a fluorescence intensity trajectory of a DNA molecule. The solid line is an exponential fit with decay rate of $65 \pm 5\ s^{-1}$. (B) Distribution of the slow DNA-XPA binding−unbinding motion rates for 50 individual complexes. A 10-fold variation of the XPA binding−unbinding motion rate is observed.

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(9) (a) Iakoucheva, L. M.; et al. *Protein Sci.* **2001**, *10*, 560. (b) Fluorescence anisotropy titrations, fluorescence intensity titrations, and stop-flow fluorescence measurements concluded that the XPA forms 1:1 complexes with DNA; binding rate constant of $(9.0 \pm 0.3) \times 10^8\ M^{-1}\ s^{-1}$ and dissociation equilibrium constant of 24 ± 3 nM. (Iakoucheva, L. M.; et al. Submitted for publication.)

(10) DNA-XPA complexes were prepared by mixing 10 nM fluorescein-labeled DNA and 20 to 50 nM XPA in 50 mM Tris-acetate (pH 7.5), 100 mM potassium acetate, 10% glycerol, 1 mM EDTA, and 5 mM DTT, at room temperature. The DNA control (absence of XPA) was prepared in the same buffer.

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(12) The interaction between single DNA-XPA complexes and the agarose gel matrix was examined by polarization modulation (0.1 to 100 Hz). Fluorescence intensity change is evident in a trajectory recorded from a single DNA-XPA complex in a dry agarose gel matrix, while there is no intensity modulation in 0.5% agarose. Single complexes in agarose gel freely rotate at a faster rate than the modulation rate of the excitation light. The translational motion of individual DNA-XPA complexes does not extend beyond the laser diffraction limited focus spot.

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collected from the single complexes did not show significant spectral fluctuation, which is consistent with the ensemble-averaged results.[13]

To identify the conformational change coordinates, we measured the fluorescence intensity trajectories from single DNA molecules[10] in the absence of XPA, and only the fast fluctuations were observed (Figure 1A, inset). They can be due either to intrinsic conformational changes at the fluorescent site including the motion of the alky chain connecting the label to the DNA or to the interaction between fluorescein and DNA bases.[2,6,7] After addition of XPA,[10] more than 70% of the trajectories gave biexponential autocorrelation functions with additional slow decays. We thus attribute the fast conformational change coordinates to be associated with DNA. The slow conformational coordinates are most likely associated with interactive motions of the DNA-XPA complex at the binding site. Our rate calculations[14] show that slow fluctuations are not due to dissociation of the single complexes because (1) dissociation would be a rare event, (2) the subsequent reassociation would occur in less than a microsecond, and (3) conformational dynamics showed no change upon variation of agarose concentration (0.2−2.5%).[14] The slow intensity fluctuation amplitude change is comparable to that of the bound and unbound states of XPA-DNA observed by conventional spectrofluorimetry.[9] In single-molecule experiments, fluorescence fluctuations reflect conformational changes between the bound and loosely bound states while the overall complex is still associated. We postulate that the loosely bound states are a subset of conformations with deviated nuclear displacements from equilibrium. These states partially restore the local environment of the fluorescein, but do not completely lose the subnanometer long-range interactions[15,16] between XPA and DNA. Slow and large-amplitude conformational motions by DNA-XPA complexes have not been reported previously and this finding is consistent with XPA's ability to recognize a wide variety of DNA lesions.[8] To summarize, we attribute the fast fluctuations to conformational changes in DNA and the slow fluctuations to the DNA-XPA interactive motions. Anisotropy measurements were unable to distinguish whether the slow interactive motions of XPA are clamping or sliding. Identifying exact conformational nuclear coordinates awaits further studies, perhaps eventually by single-molecule Raman spectroscopy.[3]

Approximately 10-fold variation occurs in the rates of the slow interactive conformational motions among the individual complexes (Figure 1B).[17] This inhomogeniety is most likely associated with the existence of different subsets of protein conformations seeking an induced-fit to a conformationaly fluctuating DNA lesion. This is consistent with XPA having a highly disordered tertiary structure that can adopt inhomogeneous conformations in solution.[9] The inhomogeneous rates of slow spontaneous interactive motions are presumably determined by the rugged free-energy landscape[16,18] of DNA-XPA interactions involving inhomogeneous conformations, which is extremely difficult to directly measure by ensemble-averaged experiments at room temperature.

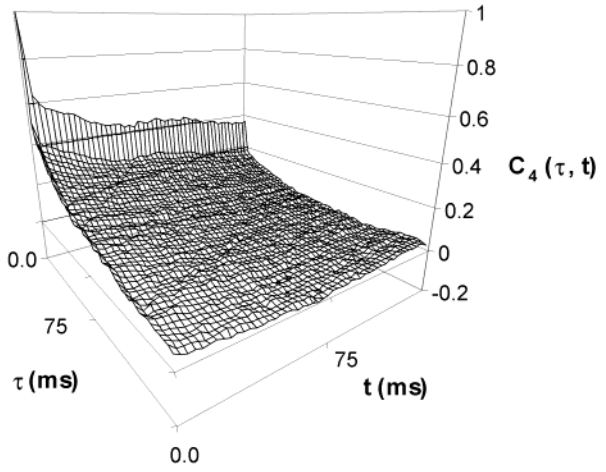

Figure 2. Fourth-order correlation function, $C_4(\tau,t)$, calculated from the same single-molecule fluorescence intensity trajectory as in Figure 1A. The decay along both the τ-axes and t-axes is evident.

The fluctuation dynamics of DNA-XPA interactions was further evaluated by calculating the 4th order correlation functions of the single-molecule trajectories, $C_4(\tau,t) = \langle \Delta I(0)\Delta I(\tau)\Delta I(t+\tau)\Delta I(t+2\tau)\rangle / \langle \Delta I^4 \rangle$. Figure 2 shows that decay is evident in both the τ and t axes. Dependence of $C_4(\tau,t)$ along the t axis for ~70% of the single XPA-DNA complexes was observed. This suggests the interaction dynamics may be essentially fit to a two-state model[4] assuming the single-complex intensity fluctuations reflect the state population[4] changes. We cannot, however, rule out the possible memory effect,[4,18] i.e., multiple states associated with rate fluctuations, because $C_4(\tau,t)$ dependence on t is apparently nonexponential. More sophisticated models involving the conformational dimensionality reduction,[16,19] fly casting driven by induced-fit,[16,20] protein interior packing relaxation,[20,21] and hydrogen bond formations[15] are likely to provide insights for the fluctuating interactions that could be crucial in DNA-XPA interaction dynamics and intrinsically pertinent to the DNA-protein complexes in other DNA damage recognition processes.[22]

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(13) The emission spectrum does not show significant shift but the emission quantum efficiency changes upon DNA-XPA complex formation. If any single-molecule spectral fluctuation occurs, then it is at a time scale shorter than the measurement resolution.

(14) Calculation based on evaluating the equilibrium constant and the rate constant of complex formation[9] and the free space in agarose gel.[12] We have obtained the SEM images of the agarose gel using super-critical point sample preparation at different concentrations of agarose gel from 0.2% to 2%. The pore sizes that may confine DNA-XPA complexes are on the averaged 50 to 300 nm in diameter.

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(22) This hypothesis is currently being evaluated by using different types of DNA with site-specific lesions (lesion in the middle of ds and plasmid DNAs) and XPA mutants with targeted modifications.