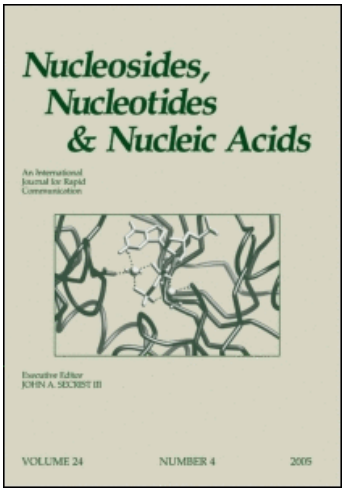

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EXONUCLEASE DEGRADATION OF DNA STUDIED BY FLUORESCENCE CORRELATION SPECTROSCOPY

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ABSTRACT. Here we developed an accurate method for kinetic analysis of enzymatic degradation processes of double and/or single-stranded DNA/oligonucleotides using fluorescent reporter dyes. 217-bp DNA fragments were produced by polymerase chain reaction and cleaved by the 3' to 5' exonuclease activity of T7-DNA polymerase. The analysis of the products was performed by Fluorescence Correlation Spectroscopy measuring autocorrelation amplitudes and diffusion times. We give proof of: (i) complete enzymatic degradation, (ii) retardation of complete enzymatic degradation by internally labelled Rhodamine-4-nucleotides and Cy5-nucleotides, respectively. Data evaluation by global analysis indicated first-order reaction kinetics with full-length DNA and free fluorescent nucleotides in the time window of measurements used.

INTRODUCTION

One of the advanced technological developments of the last 20 years has been the detection of a single molecule by laser-induced fluorescence correlation spectroscopy (FCS). This methodology employs extremely small open volume elements (10^{-15} l and below) combined with confocal epi-illumination, therefore the signal-to-noise ratio could be increased by 10^6 [1-4]. Now FCS allows the analysis of molecular interactions and kinetics at the level of single molecules. We have applied FCS to study the enzymatic degradation of DNA by the 3' to 5' exonuclease activity of T7-DNA polymerase (wild type) in the absence and presence of fluorescent dyes proposed for single-molecule DNA sequencing. Rapid DNA sequencing [5,6] is based on the idea of complete enzymatic degradation. For the first time we give proof of complete degradation of internally labelled fluorescence nucleotides. Our method developed here for studying enzymatic degradation is of general interest because we gain access to molecular parameters like the turnover number of enzyme molecules.

EXPERIMENTAL AND THEORETICAL METHODS

PCR. PCR was performed in 60 µl with 22 mM Tris-Cl (pH 8.3 at 25°C), 42 mM KCl, 1.7 mM $MgCl_2$, 166.7 µM dNTPs, 2 x 2.5 pmole primer (5'-dAA AGG GGG ATG TGC TGC AAG GCG, 5'-dGC TTC CGG CTC GTA TGT TGT GTG), 0.1 ng

M13mp18$^+$ DNA (Pharmacia), with and without 8 x 10^{-5} % (v/v) Tween^{R}20, 5 U Taq DNA polymerase (Pharmacia). The 217-bp model gene was amplified using primers with and without fluorogenic reporter dye Cy5 (or Tetramethyl-Rhodamine or Rhodamine-Green) in the presence and absence of 30 μM Rhodamine-4-dUTP, or 30 μM Cy5-dUTP and 30 μM Cy5-dCTP (Amersham). Thermocycling was done in the GeneAmp^R PCR System 2400 from Perkin Elmer: 95°C/3 min; 25 cycles with 96°C/0.5 min, 65°C/1 min, 72°C/2 min, held at 4°C until the PCR product was used or frozen at -20°C theoretically giving 0.72 μg DNA. Up to now there are no other biochemical methods available for a quantitative and specific preparation of fluorescently labelled DNA that are compatible with the detection process in FCS [7]. After PCR the excess of labelled primers and dNTPs was removed by MicroSpin^{TM} S-200 HR Columns, Pharmacia Biotech, Sweden.

Degradation by T7-DNA polymerase. The T7-DNA polymerase (wild type) was prepared as previously described [8]. The exonucleolytic degradation was done under the experimental conditions found here to provide full 3' to 5' exonuclease activity within the time window of analysis. For example, 250 nM enzyme (stored at -80°C) was preincubated with 330 μM DTT and 12.5 μM E.coli thioredoxin in 100 mM Tris-Cl (pH 7.5 at 25°C). The degradation assay contained in 25 μl: 100 mM Tris-Cl (pH 7.5 at 25°C), 10 mM $MgCl_2$, 2.5 mM DTT, 2.5 μl PCR product after spinning down, 10 nM enzyme for kinetic study and 200 nM enzyme for detemination of the number of incorporated labels, respectively. The kinetic experiments were done at 16°C.

FCS. Most of the measurements were carried out with the Fluorescence-Correlation-Spectrometer Axiovert 135 TV (prototype) from Zeiss, Germany, at 633 nm (absorption) and 685 nm (emission), or 488 nm (absorption) and 540 nm (emission). We measured the fluorescence intensity, the correlation time and the number of molecules in the sample. For the kinetic experiments a new measurement started at each 25 s and had a duration of 15 s. Data evaluation was performed with a 3 component model (Eqn. (1)) for the normalized theoretical autocorrelation function

$$G(t) = \frac{1}{N}\left[\frac{1-y-z}{\left(1+t/\tau_x\right)\sqrt{1+t/\tau_x\left(\frac{\omega_1}{\omega_2}\right)^2}} + \frac{y}{\left(1+t/\tau_y\right)\sqrt{1+t/\tau_y\left(\frac{\omega_1}{\omega_2}\right)^2}} + \frac{z}{\left(1+t/\tau_z\right)\sqrt{1+t/\tau_z\left(\frac{\omega_1}{\omega_2}\right)^2}}\right] + D_C \quad , \qquad (1)$$

where in global analysis individual parameters were y , z , N , D_C and global parameters were τ_x , τ_y , τ_z , ω_1/ω_2. N = number of particles, x = fraction of free nucleotides, y = for example, fraction of primers or oligomeric species, z = fraction of full-length DNA, $\tau_{x,y,z}$ = diffusion times for the free nucleotides, primers, full-length DNA, ω_1/ω_2 = ratio of the dimensions in the volume element of the laser beam, D_C = background constant for the autocorrelation function. The individual parameters were specified for each measurement. The data evaluation was carried out using the Marquardt nonlinear least squares minimization [9] and vectorized maximum likelihood estimates (global analysis).

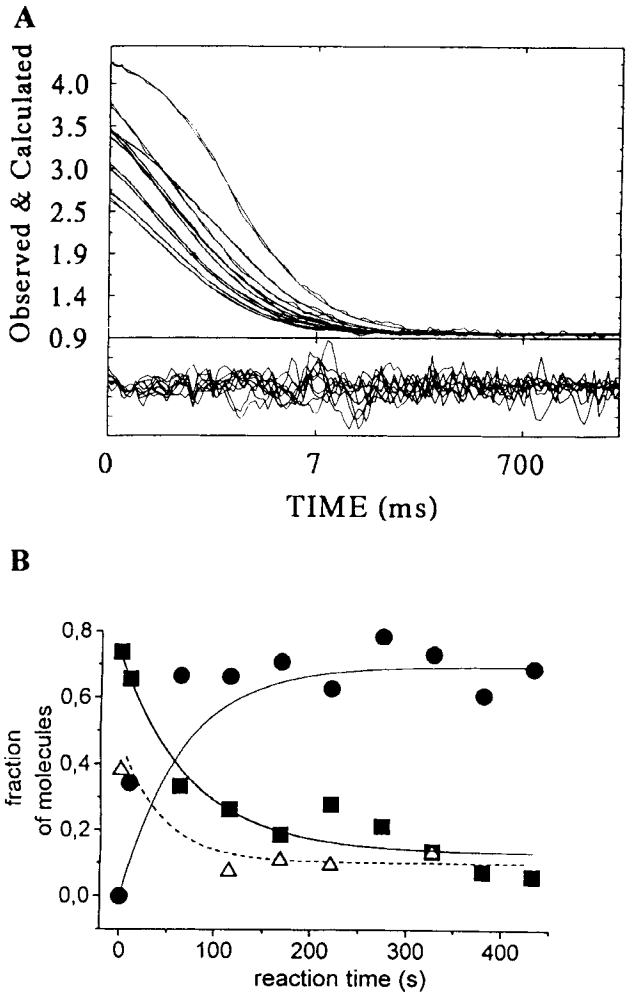

Fig. 1. Degradation of 5-primed Cy5-reporter dye dsDNA.

A: Measured and calculated autocorrelation curves. One run of global analysis including 10 measurements is shown. The lower part shows the difference between the measured and the calculated autocorrelation function.

B: Calculated fraction of full-length dsDNA (217-bp) as function of reaction time (solid square). The first-order reaction rate constant of complete degradation is $k = 5.7 \times 10^{-3}\ s^{-1}$. Solid circle: free Cy5-reporter nucleotide. Open up-triangle: labelled primer.

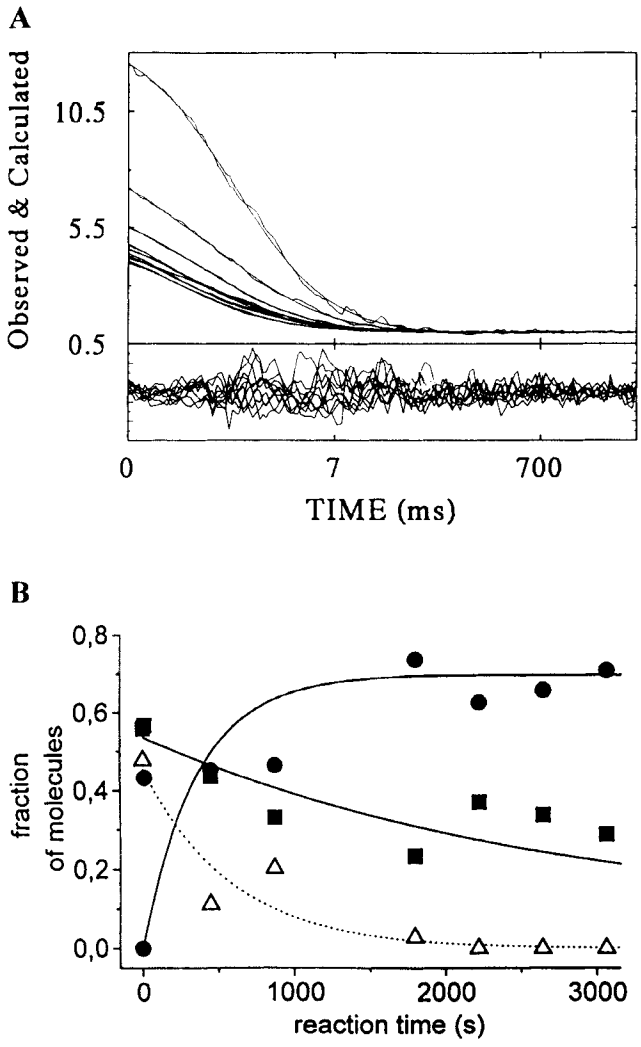

Fig. 2. Degradation of 5-primed Cy5-reporter dye dsDNA polymerized in the presence of Rho-4-dUTP.

A: Measured and calculated autocorrelation curves. One run of global analysis including 10 measurements is shown. The lower part shows the difference between the measured and the calculated autocorrelation function.

B: Calculated fraction of full-length dsDNA (217-bp) as function of reaction time (solid square). The first-order reaction rate constant of complete degradation is $k = 3.3 \times 10^{-4}\ s^{-1}$. Solid circle: free Cy5-reporter nucleotide. Open up-triangle: labelled primer.

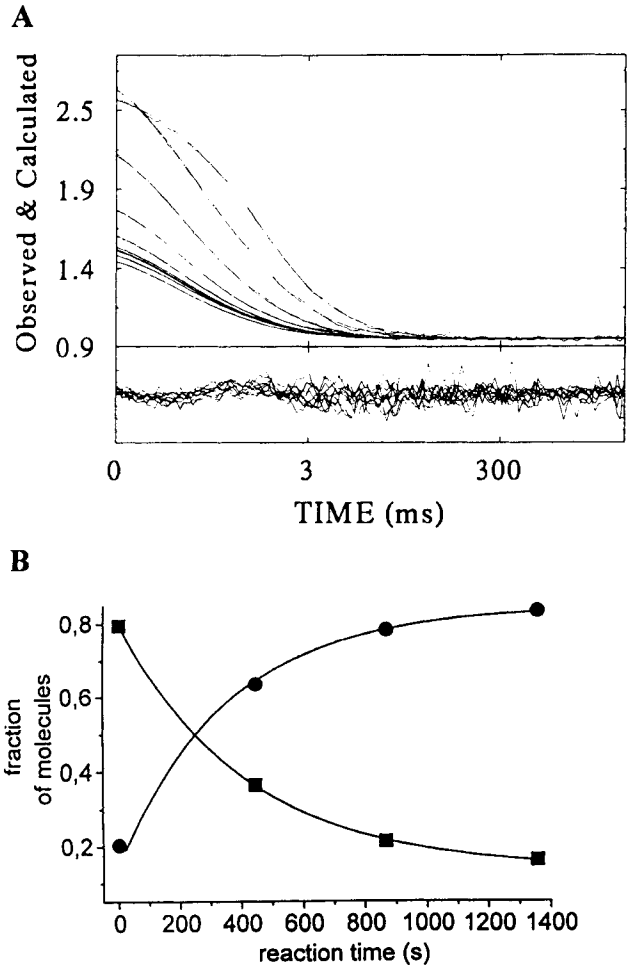

Fig. 3. Degradation of internally Cy5-dUTP and Cy5-dCTP labelled dsDNA.

A: Measured and calculated autocorrelation curves. One run of global analysis including 10 measurements is shown. The lower part shows the difference between the measured and the calculated autocorrelation function.

B: Calculated fraction of full-length dsDNA (217-bp) as function of reaction time (solid square). The first-order reaction rate constant of complete degradation is $k = 1.2 \times 10^{-3}\ s^{-1}$. Solid circle: free Cy5- nucleotides.

RESULTS AND DISCUSSION

Figs. 1 and 2 give proof of complete enzymatic degradation of DNA. As a reporter system 5-primed Cy5 was used and incorporated into the upper DNA strand by 5'-labelled reverse primer via PCR. At high enzyme concentration (nM range) full exonucleolytic activity is provided during the time window of measurements taken for the analysis. As model substrate we use here double-stranded DNA to follow the degradation; this is a first approximation to the exonucleolytic situation of rapid DNA sequencing. The concentration of full-length product as well as of Cy5-nucleotide release could be observed by FCS (λ_{ex} = 633 nm) in the absence (Fig.1) and presence (Fig.2) of incorporated Rhodamine-4-dUTP.

The data files for global analyses of the kinetic breakdown were organized in a time frame regime covering 1h period of observation. We found that the model Eqn. (1) evaluated by global analysis describes well the diffusion times of full-length polymer and cleaved nucleotides. Only random differences between these mean values could be detected. Next, we checked whether the mean values of the calculated diffusion times depend on the time frame of data organization for running global analysis. From the biochemical viewpoint the observation of oligomeric reaction intermediates would result in a monotoneous decrease in diffusion time within the time course of measurements. We used global analysis for the first 10 and last 10 measurements. All of the analyses indicated constant diffusion times. We conclude that under the experimental conditions of enzymatic degradation we have full-length product and cleaved nucleotides from complete degradation. No intermediate oligomeric reaction species of the degradation process were found. This is consistent with a first-order overall reaction scheme. An outcome of the global analysis is shown in Fig. 1A and Fig. 2A. There is a very good correspondence between measured and calculated autocorrelation curves. From Fig. 1B and Fig. 2B we estimated a decrease of first-order reaction rate constant by factor of about 17 in the presence of incorporated Rhodamine-4-dUTP. The retardation of the complete degradation is due to steric hindrance by the internal dye. From the experimental and theoretical analysis shown in Fig. 3 it is evident that Cy5-nucleotides are also completely released when the DNA was internally labelled. The first-order reaction rate constant for the breakdown of the DNA is slower than that for degradation of internally unlabelled DNA (see Fig. 1B) but higher than that for degradation of internally rhodamine-labelled DNA (see Fig. 2B). An explanation for the different kinetic behaviour of the internally labelled Cy5-dye DNA is that the total number of incorporated Cy5-nucleotides was much less (2 nucleotides) than the total number of incorporated Rhodamine-4-nucleotides (11 nucleotides).

From the data in the literature we estimated K_M for the DNA substrate of the 3′ to 5′ exonuclease activity of T7-DNA polymerase to be about 4×10^{-6} M. The estimation of the catalytic cleavage rate constant k^*_C [10] is given for the studied 5-primed Cy5-dye DNA (Fig.1), 5-primed Cy5-dye DNA with incorporated Rho-4-dUTP (Fig. 2), and DNA with incorporated Cy5-dUTP and Cy5-dCTP (Fig. 3) as follows: $k^*_{C1} = 480\ s^{-1}$, $k^*_{C2} = 28\ s^{-1}$ and $k^*_{C3} = 100\ s^{-1}$, respectively. Knowing the average cleavage rates of internally labelled DNA (k^*_{C2} and k^*_{C3}) and of internally unlabelled DNA (k^*_{C1}) the intrinsic cutting rate of the labelled nucleotide can be calculated. We obtained for Rho-4 and for Cy5 at 16°C 1.5 s^{-1} and 1.3 s^{-1}, respectively. For DNA sequencing the intrinsic cutting rate of the labelled nucleotide can be increased by increasing the temperature.

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