Real time kinetics of restriction endonuclease cleavage monitored by fluorescence resonance energy transfer

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ABSTRACT

The kinetics of *Pae*R7 endonuclease-catalysed cleavage reactions of fluorophor-labeled oligonucleotide substrates have been examined using fluorescence resonance energy transfer (FRET). A series of duplex substrates were synthesized with an internal CTCGAG *Pae*R7 recognition site and donor (fluorescein) and acceptor (rhodamine) dyes conjugated to the opposing 5' termini. The time-dependent increase in donor fluorescence resulting from restriction cleavage of these substrates was continuously monitored and the initial rate data was fitted to the Michaelis – Menten equation. The steady state kinetic parameters for these substrates were in agreement with the rate constants obtained from a gel electrophoresis-based fixed time point assay using radiolabeled substrates. The FRET method provides a rapid continuous assay as well as high sensitivity and reproducibility. These features should make the technique useful for the study of DNA-cleaving enzymes.

INTRODUCTION

Restriction endonucleases are essential enzymes in recombinant DNA procedures for the molecular cloning and analysis of nucleic acids (1–3). In addition, these enzymes have been used extensively for finding restriction fragment length polymorphisms (RFLPs) (4) in allelic genomic regions. The discovery of RFLPs as genetic markers has been immensely useful in genetic linkage analysis, determination of patterns of inheritance for genetic diseases and mapping of genes to specific chromosomal loci.

Traditional methods for assaying enzymatic activity of restriction endonucleases have relied on gel electrophoretic analysis of cleaved macromolecular DNA substrates (e.g. plasmids) (2,5). Synthetic oligonucleotides have also found application in a number of restriction endonuclease kinetic studies, in which product formation has been monitored by gel electrophoresis, filter binding, homochromatography or HPLC (6–8). All these methods share the feature of being discontinuous and entail sample handling at fixed time points followed by a separation step for resolution of the reaction mixture. Often,

radiolabeling of the substrates is required to afford the sensitivity necessary for detecting strand scission at low substrate concentrations. A continuous spectroscopic assay for endonucleases has been reported, and is based on the hyperchromic effect resulting from turnover of a duplex oligonucleotide substrate to single-stranded DNA products (9). Though this technique circumvents the major disadvantages of the earlier methods, its scope is limited by its narrow dynamic range and range of substrate concentrations. Recently, a sensitive non-isotopic detection approach using an ELISA-based format has been described for monitoring DNA cleavage reactions (10). However, the method is discontinuous and requires extensive sample handling for the detection step.

A major focus of our research has been the application of fluorescence resonance energy transfer (FRET) techniques for studying molecular interactions involving nucleic acids. FRET is the transfer of electronic excitation energy from a fluorescent donor chromophore to an unexcited acceptor chromophore resulting from the dipole–dipole coupling of the donor and acceptor molecules. The transfer efficiency depends on the orientation of the donor and acceptor transition dipoles, the distance between the donor and acceptor molecules and the spectral properties of the donor and acceptor. FRET has been widely used to estimate intramolecular distances in biomolecules (11,12). In addition, the technique has found utility in enzymology for measuring hydrolytic activity of proteases (13–17). Early applications of FRET to nucleic acids have probed proximity relationships in tRNA and its solution conformation (18,19). The advances in automated oligonucleotide synthesis and the convenient site-specific labeling of synthetic oligonucleotide analogues with fluorophors (20–22) have spurred a renewed interest in FRET applications to nucleic acids (23). FRET has been used to examine the structure and conformational distributions of dye-labeled four-way DNA junctions (24–26), deduce helical geometry of double-stranded DNA in solution (27) and for measuring distances and distance distributions along the helix backbone in dye-linked DNA (28,29). Other FRET studies have investigated nucleic acid hybridization (30), DNA–protein complexes (31–33) and polymerase–substrate interactions (34).

In this report, we describe the application of FRET for studying

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enzyme-catalysed cleavage of double-stranded nucleic acids. Oligonucleotide duplexes have been synthesized containing donor (fluorescein) and acceptor (tetramethylrhodamine) chromophores attached to opposing 5' termini. The relatively close proximity of the donor and acceptor groups within the short oligonucleotide duplexes facilitated efficient energy transfer from the donor to acceptor, thereby reducing the fluorescence emission intensity of the donor moiety. Cleavage of the duplex molecules will separate the donor and acceptor dyes, leading to a recovery of the donor emission intensity to its intrinsic unquenched value. Consequently, the extent of cleavage is reflected in the emission intensity of the donor moiety, which will increase over time during the course of a cleavage reaction. This technique has been used to continuously monitor the *PaeR7* endonuclease-catalysed cleavage of fluorophor-labeled oligonucleotide substrates by spectrophotometric means, and the kinetic rate constants have been determined from initial rates. The utility of using FRET for investigating endonuclease action is demonstrated by a comparative study with the corresponding radiolabeled substrates, and parameters for FRET substrate design are discussed.

MATERIALS AND METHODS

PaeR7 endonuclease was purified to homogeneity as previously described (35) and was a gift from Dr J. Brooks of New England Biolabs. T_4 polynucleotide kinase (EC 2.7.1.78) was obtained from New England Biolabs, and bovine serum albumin fraction V (BSA) from Sigma. [γ - 32 P]ATP was purchased from Du Pont and rhodamine *N*-hydroxysuccinimide ester was purchased from Molecular Probes. Phosphoramidites and Aminolink 2TM reagent used in oligonucleotide synthesis were purchased from Applied Biosystems and acetonitrile was from Burdick and Jackson. All other chemicals were purchased from Aldrich. Radioactivity was measured by Cerenkov counting with a Beckman LS 7800 liquid scintillation counter. Measurement of absorbances for thermal melting curves of oligonucleotide duplexes were taken in a Beckman DU 7400 UV/Vis spectrometer. The spectrometer's thermostated cells were fitted with a cuvette temperature probe. Linear regression analysis of the data was carried out using the KaleidaGraph program (Abelbeck Software).

Oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer using standard phosphoramidite chemistry. The hexylamine-linked oligonucleotides were prepared by using the Aminolink 2TM reagent in the last coupling step of the automated synthesis. The tritylated and 5'-aminoethyl oligonucleotides were purified by reverse-phase HPLC as described elsewhere (36,37). The purified oligonucleotides migrated as single bands on a 15% denaturing polyacrylamide gel. Oligonucleotides were phosphorylated at their 5'-termini using T_4 polynucleotide kinase and [γ - 32 P]ATP (3).

Synthesis of fluorophor-labeled oligonucleotides

5'-Aminoethyl oligonucleotides (50 nmol) were treated with a 200-fold excess of fluorescein isothiocyanate isomer 1 (FITC) in 250 μ l of a buffer containing 80 mM NaHCO₃ and supplemented with 20% DMF (reaction buffer). The mixtures were incubated in the dark for 16 h at room temperature. The 14-mer derivative was separated from excess FITC by passage through a 10 ml Sephadex G-10 column using 0.1 M triethylammonium acetate (TEAC), pH 6.8, as eluant. Size

exclusion on G-25 Sephadex columns removed unreacted FITC from reaction mixtures containing the 16- and 19-base length oligonucleotides. The nucleic acid-containing fractions were pooled and concentrated *in vacuo*. The fluorescein-labeled oligonucleotides were purified by HPLC on a C18 reverse-phase column (4.6 $\times$ 250 mm) using a 11–35% acetonitrile gradient in 0.1 M TEAC over 25 min. Effluent was monitored simultaneously at 260 and 493 nm using a Waters 990 series photodiode array detector.

Rhodamine-oligonucleotide derivatives were prepared by reacting 5'-aminoethyl oligonucleotides (50 nmol) with a 20-fold excess of rhodamine succinimidyl ester in the dark for 16 h in 250 ml reaction buffer. Following Sephadex gel filtration, the conjugates were purified by reverse phase HPLC using a 14–28% acetonitrile gradient in 0.1 M TEAC over 40 min and by simultaneously monitoring the effluent at 260 and 558 nm.

Oligonucleotides and their fluorophor-labeled derivatives were quantitated on the basis of their absorbances and their extinction coefficients at 260 nm using ϵ values of 15,400, 7300, 11,300 and 8,800 M⁻¹ cm⁻¹ for the bases A, C, G and T, respectively (38).

Kinetic studies

Molarities of the *PaeR7* endonuclease are based on M_r 58,000 of the dimeric protein. Immediately prior to the kinetic assays, the enzyme stock solution was diluted with 50% glycerol in NANOpure H₂O (Barnstead) to give the requisite concentrations of the enzyme. Stock solutions of duplex substrates were prepared by adding an excess of one of the strands to ensure complete hybridization to its complementary strand. An earlier study (39) had shown that single-stranded oligonucleotides containing the *PaeR7* recognition sequence do not serve as substrates for the endonuclease. Thus, 32 P-labeled duplex substrates were prepared by annealing the radiolabeled strand with a 3.5-fold excess of unlabeled complementary strand in 0.1 M NaCl, 10 mM Tris, pH 8.3. The substrates were diluted in 10 mM Tris, pH 8.3, 1 mM EDTA prior to their addition to the reaction digests. The T_m s of the radiolabeled substrates in *PaeR7* endonuclease reaction buffer (see below) were determined by the method of Uhlenbeck *et al.* (40) and were as follows: 19 nt duplex (56°C); 16 nt duplex (52°C); 14 nt duplex (49°C). The various concentrations of fluorophor-labeled duplex substrates were prepared individually by adding a 2.5-fold excess of rhodamine-labeled strand from 10 or 20 μ M stock solutions to their complementary fluorescein-labeled oligonucleotide strand.

Fluorescence energy transfer-based cleavage assay

PaeR7 endonuclease enzyme assays were performed at 37°C in 10 mM Tris, pH 8.3, 6 mM MgCl₂, 10 mM β -mercaptoethanol, 0.1 mg/ml bovine serum albumin buffer (*PaeR7* assay buffer) and using 50 pM enzyme. Fluorescence measurements were performed in a quartz cuvette (0.5 $\times$ 1.0 cm) on a SLM SPF500C spectrofluorimeter with excitation at 480 nm and observation of emission at 518 nm. Substrate concentrations were 0.2–5.0 $\times$ approximate K_m , which was estimated from the initial velocities at two different substrate concentrations (41). For each substrate concentration, measurement of the fluorescein emission of the fluorescein-labeled strand before and after addition of the rhodamine-labeled strand provided the final and initial intensity values of the cleavage reaction (see Figure 2A). Cleavage of the substrate was followed by monitoring the increase in fluorescence

emission at 518 nm with time over ~20 min. Initial velocities of each reaction were determined from the slope of the intensity trace, and the values were used to calculate k_{cat} and K_m from Lineweaver–Burk plots.

Radioisotope-based cleavage assay

The fixed time point procedure described by Ghosh *et al.* (39) was used to follow the cleavage of ^{32}P -labeled substrates. The amount of ^{32}P -labeled duplex oligonucleotide was kept constant ($\sim 3\text{--}5 \times 10^5$ cpm) and was supplemented with unlabeled duplex oligonucleotide to give the desired substrate concentrations. The digests were carried out in a total volume of 30 μl in *PaeR7* assay buffer at 37°C and using 50 pM of the endonuclease. The reactions were quenched after 15 min by addition of 12 μl stop reagent (0.33M EDTA, 0.2% bromophenol blue, 27% sucrose). Aliquots of each reaction (7 μl) were

removed and the nucleic acids were resolved by electrophoresis on a 20% non-denaturing polyacrylamide gel. After autoradiography, the product and unreacted substrate bands were excised. The radioactivity associated with the bands were determined by Cerenkov counting and used to calculate the rates of the cleavage reaction. Less than 15% cleavage was observed for the various concentrations of the three substrates using the reaction conditions described above.

RESULTS AND DISCUSSION

Three dye-labeled oligonucleotide duplexes, which contained an internal CTCGAG *PaeR7* recognition site, were studied as substrates for *PaeR7* endonuclease using the FRET technique. The duplex substrates, 14, 16 and 19 nucleotides (nts) in length, were 5'-labeled with fluorescein and rhodamine on opposing strands

Figure 1. Substrates for the FRET study. The substrates were prepared by annealing an excess of the rhodamine-labeled (Rh) strands with their complementary fluorescein-derivatized (Fl) sequences (see Materials and Methods). The *PaeR7* endonuclease recognition sequence in the duplexes is highlighted and shown with the cleavage sites. The radiolabeled substrates were labeled with ^{32}P at the 5' termini of the oligonucleotides corresponding to the fluorescein-labeled strands.

Figure 2. Principle of the FRET endonuclease assay. (A) The emission spectrum of a 16 nt donor strand (60 nM, solid line) is quenched upon addition of 150 nM of its complementary rhodamine-labeled strand (dashed line); the fluorescence spectrum (dotted line) taken 2 h after addition of *PaeR7* endonuclease (50 pM final concentration) to the above mixture shows full recovery of initial emission intensity of the donor strand, indicating complete cleavage of duplex substrate. The peak at 580 nm in the emission spectra of duplex samples reflects rhodamine fluorescence. (B) Time course of a typical FRET assay: increase in fluorescence emission intensity at 514 nm resulting from cleavage of 16 nt fluorophore-labeled substrate (60 nM) with 50 pM *PaeR7* endonuclease.

(Figure 1). Initially, fluorescence measurements were carried out to establish conditions under which fluorescein-labeled oligonucleotide (donor) strands were completely annealed to their complementary rhodamine-derivatized DNA (acceptor) strands. The donor strands were titrated with increasing amounts of the acceptor strands while monitoring the fluorescence emission intensity of the donor dye. The extent of donor quenching reached a plateau value after approximately 1.5 molar equivalents of acceptor strand were added, indicating complete duplex formation. In the FRET study, each substrate was prepared by using an additional molar equivalent of the acceptor strand (2.5-fold excess) to ensure that the donor strand was entirely in duplex form for all substrate concentrations. Predictably, the extent of fluorescence quenching decreased with increasing DNA duplex size, reflecting a greater average distance between the donor and acceptor.

Cleavage of the dye-labeled substrates resulted in an increase in fluorescence emission intensity at 518 nm, and which over time recovered the initial intensity value of the donor strands (Figure 2A). Strand scission could be observed continuously over a wide range of substrate concentrations by monitoring the time-dependent increase in fluorescence emission intensity (Figure 2B). Saturation kinetics was observed with increasing concentrations of the substrates (Figure 3 inset). The initial velocities for a range

Figure 3. Lineweaver-Burk plot of *PaeR7* endonuclease cleavage of the 16 nt FRET substrate. The enzyme (50 pM) was incubated with 0.012–0.28 μ M substrate and the cleavage reaction was followed by monitoring the increase in fluorescence emission at 518 nm. Initial rates, determined from the first 10% of the cleavage reactions, were used to estimate the kinetic parameters using a non-linear least square fit of the data. Inset shows cleavage velocities as a function of increasing substrate concentration.

of substrate concentrations were fitted to the Michaelis-Menten equation. An excellent agreement with the rate equation was obtained for the three substrates, as shown by the representative Lineweaver-Burk plot for the 16 nt fluorophore-labeled substrate (Figure 3).

An analysis of the kinetics of interactions of the three duplexes with *PaeR7* endonuclease (Table 1) indicated that they behave very much like other endonuclease substrates of similar lengths. The k_{cat}/K_m values ($2.5\text{--}9.9 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$) were comparable to those reported for substrates of *EcoR1* (6,7) and for *PaeR7* substrates used in an earlier study (39).

A further validation of the applicability of the FRET method for studying DNA cleavage reactions was obtained from a comparative study using the corresponding ^{32}P -labeled substrates. The substrates for the radioisotopic assay were prepared by hybridizing 5' ^{32}P -labeled strands (corresponding to the fluorescein-derivatized sequences) with a 3.5-fold excess of their unmodified complementary oligonucleotides. The kinetics of *PaeR7* endonuclease-catalysed cleavage of the radiolabeled substrates were determined using a discrete time point assay (39). In this procedure, the extent of enzymatic cleavage of substrate after a 15 min enzymatic digest is estimated from the radioactivity associated with the product and unreacted substrate bands after resolution of the reaction mixture by gel electrophoresis. The 16 and 19 nt length FRET and radiolabeled substrates were cleaved with similar efficiencies by the endonuclease (Table 1). This suggests that the presence of dyes at the 5' termini of these FRET substrates appear to have no adverse effect on the interaction of the endonuclease with these duplexes and in catalysis. Minor differences in kinetic parameters obtained by the two methods probably reflect errors associated with the radioisotopic assay, which involves an indirect measurement of the cleavage reaction. Interestingly, the 14 nt FRET duplex was found to be the best substrate. The increased cleavage efficiency for this substrate was primarily due to a 4-fold decrease in its K_m value compared to its ^{32}P -labeled counterpart. We postulate that the dye molecules in this shorter substrate may be involved in energetically favourable hydrophobic interactions with the endonuclease, thereby resulting in tighter binding. Our results imply that the endonuclease's footprint may have to be considered as a criterion for the design of FRET substrates in order to render them equivalent to the traditional ^{32}P -labeled forms, and that the substrate size should be longer than the footprint to minimize dye-enzyme interactions.

The FRET method permits the cleavage reaction to be monitored continuously in real time, and offers high sensitivity and reproducibility. It requires little sample handling and can be carried out using conventional spectrofluorimeters. Importantly, the method can be used to investigate DNA cleaving reactions under a variety of reaction conditions and can be easily adapted to study rapid reaction kinetics using stopped flow techniques.

Table 1. Kinetic parameters for *PaeR7* endonuclease substrates based on FRET and ^{32}P assays

Substrate	Method	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}/\text{M}^{-1}$)
14-mer	FRET	43 ± 5	42.7 ± 1.7	9.9 ± 1.6
	^{32}P	166 ± 38	39.1 ± 1.6	2.4 ± 0.6
16-mer	FRET	75 ± 2	34.8 ± 2.9	4.6 ± 0.5
	^{32}P	74 ± 24	16.1 ± 2.5	2.3 ± 0.6
19-mer	FRET	72 ± 8	18.1 ± 0.7	2.5 ± 0.4
	^{32}P	101 ± 16	20.1 ± 2.6	2.0 ± 0.3

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