A real-time assay for monitoring nucleic acid cleavage by quadruplex formation

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ABSTRACT

Direct and straightforward methods to follow nucleic acid cleavage are needed. A spectrophotometric quadruplex formation assay (QFA) was developed, which allows real-time monitoring of site-specific cleavage of nucleic acids. QFA was applied to study both protein and nucleic acid restriction enzymes, and was demonstrated to accurately determine Michaelis–Menten parameters for the cleavage reaction catalyzed by EcoRI. QFA can be used to study the mechanisms of protein–nucleic acid recognition. QFA is also a useful tool for dissecting individual nicking rates of a double-stranded cleavage.

INTRODUCTION

Site-specific cleavage of DNA or RNA molecules has important applications in a variety of fields ranging from biotechnology to pharmacology (e.g. mapping and manipulation of genomes, targeting of specific genes or mRNA). In addition, restriction endonucleases (REases), which recognize and cleave DNA sequences with very high specificity, are traditionally used as model systems in mechanistic studies of DNA–protein interactions. Cleavage of DNA or RNA substrates is usually monitored using gel electrophoresis. In these studies the accumulation of the cleavage products from radio-labeled substrates are monitored as a function of time by quenching the reaction at the appropriate time, separation, visualization and quantification of the products. It is obvious that real-time monitoring of a cleavage reaction has many advantages over discontinuous electrophoresis assays (1–3). This is particularly true for the spectrophotometry which has prominent advantage of being simple and rapid to perform. Real-time spectroscopic assay, developed earlier (1), is based on the hyperchromicity of DNA oligonucleotides; upon cleavage the short DNA substrate falls apart, which induces an increase in optical density at ∼260 nm. However, since this assay requires dissociation of the DNA substrate, it is limited to short substrates (∼15 bp) and consequently to enzymes that cleave DNA within immediate vicinity of binding sites. In addition, spectrophotometric measurements based on changes at 260 nm are limited to solutions with optical densities <1, and therefore, this assay must be conducted within a narrow range of substrate concentrations limiting Michaelis–Menten analysis and mechanistic studies of DNA–protein recognition which is usually studied in the presence of non-specific DNA.

To overcome these problems, a real-time spectrophotometric method has been developed that is based on optical changes at 300 nm due to formation of a DNA quadruplex (4). A key feature of this quadruplex formation assay (QFA) is that some guanine-rich oligonucleotides are able to form quadruplexes with strong circular dichroism (CD) and optical density signals in long-wavelength range of UV region (4–6). For instance, DNA oligonucleotide (G₂T₂G₂TGTG₂T₂G₂), so called thrombin-binding aptamer (TBA) (7), folds into monomolecular quadruplex in the presence of K⁺, Sr²⁺, Ba²⁺ or Pb²⁺ (6,8). TBA incorporated into a double-stranded substrate does not absorb light at these wavelengths (4). Upon cleavage the released sequence folds into the quadruplex and serves as a reporter of the reaction.

MATERIALS AND METHODS

All oligonucleotides were purchased from Integrated DNA Technologies and desalted by dialysis against water at 4°C using dialysis tubing with a molecular weight cutoff of 500 Da. The concentrations of the oligonucleotides were determined as described earlier (4). EcoRI was purchased from New England Biolabs (Beverly, MA). The concentration of EcoRI (62 kDa dimer) was determined using Bio-Rad protein assay using BSA as a standard.

UV absorption experiments were conducted on a GBC 918 spectrophotometer equipped with thermoelectrically controlled cell holder. CD spectra were obtained with a JASCO J710 spectropolarimeter equipped with a water-jacketed cell holder.

EcoRI steady-state experiments were initiated by adding enzyme solution into the optical cell with substrate in the reaction buffer (100 mM NaCl, 10 mM MgCl₂, 10 mM Na-HEPES, pH 7.5, and 2 mM SrCl₂). Usually the reactions were monitored over 60 min and the rates were determined from the initial portion of the graphs. Single-turnover kinetics for 17E DNAzyme cleavage (2.6 μM substrate and 14 μM enzyme) in 100 mM NaCl, 10 mM Na-HEPES, pH 7.5, at
25°C was initiated by adding metal-cofactors, 10 mM Mg$^{2+}$ or 7 μM Pb$^{2+}$. In case of Mg$^{2+}$ the solution contained 2 mM Sr$^{2+}$ as a quadruplex forming agent. All kinetic measurements were performed in 1 cm optical cell of 250 μl volume.

RESULTS AND DISCUSSION

Principle of QFA

The reactions shown in Figure 1A were designed to test the principle of QFA using cleavage by EcoRI as a model system. As expected, the absorption difference spectrum (product minus substrate) of EcoRI cleavage in the absence of Sr$^{2+}$ (reaction 2) reveals only duplex unfolding with typical hyperchromicity at ~260 nm (Figure 1B, blue line). In the presence of Sr$^{2+}$ (reaction 1), we observe two maxima of similar intensity ~260 and 300 nm (Figure 1B, red line). The latter peak is due to quadruplex formation of TBA (Figure 1B, black line). Note that quadruplex formation does not affect the absorbance at 260 nm, and in the absence of quadruplex formation (no Sr$^{2+}$), digestion of the duplex and liberation of the single strands does not lead to a hyperchromicity at 300 nm. Thus, one can monitor cleavage by two simultaneous and separate assays, hyperchromicity (1) and QFA, with both methods showing the same time dependence (Figure 2) excluding impact of quadruplex folding on the cleavage kinetics.

The Michaelis–Menten parameters obtained by QFA for cleavage of the substrate shown in Figure 1A by EcoRI...
Figure 3. Michaelis–Menten plot for cleavage of the substrate (23–10000 nM) shown in Figure 1A by EcoRI (0.41 nM) in 100 mM NaCl, 10 mM MgCl₂, 2 mM SrCl₂ and 10 mM Na-HEPES, pH 7.5, at 37°C. Kinetics were monitored by optical density at 300 nm. The solid line is a fit of the experimental points to the Michaelis–Menten equation, which resulted in the kinetic parameters indicated on the plot.

Figure 4. CD spectra of ACTCACTATrAG₂T₂G₂TGTG₂T₂G₂ without (dashed line) and with (solid line) 2 mM SrCl₂ in 100 mM NaCl, 10 mM MgCl₂ and 10 mM Na-HEPES, pH 7.5, at 20°C.

(QFAs) are consistent with earlier studies using short oligonucleotides (9–11). We would like to emphasize that the highest substrate concentration used in the Michaelis–Menten analysis, 10 μM, is not the upper limit of the assay. QFA can accurately monitor cleavage at any higher concentration of substrate, since (i) optical density raises from near zero level, (ii) to determine the cleavage rate under steady-state conditions only initial part of the reaction is enough, and (iii) one can use quartz cells with a lower pathlength (1–2 mm or less) which would allow working at even higher concentrations.

QFA can be employed essentially for any REase

It is clear that QFA can be used for the enzymes that cleave outside (e.g. type IIB and IIS) or in the immediate vicinity of specific recognition sites (e.g. MaeIII or MboII). It is also a good tool for studying REases that can cleave within a particular recognition site and that can accommodate the terminal nucleotides of TBA (e.g. EcoRI shown in Figure 1A or Ball with recognition sequence TGG(CCA). For REases that do not meet these requirements, one can attach the TBA sequence directly to the 3’ or 5’ end of the target sequence. Upon cleavage of these substrates, the release of TBA with a few extra nucleotides at the end can still accurately monitor cleavage reactions, since short ‘tails’ do not interfere with quadruplex folding (12,13). We performed CD study of elongated version of TBA, ACTCACTATrAG₂T₂G₂TGTG₂T₂G₂ (Figure 4), which has a potential to form alternative hairpin structure with four perfect and one G·T wobbling base pairs (14). As predicted, in the absence of Sr²⁺ we observe conservative CD spectrum characteristic to B-DNA duplex (positive band at 275 nm and negative band at 245 nm) (Figure 4). The changes induced by Sr²⁺ are typical for quadruplex formation indicating that TBA even with 10 extra nucleotides can be used as a reporter of cleavage. Keeping in mind that upon REase cleavage a maximum of 3–4 extra nucleotides are expected, it is clear that QFA could be employed for any enzyme. In addition, to better design the recognition sites of enzymes under investigation, one can introduce certain modifications in TBA sequence without compromising its secondary structure (13) or use other quadruplex forming molecules [e.g. (G₃TTA)₃G₃ or (G₄T₄)₃G₄] (5) as the reporter molecules.

Mechanistic studies of protein–nucleic acid recognition

QFA can also be used to study the mechanism of target site location by restriction enzymes such as EcoRI. Figure 5 shows three different substrates containing either one (Sub1-2) or two (WT Sub1-2 and Mut Sub1-2) cleavage sites. After cleaving the first target of WT Sub1-2, EcoRI can either remain associated with the substrate and ‘slide’ to the next site, or dissociate and bind to the next site in a random manner (i.e. ‘hopping’ mechanism). Since, product dissociation is expected to be rate-limiting, and diffusion of the protein along DNA is fast (15), the sliding mechanism would predict that cleavage of recognition sites in WT Sub1-2 would be twice as fast as the rate of cleavage of a similar substrate with a single site (e.g. Sub1-2 in Figure 5) (16). However, our experiments reveal that the cleavage rate of both substrates is the same (Figure 5), suggesting that cleavage of the second site is independent of the first site. These data support the dissociation or ‘hopping’ of EcoRI between cleavage sites. Cleavage of Mut Sub1-2, which is identical to WT Sub1-2 but with one of the sites ‘silenced’ by a single G to T mutation was also investigated. The presence of an extra QFA-inactive recognition site is expected to result in a 2-fold decrease in the cleavage rate of site 2 in the case of a hopping mechanism, and has no effect in the
case of a sliding mode. The cleavage rate of Mut Sub1-2 is 2-fold slower than that of WT Sub1-2 (Figure 5), which again supports a hopping mechanism of EcoRI. This conclusion is in general agreement with earlier studies of EcoRI cleavage site recognition (16,17).

Cleavage by NA enzymes

To investigate whether QFA is suitable for studying the catalytic activity of nucleic acid enzymes, single-turnover kinetic assays of the 17E DNAzyme (18) were conducted (Figure 6). This enzyme cleaves DNA substrates containing one ribonucleotide located immediately upstream from a G>T mismatch. The reaction studied in the presence of two different metal-cofactors, Mg$^{2+}$ and Pb$^{2+}$, revealed good agreement with previous determinations (18).

Other applications

Although perfectly palindromic recognition sites are cleaved at equal rates in each strand, asymmetric modifications (e.g. 1 bp exchange within a site) result in cleavage processes with different nicking rates (19). Since the TBA sequence can be selectively ‘silenced’ (4), QFA is a perfect tool for dissecting individual nicking rates. Another advantage of QFA is that reactions may be studied over a broad range of concentrations and solution conditions (e.g. pH, temperature and ionic strength), since varying these parameters does not have significant effects on quadruplex formation.

CONCLUSION

A versatile, straightforward and accessible method for studying site-specific cleavage of DNA or RNA substrates has been developed. The potential to investigate mechanisms governing DNA–protein recognition by QFA has been demonstrated here. This method enables rapid characterization of a wide variety of protein and nucleic acid restriction enzymes.

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REFERENCES

1. Waters,T.R. and Connolly,B.A. (1992) Continuous spectrophotometric assay for restriction endonucleases using synthetic oligodeoxynucleotides and based on the hyperchromic effect. Anal. Biochem., 204, 204–209.
2. Kettling,U., Koltermann,A., Schwille,P. and Eigen,M. (1998) Real-time enzyme kinetics monitored by dual-color fluorescence cross-correlation spectroscopy. Proc. Natl Acad. Sci. USA, 95, 1416–1420.
3. Perona,J.J. (2002) Type II restriction endonucleases. Methods, 28, 353–364.
4. Kankia,B.I. (2004) Optical absorption assay for strand-exchange reactions in unlabeled nucleic acids. Nucleic Acids Res., 32, e154.
5. Mergny,J.L., Phan,A.T. and Lacroix,L. (1998) Following G-quartet formation by UV-spectroscopy. FEBS Lett., 435, 74–78.
6. Kankia,B.I. and Marky,L.A. (2001) Folding of the thrombin aptamer into a G-quadruplex with Sr(2$^{+}$): stability, heat, and hydration. J. Am. Chem. Soc., 123, 10799–10804.
7. Bock,L.C., Griffin,L.C., Latham,J.A., Vermaas,E.H. and Tooze,J.J. (1992) Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Nature, 355, 564–566.
8. Smirnov, I. and Shafer, R.H. (2000) Lead is unusually effective in sequence-specific folding of DNA. *J. Mol. Biol.*, **296**, 1–5.

9. Brennan, C.A., Van Cleve, M.D. and Gumport, R.I. (1986) The effects of base analogue substitutions on the cleavage by the EcoRI restriction endonuclease of octadeoxyribonucleotides containing modified EcoRI recognition sequences. *J. Biol. Chem.*, **261**, 7270–7278.

10. Nordlund, T.M., Andersson, S., Nilsson, L., Rigler, R., Graslund, A. and McLaughlin, L.W. (1989) Structure and dynamics of a fluorescent DNA oligomer containing the EcoRI recognition sequence: fluorescence, molecular dynamics, and NMR studies. *Biochemistry*, **28**, 9095–9103.

11. Jeltsch, A., Fritz, A., Alves, J., Wolfes, H. and Pingoud, A. (1993) A fast and accurate enzyme-linked immunosorbent assay for the determination of the DNA cleavage activity of restriction endonucleases. *Anal. Biochem.*, **213**, 234–240.

12. Ren, J., Qu, X., Trent, J.O. and Chaires, J.B. (2002) Tiny telomere DNA. *Nucleic Acids Res.*, **30**, 2307–2315.

13. Smirnov, I. and Shafer, R.H. (2000) Effect of loop sequence and size on DNA aptamer stability. *Biochemistry*, **39**, 1462–1468.

14. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.*, **31**, 3406–3415.

15. Erskine, S.G., Baldwin, G.S. and Halford, S.E. (1997) Rapid-reaction analysis of plasmid DNA cleavage by the EcoRV restriction endonuclease. *Biochemistry*, **36**, 7567–7576.

16. Wright, D.J., Jack, W.E. and Modrich, P. (1999) The kinetic mechanism of EcoRI endonuclease. *J. Biol. Chem.*, **274**, 31896–31902.

17. Langowski, J., Alves, J., Pingoud, A. and Maass, G. (1983) Does the specific recognition of DNA by the restriction endonuclease EcoRI involve a linear diffusion step? Investigation of the processivity of the EcoRI endonuclease. *Nucleic Acids Res.*, **11**, 501–513.

18. Brown, A.K., Li, J., Pavot, C.M. and Lu, Y. (2003) A lead-dependent DNAzyme with a two-step mechanism. *Biochemistry*, **42**, 7152–7161.

19. Lesser, D.R., Kurpiewski, M.R. and Jen-Jacobson, L. (1990) The energetic basis of specificity in the Eco RI endonuclease–DNA interaction. *Science*, **250**, 776–786.