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Fluorophore-Quencher Pair For Monitoring Protein Motion

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Abstract

A fluorophore/quencher pair capable of detecting conformational changes of DNA-protein complexes is described. The system employs a fluorescent nucleoside analog 1,3-diaza-2-oxophenothiazine (tC) within duplex DNA and a non-fluorescent quencher (TEMPO) attached to an engineered cysteine residue of the protein. The straightforward labeling methodology allows for the placement of the fluorophore and quencher moieties at specific positions suited to studying the conformational change of interest. To illustrate the utility of the tC-TEMPO pair, we have monitored nucleotide-induced conformational changes of the Klenow fragment (KF) polymerase bound to duplex DNA. In this system, tC was incorporated in the primer strand of the duplex, adjacent to the 3' end, while TEMPO was positioned at the end of the O-helix within the fingers domain of KF. Using steady-state fluorescence spectroscopy, we measured the quenching efficiency in a binary complex of tC-modified DNA and TEMPO-labeled KF and in ternary complexes containing cognate or non-cognate dNTP substrates. The quenching efficiency is significantly enhanced in the presence of a cognate dNTP, indicating that the O-helix has moved closer towards the DNA. In contrast, no significant tC quenching is observed in the presence of a non-cognate dNTP, indicating that the O-helix remains in a position that is beyond the distance reporting range of the tC-TEMPO pair. These results demonstrate that a cognate dNTP substrate induces a large conformational change of the O-helix, which can be sensitively detected using the tC-TEMPO pair. This fluorophore/quencher pair may be useful to study conformational changes associated with other DNA-enzyme complexes.

Keywords

DNA polymerase; Klenow fragment; fluorescent base analog; TEMPO quencher

Introduction

Fluorescent DNA base analogs provide a useful means to study the interactions of nucleic acids with proteins. A recent perspective of fluorescent DNA bases concludes that these analogs are better able than tethered fluorophores to provide a direct response to physical changes because of their defined location and orientation within a DNA duplex [1]. Previous experiments with a tricyclic cytosine analog, 1,3-diaza-2-oxophenothiazine (tC, Figure 1a) [2], have shown that it does not disturb base stacking, does not distort the DNA backbone [3], forms hydrogen bonds with G in the complementary strand [4] and its fluorescence is relatively insensitive to

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interaction with neighboring bases [5]. Hence, tC is a non-perturbing fluorescent probe that becomes an integral part of the surrounding DNA structure. Here we explore the possibility of using tC as a reporter of large scale conformational changes of DNA or a DNA-protein complex, wherein the fluorescence of tC is modulated in a distance-dependent fashion by a remote quenching moiety, such as the stable nitroxide radical TEMPO. TEMPO has been used in combination with dyes to measure the distance dependence of fluorescence quenching. As an example, TAMRA and TEMPO were attached to a series of oligonucleotide duplexes with varying distances between them. Deniz et. al. showed that TEMPO could be used to measure dye-quencher distances in the 10-30 Å range [6].

In this study, we use tC and TEMPO to establish a fluorophore-quencher system to detect conformational changes of a protein-DNA complex. We illustrate the method using the Klenow fragment (KF) of *E. coli* DNA polymerase I, a DNA-dependent DNA polymerase. In common with many other DNA polymerases, KF undergoes a significant conformational change during its nucleotide incorporation cycle. Thus, when a binary polymerase-DNA complex binds a cognate nucleotide substrate, it converts from an open inactive form to a closed active form. Crystal structures of the open and closed forms of ternary complexes of Klenoq, which is structurally similar to KF, show that the fingers domain is reoriented in the two forms, which significantly changes the positioning of the O-helix. In the closed form, the O-helix is significantly closer to the DNA than in the open complex [7].

To monitor this conformational change in solution, we incorporated tC within a duplex DNA substrate and attached TEMPO to an engineered cysteine residue at the end of the O-helix of KF. We measured the fluorescence intensity and quenching efficiency of tC in a binary DNA-KF complex and in ternary complexes containing either cognate or non-cognate dNTP substrates. The results establish the tC-TEMPO pair as a sensitive reporter of enzyme conformational changes specifically induced by a correct incoming nucleotide substrate.

Materials and Methods

The fluorescent tC and an oligonucleotide containing tC were synthesized according to literature procedure [5]. The 11-mer primer tC strand, which was dideoxy terminated to prevent incorporation of nucleosides, was annealed with a 17-mer oligonucleotide to form a primer/template duplex (Figure 1b). A plasmid encoding the D424A/C907S/S751C KF mutant, a mutant lacking the 3'-5' exonuclease activity, with its single cysteine removed and another cysteine introduced at position 751 within the O-helix, was provided by Dr. Catherine Joyce (Yale University). DNA sequencing of the complete KF gene confirmed the presence of the desired mutations. Expression and purification of the KF mutant was carried out as described [8].

TEMPO-labeled KF was prepared by coupling TEMPO maleimide to the cysteine at position 751. After reduction with DTT, KF was reacted with a 15-fold molar excess of TEMPO maleimide in 50 mM phosphate buffer, pH 7.0 containing approximately 5% DMSO. The excess TEMPO maleimide was removed on a Sephadex-G25 gel filtration column and the KF-751 TEMPO was purified by FPLC. The unlabeled and labeled KF-751 were well resolved by FPLC allowing for the collection of pure TEMPO labeled material. The results of a primer extension assay using an extendable primer/template duplex assure that the KF selectivity for the incoming cognate nucleotide is not compromised by the presence of TEMPO at position 751 or tC adjacent to the primer 3' terminus (Figure 2).

Steady-state fluorescence spectra of tC complexes were measured by exciting the tC at 393 nm and scanning the emission spectrum under magic angle conditions. Primer:template (PT) duplexes were formed in aqueous buffer (50 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 1 mM DTT,

5% (v/v) glycerol) with a 20% excess of the template strand. Binary complexes contained 500 nM PT and 1 μ M TEMPO-labeled KF. Ternary complexes contained, in addition, 250 μ M of cognate (dATP) or non-cognate (dTTP) nucleotide.

Results and Discussion

The emission spectrum of a binary complex of PT (500 nM) with KF-751-TEMPO (1 μ M), shown in Figure 3 (green), is the same as the PT duplex alone (red). This result indicates that in the binary complex, there is no significant quenching of the tC fluorescence by TEMPO. As predicted from the crystal structure of a binary complex of the homologous KlenTaq polymerase with DNA, the O-helix is likely rotated away from the tC base, keeping TEMPO too far away to exhibit any significant quenching. This result is also important because it shows that the intrinsic fluorescence of tC is insensitive to the presence and/or interaction with DNA polymerase. This lack of environmental sensitivity is in marked contrast to the fluorescent nucleotide analog 2-aminopurine (2-AP), which has been used extensively as a fluorescent probe of nucleic acid structural changes [9]. Unlike 2-AP, any changes in tC fluorescence observed in the present system can be attributed to a change in the fluorophore-quencher distance rather than a change in local environment.

When a KF binary complex binds a cognate nucleotide, the O-helix in the fingers domain is expected to move from an “open” (green, Figure 4) to a “closed” (blue) conformation. Position 751 of KF is at the outside hinge of the O-helix. Based on crystal structures of KlenTaq [9, 10], TEMPO will be brought significantly closer to the fluorescent tC in the closed complex as compared to the open complex and within the distance reporting range of TEMPO. Consistent with this expectation, we observe significant quenching of the tC emission when the cognate nucleotide (250 μ M dATP) is added to the binary complex of PT with TEMPO-labeled KF (Fig. 3, purple). By comparison with the spectrum of PT alone, tC is quenched by approximately 30 % in the presence of the cognate nucleotide. Note that the primer terminus is blocked with a 2',3'-dideoxy modification to prevent actual incorporation of the nucleotide.

In contrast, when a non-cognate nucleotide (250 μ M dTTP) is added to a binary complex of PT and TEMPO-751-KF, shown in Figure 3 (black), no significant tC quenching is observed. This result indicates that the O-helix does not rotate close enough to the tC to quench the fluorescence to any measurable extent under these conditions. Clearly, the structural rearrangement to form the closed ternary complex only occurs in the presence of the cognate nucleotide. Moreover, this conformational change is readily detected with the tC-TEMPO pair. While the distance dependence of fluorescence quenching has been established for the TAMRA-TEMPO pair [6], these results are not directly applicable to the tC fluorophore. Studies are now underway in our laboratories to correlate the degree of quenching of tC by TEMPO with the physical proximity of the fluorophore and quencher. The completion of those studies will establish a ruler to correlate tC quenching with distance changes of position 751 on KF.

In summary, we have described the use of tC and TEMPO as a fluorophore-quencher pair which can report on structural changes associated with binding of a cognate dNTP substrate by a DNA-polymerase complex. Steady-state fluorescence measurements have shown that the fluorescence of tC is unaffected by the formation of a binary DNA-KF complex or addition of a non-cognate dNTP. However, when cognate nucleotide is added to the binary complex, the tC fluorescence is reduced by approximately 30%. The change in fluorescence indicates the formation of a closed ternary complex in which the O-helix is closer to the tC than in the binary complex. The kinetics and dynamics of nucleotide selection and incorporation are areas of significant current interest [11-13]. The tC-TEMPO pair provides a sensitive spectroscopic

system to investigate the structural changes of the polymerase that occur during nucleotide selection and binding. More generally, the tC-TEMPO pair should be applicable to other DNA processing enzymes.

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Figure 1.

(a) 1 tC nucleoside. (b) Primer/template sequence (X = tC).

Figure 2.

Selective extension of primer/template complex with cognate dNTP. The assay was initiated by adding KF-751-TEMPO to a solution of primer/template duplex and dNTP so that the final concentration of KF was 45 nM, primer/template was 9 mM and 900 mM dNTP. The reaction buffer contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM DTT, and 10% (v/v) glycerol. The reaction was stopped after 30 minutes by quenching with 80% formamide containing 50 mM EDTA. The top band is the template strand and the bottom band is the primer strand. The difference in brightness is due to differential staining with SYBR gold (Invitrogen, Carlsbad, CA), based on strand length. The single base extension product is only observed in the presence of the cognate nucleotide dATP.

Figure 3. Fluorescence emission spectra (uncorrected) of primer/template (PT), binary complex (PT + KF-751-TEMPO) and ternary complexes containing cognate (PT + KF-751-TEMPO + dATP) and non-cognate (PT + KF-751-TEMPO + dTTP) dNTPs.

Figure 4.

Overlay of crystal structures of primer/template bound to KlenTaq in open binary (PDB accession code 2KTQ) and closed ternary complexes (3KTQ), emphasizing the movement of the 751 residue (numbered according to the KF sequence) in red (binary/green; ternary/blue; tC position/yellow)[7].