Sensitized photomodification of mammalian DNA polymerase β. A new approach for highly selective affinity labeling of polymerases

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Abstract To enhance the specificity of polymerase photoaffinity labeling, a novel approach based on sensitized photomodification has been developed. A base-substituted analog of TTP containing a pyrene group (PyrdUTP) was synthesized and used as an active site-bound photosensitizer for photoaffinity modification of DNA polymerase β (pol β). 5′-[^{32}P]-labeled primer was elongated in situ by pol β with a photoreactive analog of TTP (FAB-4-dUTP). The pyrene sensitizer (PyrdUTP), excited by light (365–450 nm), can activate the photoreagent, crosslinking it to pol β as a result of fluorescence resonance energy transfer. The initial rate of pol β photomodification was shown to increase by a factor of ten. The selectivity of pol β photosensitized modification was proved by adding human replication protein A.

Key words: Photoaffinity labeling; dNTP analog; Photosensitization

1. Introduction

Photoaffinity labeling is a useful technique for studying macromolecular interactions in biochemical systems. An advanced approach for increasing the efficiency and specificity of photoaffinity labeling is the highly effective technique of sensitized photomodification [1,2]. The sensitized photomodification of target DNA by a binary system of oligonucleotide reagents was described earlier [3,4]. One oligonucleotide, complementary to the DNA template, carried a photoreagent *p*-azidotetrafluorobenzamide, and the other carried a pyrene group as photosensitizer. Binding of the oligonucleotides to adjacent sequences in the target nucleic acid brings these groups in contact, which allows the initiation of crosslinking of the reagent to the target. The rate of sensitized photomodification under long wave UV light irradiation was 100- to 1500-fold higher than that of site-specific modification in the absence of the sensitizer [3]. Here we adapt this approach to carry out highly effective sensitized photomodification of DNA polymerases.

We have previously developed photoaffinity labeling of DNA polymerases by using the substrate activity of base-substituted dNTP analogs carrying a photoreactive group [5–10]. Photoreactive dNTP analogs were used as substrates for the elongation of 5′-[^{32}P]-labeled primer-template. Subsequent UV irradiation results in crosslinking the photoreactive primer to DNA polymerases [5–10]. This procedure has been used for photoaffinity labeling of RPA [10].

In the present study, FAB-4-dUTP (see Fig. 1) was used as a substrate for primer elongation by pol β. The photosensitizer was an analog of dUTP carrying a pyrene group at position 5 of the uridine ring (PyrdUTP, Fig. 1).

The main idea of the present approach is the following. A photoreactive primer, synthesized in situ with DNA polymerase, locates in the primer-template binding site of the enzyme and PyrdUTP binds to the dNTP binding site (Fig. 2A). During irradiation (365–450 nm), the energy initially absorbed by photosensitizer is then transferred to the arylazido group within the photoreagent-pol β-photosensitizer complex (Fig. 2B). This results in covalent attachment of the primer to the polymerase (Fig. 2C). The initial rate of sensitized photomodification is extremely sensitive to the distance between the sensitizer and the photoreagent because the efficiency of the fluorescence-resonance-energy transfer depends on the inverse sixth power of this distance [11]. Photodecomposition of the reagent in solution was not observed at the reagent and photosensitizer concentrations and irradiation time used. To test the specificity of pol β labeling sensitized by PyrdUTP, we added another DNA binding protein, RPA, as an alternative target for photoreactive DNA (Fig. 2D). The results are in favor of specificity of the DNA polymerase modification induced by PyrdUTP.

2. Materials and methods

1-Pyrenebutyric acid was from Fluka. FAB-4-dUTP was synthesized earlier [12]. 5-(amino-*trans*-propenyl-1)-2′-deoxyuridine-5′-triphosphate (I) was synthesized according to [13]. The *N*-hydroxysuccinimide ester of 1-pyrenebutyric acid (II) was obtained according to standard procedure [14].

^{31}P and ^{1}H NMR spectra were recorded on a Bruker AC-200 (200 132 MHz) spectrometer. ^{1}H and ^{31}P chemical shifts are reported relative to TMS and 85% H_3PO_4, respectively. UV absorption spectra were recorded on a Shimadzu UV-2100 spectrophotometer (Japan). Analytical TLC was carried out on silica gel plates (Merck, DC-Alufolien Kiselgel 60 F_{254}).

Synthesis of 5-[*N*-(4-(1-pyrenyl)-butylcarbonyl)-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate, tetralithium salt (PyrdUTP), was carried out from nucleotide I and ester II according to the procedure described for synthesis of 5-substituted dUTP derivatives [13]. The yield of PyrdUTP was 83%. R_f: 0.58 (dioxane/NH_4OH/water, 6:1:4 (v/v)). UV (H_2O) λ_{max}, nm (ε, M^{-1} cm^{-1}): 242 (57 800), 266 (28 300), 276 (44 000), 328 (24 700), 344 (31 500); λ_{min}, nm (ε, M^{-1} cm^{-1}): 215 (20 400), 258 (19 300), 269 (25 000), 292 (10 100), 318 (14 200), 335 (17 200). ^{31}P NMR (D_2O) δ: −19.93 (t, J = 20 Hz, P_β, 1P), −10.68 (d, J = 20 Hz, P_α, 1P), −6.92 (d, J = 20 Hz, P_γ, 1P). ^{1}H NMR (D_2O) δ: 2.40 (m, H10, H11, 4H), 2.45 (m, H2′, 2H), 3.51 (t,

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Abbreviations: pol β, mammalian DNA polymerase β; RPA, human replication protein A; FAB-4-dUTP, 5-[*N*-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate; PyrdUTP, 5-[*N*-(4-(1-pyrenyl)-butylcarbonyl)-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate

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J_{H11} = 7.5 Hz, H12, 2H), 4.05 (d, J_{H8} = 5 Hz, H9, 2H), 4.25 (m, H3′, H4′, H5′, 4H), 6.34 (m, H1′, H7, H8, 3H), 7.28 (s, H6, 1H), 8.00–8.45 (m, H (13–21), 9H).

Recombinant pol β was purified according to [15]. Synthetic DNA template 5′-GGTTCGATATCGTTGTTCCACTGTATAGCCCCT-ACC-3′ and primer 5′-GGTAGGGGCTATACAG-3′ were prepared using the LKB Gene Assembler. Dephosphorylated primer was 5′-phosphorylated as described [16].

2.1. Photoaffinity labeling experiments

Reaction mixtures (10 μl) contained 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 50 mM KCl, 1.4 μM pol β, 0.5 μM 5′-[^{32}P] primer-template, 1 μM FAB-4-dUTP. After complete primer elongation (25°C, 20 min), PyrdUTP or 1-pyrenebutyric acid was added to the reaction mixtures to a final concentration of 10 μM. When present, RPA was added to a final concentration of 0.5 μM. Photolysis was induced by light using a DRK-120 VIO-1 UV lamp ('LOMO', Saint Petersburg) at a distance of 110 mm; supplemented with glass filters FS-1 and BS-7 (365–450 nm) or WG-280 (> 280 nm). Reactions were stopped by adding Laemmli buffer and heating.

The photochemical crosslinked protein-DNA samples were separated by SDS-PAGE [17]. Dried gels were subjected to autoradiography.

3. Results and discussion

Synthesis of PyrdUTP was carried out according to the standard procedures described for synthesis of 5-substituted dUTP analogs [13]. The structure of the compound was proved by UV and NMR (^{1}H, ^{31}P) spectra.

Both dUTP analogs were shown to be substrates of pol β. K_m values were 0.1 μM for FAB-4-dUTP, 0.2 μM for PyrdUTP and 4.5 μM for TTP. Taking into account the K_m values for the analogs, we used 1 μM FAB-4-dUTP and 10 μM PyrdUTP in order to oust FAB-4-dUTP from the dNTP binding pocket of pol β after primer elongation.

When pol β elongates primer using TTP or FAB-4-dUTP as substrate (Fig. 3, lanes 2 and 3, respectively), only a single nucleotide moiety was introduced into the 3′-end of the 5′-[^{32}P]-labeled primer. We did not observe further elongation of the photoreactive primer with PyrdUTP after completing primer synthesis with FAB-4-dUTP (Fig. 3, lane 4). Photoaffinity labeling of pol β was therefore carried out using a photoreactive primer with a single *p*-azidotetrafluorobenzamide residue at the 3′-end.

The kinetics of photoreactive DNA substrate crosslinking to pol β are shown in Fig. 4. The initial rate of photomodification was 10-fold higher in the presence of PyrdUTP (curve III) than in the case of direct modification by photoreactive primer (curve I). In the presence of 1-pyrenebutyric acid (curve II) the initial rate of photomodification was 2-fold higher than in its absence (curve I). The increase in the photomodification rate in this case may be explained by the binding of 1-pyrenebutyric acid to hydrophobic domains of polymerases, followed by activation of the arylazido photoreagent

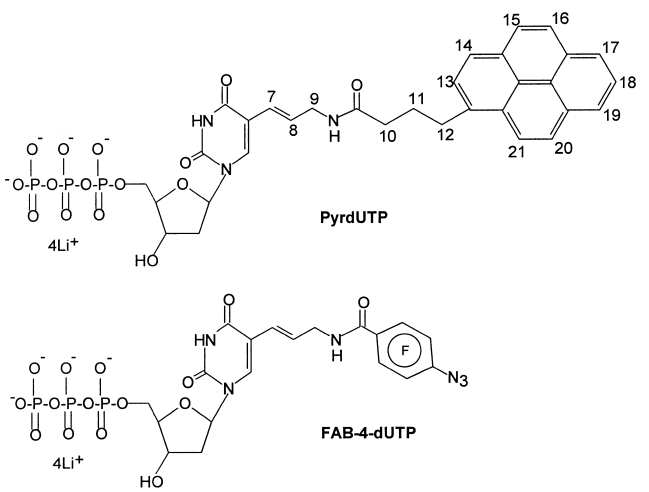

Fig. 1. Structural formulae of FAB-4-dUTP and PyrdUTP.

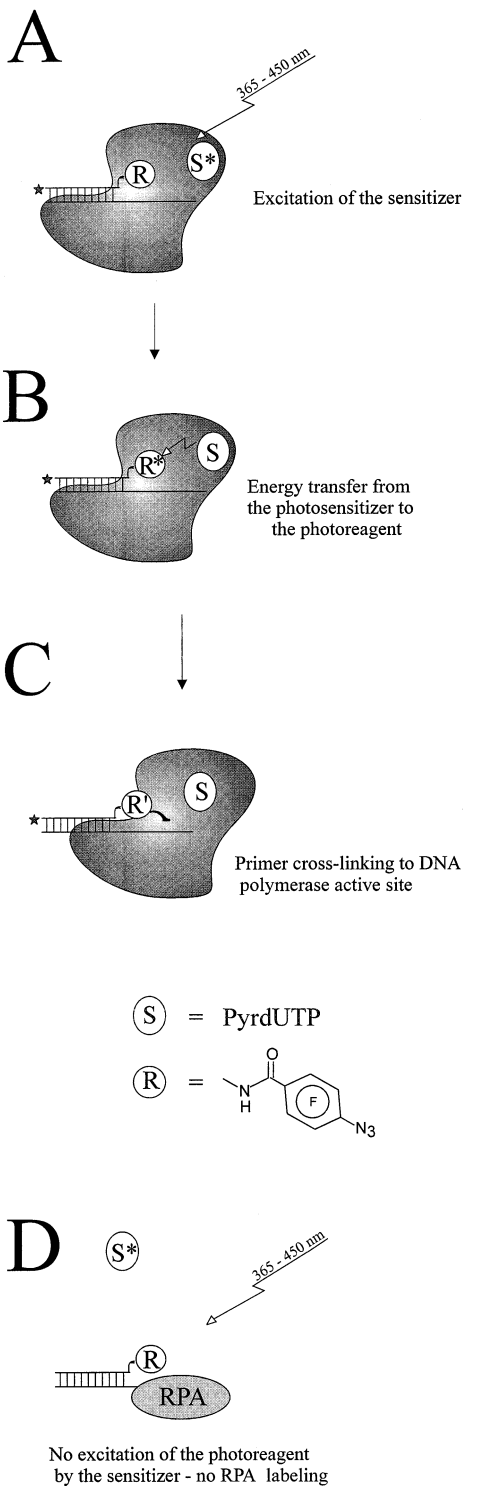

Fig. 2. Scheme of labeling of DNA polymerases by photoreactive primer photosensitized by PyrdUTP.

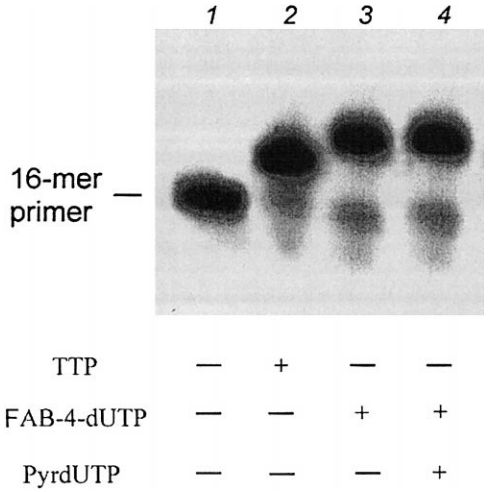

Fig. 3. Substrate properties of FAB-4-dUTP in reactions catalyzed by pol β. All reaction mixtures contained 0.7 μM 5′-[^{32}P] primer-template duplex, 1.4 μM pol β, 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 50 mM KCl. Lane 1 was without dNTP, lane 2 contained 1 μM dTTP, lanes 3 and 4 contained 1 μM FAB-4-dUTP. Reaction mixtures were incubated for 20 min at 25°C. PyrdUTP (10 μM) was added to reaction mixture 4 and incubated for 30 min at 25°C. The reactions were stopped by adding 10 μl 90% formamide, 50 mM EDTA, 0.1% Bromophenol Blue. The mixtures were heated for 3 min at 80°C and the products were analyzed by 20% PAGE followed by autoradiography.

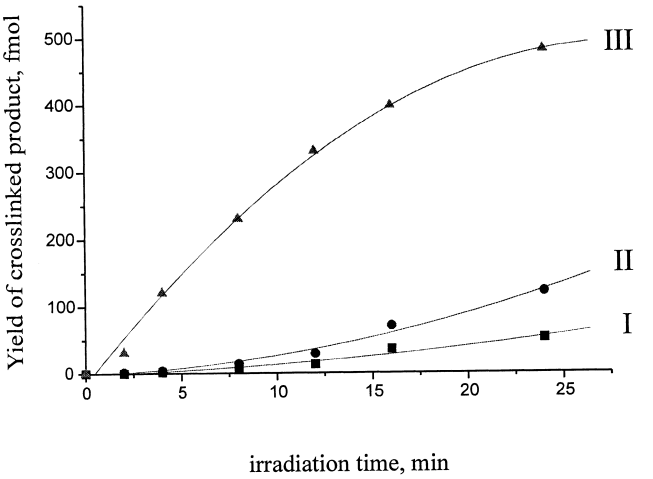

Fig. 4. Kinetics of pol β photosensitized modification. Reaction mixtures contained 0.5 μM 5′-[^{32}P] primer-template duplex, 1 μM FAB-4-dUTP, 1.4 μM pol β (for other conditions, see Section 2). After primer elongation by FAB-4-dUTP, 1-pyrenebutyric acid (curve II) or PyrdUTP (curve III) was added to the reaction mixtures. Control mixtures do not contain 1-pyrenebutyric acid or PyrdUTP (curve I). Photochemical crosslinking was induced with light of 365–450 nm. Crosslinking products were separated by 12% SDS-PAGE. Bands corresponding to the products of polymerase labeling were cut and analyzed quantitatively by Cherenkov counting.

during irradiation. The considerable increase of the initial rate of pol β photomodification in the presence of PyrdUTP proves our idea that PyrdUTP is bound to the enzyme more specifically and/or with higher affinity, since the dUTP moiety of PyrdUTP directs the pyrene residue towards the dNTP binding site of pol β. Based on the data in Fig. 4, we irradiated for 10 min (365–450 nm) in the following experiments to avoid any possible influence of direct activation of the photoreagent.

We found that the specificity of pol β photomodification is higher in the presence of PyrdUTP. Indeed, in the presence of photosensitizer only one product of pol β labeling was identified with PAGE (Fig. 5, lane 1), whereas direct modification (UV irradiation > 280 nm) gave an additional band of lower mobility (Fig. 5, lanes 3, 4). This band could be a product of pol β modification by photoreactive DNA, independent of pol β interaction with a dNTP.

To verify the influence of the photosensitizer on the specificity of polymerase labeling, another DNA binding protein, RPA, was added to the reaction mixture. In the presence of RPA, sensitized photomodification results in the labeling of only pol β (Fig. 5, lane 2), whereas direct photomodification results in the labeling of the p70 and p32 RPA subunits and pol β (Fig. 5, lane 4). These data are in agreement with the suggestion that, in the presence of photosensitizer, modification occurs only in the reagent-pol β-photosensitizer complex, and no modification of RPA was detected. Indeed, RPA can bind only photoreactive DNA substrate but not photosensitizer (PyrdUTP) (Fig. 2D). Similar results were obtained with the DNA polymerase from *Thermus thermophilus* (data in preparation).

The data presented here strongly support high specificity of photoaffinity labeling of the active site of DNA polymerase, induced by its binding to a dNTP carrying a pyrene residue. In other words, the method elaborated in this study seems appropriate for highly selective labeling of DNA polymerases and might be applicable to DNA polymerase labeling in crude cell extracts. This method can easily be adapted to the labeling of RNA polymerases.

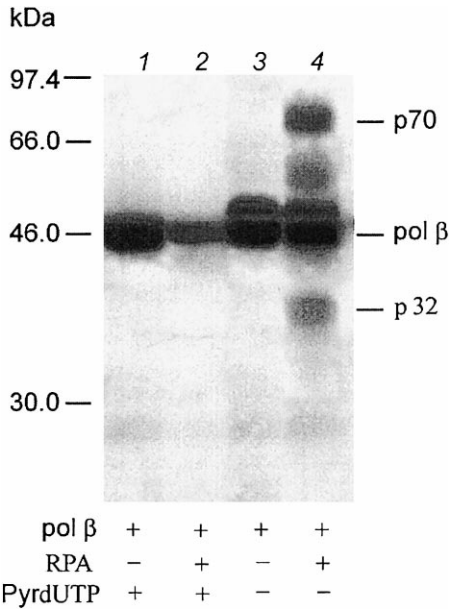

Fig. 5. Photoaffinity labeling of RPA and pol β with primers synthesized in situ. Reaction mixtures were composed of 1.4 μM pol β, 0.7 μM 5′-[^{32}P] primer-template duplex, and 1 μM FAB-4-dUTP (for other components, see Section 2). In addition lanes 2 and 4 contained 0.46 μM RPA. After complete primer elongation, the reaction mixtures were irradiated for 10 min (365–450 nm) in the presence of 10 μM PyrdUTP (lanes 1 and 2), or ($\lambda > 280$ nm) in the absence of PyrdUTP (lanes 3 and 4).

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