Biochemistry (Moscow), Vol. 67, No. 7, 2002, pp. 807-814. Translated from Biokhimiya, Vol. 67, No. 7, 2002, pp. 973-981.

Highly Efficient Labeling of DNA Polymerases by a Binary System of Photoaffinity Reagents

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Received June 12, 2001
Revision received February 5, 2002

Abstract—A binary system of photoaffinity reagents was proposed earlier for highly efficient labeling of DNA polymerases by 5′-[^{32}P]DNA primers. In the present study we demonstrate the feasibility of this approach to increase the efficiency of DNA polymerase labeling. A photoactive 2,3,5,6-tetrafluoro-4-azidobenzoyl (FAB) group was incorporated at the 3′-end of 5′-[^{32}P]DNA primers synthesized by DNA polymerase β or *Tte* in the presence of one of the dTTP analogs—FAB-4-dUTP, FAB-9-dUTP, or FAB-4-ddUTP. The reaction mixture was irradiated by light with wavelength of 334-365 nm (direct labeling) or 365-450 nm in the presence of photosensitizer, one of dTTP analogs containing a pyrene moiety, Pyr-6-dUTP or Pyr-8-dUTP. In the case of the binary system of photoaffinity reagents, a FAB group is activated by energy transfer from sensitizer localized in the dNTP-binding site of DNA polymerase in the triple complex, comprised by reagent, DNA polymerase, and Pyr-6(8)-dUTP. Direct activation of the FAB group under these conditions is negligible. The most efficient photolabeling of DNA polymerases was observed with a primer containing a FAB-4-dUMP group at the 3′-end, and Pyr-6-dUTP as a photosensitizer. Using 10-fold molar excess of photoreagent to DNA polymerase β, the labeling efficiency was shown to achieve 60%, which is 2-fold higher than the efficiency of the direct DNA polymerase labeling under harsher conditions (334-365 nm).

Key words: photoaffinity labeling, photoreactive dNTP analogs, binary system of photoaffinity reagents

The affinity modification technique is widely used for investigation of structure and functions of DNA polymerase active sites [1] as well as for identification of contacts between the catalytic subunits of DNA polymerases and primer in multicomponent systems [2-4]. Photoactivated arylazide derivatives of nucleic acids are affinity reagents suitable for modification of DNA polymerases and other proteins, since the reactive group remains inert until irradiated with light, and a photolabeling of the target can be performed after the correct enzyme–substrate (protein–ligand) complex is formed. However, these derivatives demonstrate relatively low cross-linking efficiency. This hampers the identification and sequencing of the modified peptides by the Edman method or by MALDI mass-spectrometry. The low efficiency of covalent cross-linking in arylazide derivatives of nucleic acids complicates the identification of DNA polymerases and attendant proteins in the cellular and nuclear extracts [5]. Therefore, the further application of photoaffinity modification technique for the investigation of replication complex requires the increase in both the efficiency and selectivity of reactive DNA derivative cross-linking to DNA polymerases.

A binary system of photoaffinity reagents was developed and applied earlier for highly efficient modification of DNA targets [6-11]. Some binary systems of photoaffinity reagents based on oligonucleotides allowed significant increase in the efficiency of DNA target modification, where modification efficiency of 98-99% was achieved when excess of the reagent was used [10]. In this work a binary system of photoaffinity reagents, consisting of a photoreactive primer with arylazide group at the 3′-end, and dNTP analog containing a photosensitizing

Abbreviations: DNA polymerase *Tte*) DNA polymerase from *Thermus thermophilus* B35; FAB-4-dUTP) 5-[N-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-3-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate; FAB-4-ddUTP) 5-[N-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-3-amino-*trans*-propenyl-1]-2′,3′-dideoxyuridine-5′-triphosphate; FAB-9-dUTP) 5-[N-[[(2,3,5,6-tetrafluoro-4-azidobenzoyl)-butanoyl]-amino]-3-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate; Pyr-8-dUTP) 5-[N-(4-(1-pyrenyl)-butylcarbonyl)-3-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate; Pyr-6-dUTP) 5-[N-(4-(1-pyrenyl)-ethylcarbonyl)-3-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate.

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group, is suggested to increase the DNA polymerase modification efficiency.

Previously we developed a binary system of photoaffinity reagents for highly selective modification of DNA polymerases [12, 13]. The main idea of the proposed approach is the following. Radioactively labeled photoreactive DNA primer complexed with DNA template is bound to the DNA-binding site of DNA polymerase, whereas a dTTP analog containing a pyrene residue is bound to the dNTP-binding site of the enzyme (Fig. 1a). Under light irradiation (λ = 365-450 nm) the energy is first absorbed by the photosensitizer (pyrene) and then transmitted onto the arylazide group of the reagent only within the triple complex comprising photoreactive primer, DNA polymerase, and photosensitizer (Fig. 1b). As a result, a covalent cross-linking of the primer to enzyme occurs (Fig. 1c). Energy transfer efficiency decreases as the distance between the sensitizer and a photoreactive group increases [14]; therefore, the reagent in the solution is not photolyzed under these conditions (Fig. 1b). The binary system of photoaffinity reagents was employed to perform the selective modification of rat DNA polymerase β [12], thermostable DNA polymerase of *Thermus thermophilus* [13], and a catalytic subunit (180 kD) of DNA polymerase α-primase from calf thymus (unpublished data) in the presence of another DNA-binding protein, human replication protein A. However, the use of a binary system of photoaffinity reagents resulted in lower modification efficiency compared to the direct modification by photoreactive primer during the UV irradiation (λ > 280 nm). At the same time, the application of a binary system of photoaffinity reagents provides a number of advantages over direct labeling of proteins. They are associated with a capability of using "softer" irradiation conditions during the modification, which

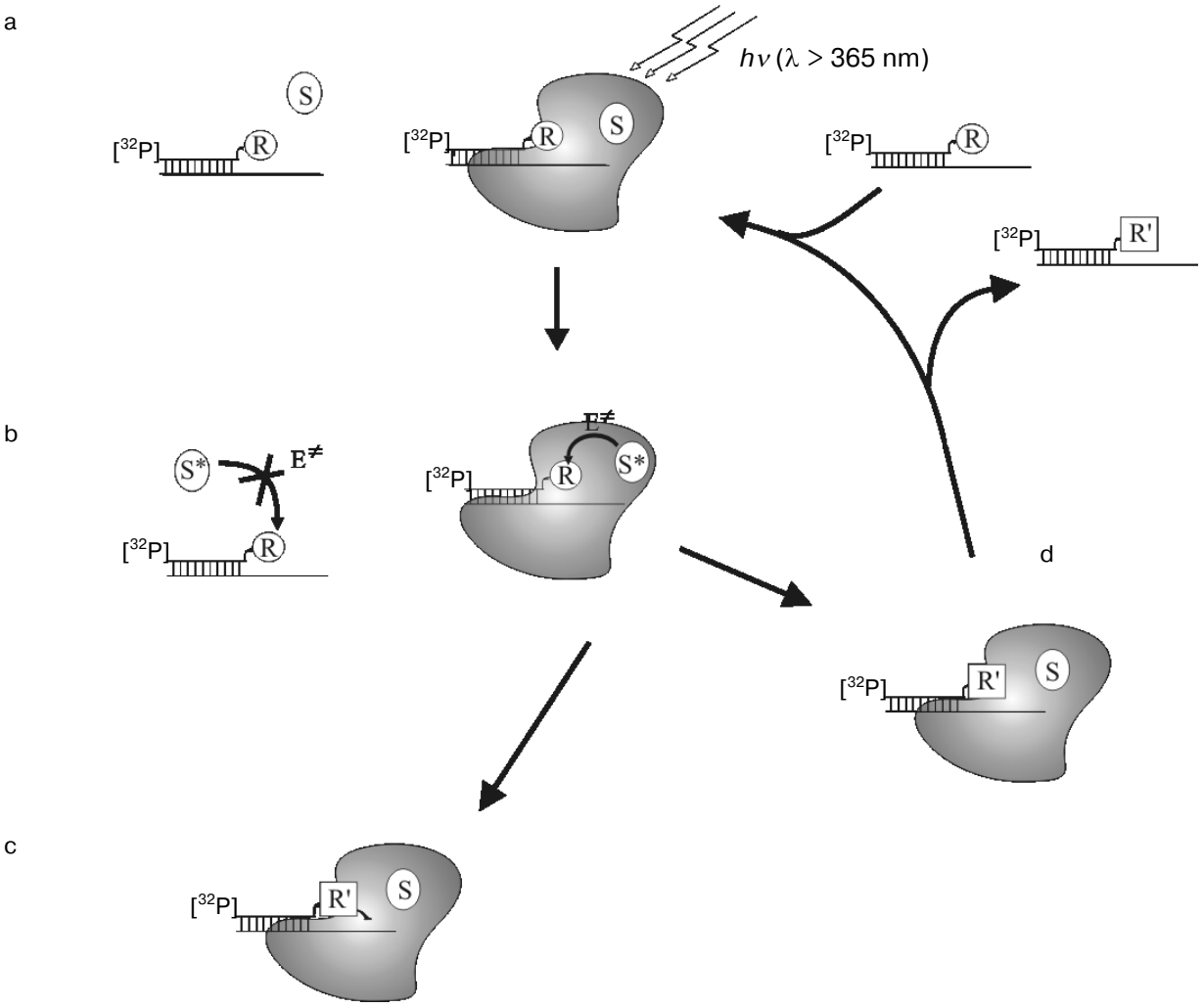


Fig. 1. Basic scheme of DNA polymerase modification by the binary system of photoaffinity reagents. a) Binding of the reagent and sensitizer to the target, excitation of the sensitizer. b) Energy transfer from the sensitizer to photoreagent during UV-irradiation. The reagent does not photolyze in solution and no energy transfer occurs. c) Covalent cross-linking of primer to enzyme. d) If the reagent has not linked to the target after the photolysis, there is a possibility of its replacement by active reagent from the solution during the irradiation.

maintains the system in a nearly native state. Degradation of the polynucleotides is totally excluded or decreased, and the formation of nonspecific complexes is reduced, i.e., the labeling selectivity increases. One of the possibilities for improving the photomodification yield is to increase the reagent excess toward the target. Under direct labeling conditions, reagent photolysis in the solution occurs at the same rate as within the complex with the target. Photolyzed reagent with a retained affinity to the target sequence acts as a competitive inhibitor for the enzyme modification reaction. When the binary system of photoaffinity reagents is employed, the reagent photolysis in the solution occurs much slower than in the triple reagent–target–sensitizer complex, and the reagent remains intact in the solution. Hence, if the reactive primer has not been covalently linked to DNA polymerase, the enzyme can bind the second reagent molecule from the solution (Fig. 1d). This leads to the increased possibility of DNA polymerase modification.

The distance between the reagent and applied sensitizer can also affect the labeling efficiency. Thus, in addition to reagents and sensitizers used earlier, synthesis and investigation of new analogs differing by length of the linker connecting the pyrene or modifying group with the heterocyclic base was performed in the present study.

Finally, since the possibility of inclusion of sensitizing nucleotide analog into the primer has to be eliminated, we for the first time synthesized and used the terminating photoreactive dTTP analog from the dideoxy group (FAB-4-ddUTP). The further primer elongation becomes impossible when this analog is used.

Thus, the present study is focused on the investigation of various approaches to increase the efficiency of DNA-dependent enzyme affinity modification by using the binary system of photoreagents. It makes possible the use of soft UV radiation, eliminating damage to both DNA and proteins, and providing highly selective affinity labeling.

MATERIALS AND METHODS

The chemicals used in this work were polynucleotide kinase T4 (Sibenzyme, Russia), [γ-^{32}P]ATP (Biosan, Russia), electrophoretic kits and buffer components (Sigma, USA), 1-pyreneacetic acid (Aldrich, USA). Other chemicals were of "extra pure" or "chemically pure" grade. N-Hydroxysuccinimide ester of 2,3,5,6-tetrafluoro-4-azidobenzoic acid was synthesized and kindly provided by T. M. Ivanova (Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences), 2′-deoxyuridine-5′-triphosphate and 2′,3′-deoxyuridine-5′-triphosphate were synthesized and kindly provided by V. S. Bogachev (Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences). 5-(*Trans*-3-aminopropenyl-1)-2′-deoxyuridine-5′-triphosphate (triethylammonium salt) (I) and 5-(*trans*-3-aminopropenyl-1)-2′,3′-deoxyuridine-5′-triphosphate (triethylammonium salt) (II) were synthesized according to [15]. N-Hydroxysuccinimide ester of 1-pyreneacetic acid was synthesized from 1-pyreneacetic acid and N-hydroxysuccinimide in compliance with the standard procedure [16]. Synthesis and photochemical properties of dTTP analogs—Pyr-8-dUTP, FAB-9-dUTP, and FAB-4-dUTP—are described earlier [12, 17, 18].

DNA templates 5′-ATATCGATATCGTAGATC-CACTGTATAGCCCCTACC-3′ (I) and 5′-ATATC-GATATCGTAGATCAACTGTATAGCCCCTACC-3′ (II) and DNA primer 5′-GGTAGGGGCTATACAG-3′ were synthesized in the Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences.

The recombinant rat DNA polymerase β was isolated from *Escherichia coli* strain according to the conventional protocol [19]. The recombinant thermostable DNA polymerase *Tte* was isolated according to [20].

Synthesis of 5-[N-(4-(1-pyrenyl)-ethylcarbonyl)-3-amino-*trans*-propenyl-1]-2′-deoxyuridine-5′-triphosphate (lithium salt) (Pyr-6-dUTP). 1-Pyreneacetic acid N-hydroxysuccinimidyl ester solution (0.2 M, 100 μl) and 57 μmol (8 μl) of triethylamine were added to 10 μmol (80 units A_{290}) of triphosphate I, dissolved in 200 μl of dimethylformamide. After the reaction was completed (1 h, 37°C), 1 ml of 2% solution of $LiClO_4$ in acetone was added to the reaction mixture. The resulting precipitate was separated by centrifugation and washed with acetone and ether. Pyr-6-dUTP was isolated by HPLC. The product was homogeneous according to TLC with R_f 0.51 (dioxane–ammonia–water 6 : 4 : 1), NMR spectra (^2H_2O), ^{31}P (δ, ppm) –4.86 (d, 1P, Pγ, $J_{P\beta}$ 20 Hz), –10.67 (d, 1P, Pα, $J_{P\beta}$ 20 Hz), –20.02 (t, 1P, Pβ, $J_{P\alpha,P\beta}$ 20 Hz), ^{1}H (δ, ppm) 3.2 (t, 2H, H2′, $J_{H1',H3'}$ 5.5), 3.7 (m, 2H, H9), 4.1-4.2 (m, 4H, H5′, H4′, H3′), 5.4-5.9 (m, 3H, H1′, H7, H8), 7.63 (s, 1H, H6), 7.8-7.9 (m, 9H, H_{11-19}).

5-[N-(2,3,5,6-tetrafluoro-4-azidobenzoyl)-3-amino-*trans*-propenyl-1]-2′,3′-dideoxyuridine-5′-triphosphate (lithium salt) (FAB-4-ddUTP) was synthesized from triphosphate II and 2,3,5,6-tetrafluoro-4-azidobenzoic acid N-hydroxysuccinimidyl ester according to the procedure described for Pyr-6-dUTP synthesis. The product was homogenous according to TLC with R_f 0.45 (dioxane–ammonia–water 6 : 4 : 1), NMR spectra (^2H_2O), ^{31}P (δ, ppm) –2.26 (d, 1P, Pγ, $J_{P\beta}$ 20 Hz), –7.77 (d, 1P, Pα, $J_{P\beta}$ 20 Hz), –17.56 (t, 1P, Pβ, $J_{P\alpha,P\beta}$ 20 Hz), 1H (δ, ppm) 2.18 (m, 3H, H2′b, H3′), 2.5 (m, 1H, H2′a), 4.2-4.4 (m, 4H, H9, H5′), 6.2-6.5 (m, 3H, H1′, H7, H8), 7.94 (s, H6, 1H).

Radioactive labeling of oligonucleotide 5′-end was performed according the method described earlier [21]. Labeled oligonucleotides were electrophoretically puri-

fied in polyacrylamide gel according to [22]. Hybridization between primer and template was performed at room temperature for 5 min.

Elongation of the radioactively labeled primer was performed using DNA polymerases β or *Tte*. The reaction mixture (10 μl) contained standard buffer components (50 mM Tris-HCl, pH 9.0 (at 25°C), 50 mM NaCl, 5 mM $MgCl_2$), 1 μM of 5′-[^{32}P]-labeled primer–template complex, 0.1 μM of DNA polymerase β or *Tte*, and 10 μM of one of dTTP analogs—FAB-4-dUTP, FAB-4-ddUTP, or FAB-9-dUTP. The mixtures were incubated for 30 min at 70°C for DNA polymerase *Tte* or at 37°C for DNA polymerase β. The reaction was terminated by adding 5 μl of 0.1% bromophenol blue and 50 mM EDTA solution in 90% formamide. The samples were heated for 5 min at 90°C. The products were separated by electrophoresis in 20% polyacrylamide gel (acrylamide/bis-acrylamide 20 : 1) with 7 M urea. Electrophoresis was performed in 100 mM Tris-borate buffer, pH 8.3.

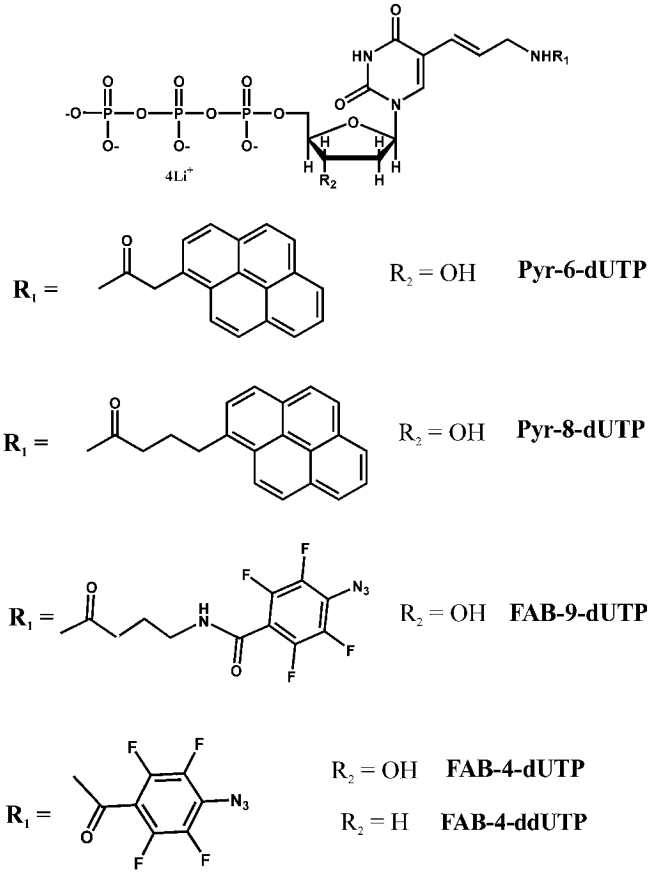
Determination of kinetic parameters for dTTP and its analogs in the reaction catalyzed by DNA polymerase β or *Tte* was performed in the reaction mixture (10 μl), containing standard buffer components (50 mM Tris-HCl, pH 9.0 (at 25°C), 50 mM NaCl, 5 mM $MgCl_2$), 0.5 μM duplex 5′-[^{32}P]-labeled primer–template, 0.01 μM DNA polymerase, 0.05-50 μM dTTP (or its analog). The mixtures were incubated for 5 min at 70°C for DNA polymerase *Tte* or at 37°C for DNA polymerase β. The reaction was terminated by adding 5 μl of 0.1% bromophenol blue and 50 mM EDTA solution in 90% formamide. The samples were heated for 5 min at 90°C. The products were electrophoretically separated in 20% polyacrylamide gel (acrylamide/bis-acrylamide 20 : 1) with 7 M urea. The positions of the original and one-step elongated primer in the gel were determined from the gel retardation image, then the corresponding regions of the gel were cut out and the radioactivity was measured by the Cherenkov technique. The values of kinetic parameters were determined according to Michaelis–Menten equation using Microcal Origin software (Microcal Software, USA).

Photoaffinity modification of DNA polymerases was performed in the reaction mixture (10 μl) containing 5 μM duplex 5′-[^{32}P]-labeled primer–template, 10 μM FAB-4-dUTP, FAB-4-ddUTP, or FAB-9-dUTP, 0.5 μM DNA polymerase β or *Tte*, and also standard buffer components described earlier. After the complete primer elongation (37 or 70°C, 30 min) 10 or 50 μM Pyr-6-dUTP or 10 μM Pyr-8-dUTP were added to the reaction mixture. The reaction mixtures were irradiated for 10 min (at 365-450 nm), or for 2 min 10 sec (at >280 nm), or for 14 min 20 sec (at 334-365 nm). Products of modification were analyzed using electrophoresis according to Laemmli procedure [23]. Bands corresponding to enzyme modification products were isolated and measured using the Cherenkov radiation technique.

UV irradiation was performed by radiation from a DRK-120 high pressure mercury lamp (light source VIO-1; Lomo, St. Petersburg, Russia; distance 110 mm) in the range >280 nm through the light filter WG-280 with incident light intensity (I) equal to $1.2 \cdot 10^{15}$ quanta·sec^{-1}·cm^{-2}, in the range 365-450 nm through the combination of light filters FS-1 and BS-7 with I equal to $5.5 \cdot 10^{14}$ quanta·sec^{-1}·cm^{-2}, and in the range 334-365 nm through the light filter UFS-6 with I equal to $8 \cdot 10^{14}$ quanta·sec^{-1}·cm^{-2}.

RESULTS AND DISCUSSION

The basic scheme showing the principles of DNA polymerase modification by the binary system of photoaffinity reagents is presented in Fig. 1. Chemical structures of dTTP analogs used in this work are listed below:



It is known that some DNA polymerases are error-prone enzymes, capable of misincorporation of nucleotides, which are not complementary to the template. In the case of using the binary system of photoaffinity reagents for DNA polymerase modification it is essential that the nucleotide-containing sensitizer is not incorporated in the primer containing the reactive group. The photoreactive analog of dideoxy nucleotide, FAB-4-

ddUTP, was synthesized in order to strictly obey this condition. After the FAB-4-ddUMP residue has been incorporated into the primer, the further elongation of a polynucleotide chain is impossible.

Substrate properties of FAB-4-ddUTP. Elongation of 5′-[^{32}P]-labeled primer was performed by DNA polymerases β and *Tte* using a primer–template system II, where two dTTP residues have to be incorporated into 3′ end of the primer according to template sequence. Figure 2 illustrates that DNA polymerase β is able to efficiently elongate the primer both in the presence of FAB-4-ddUTP (Fig. 2, lane *6*) and FAB-4-dUTP (lane *7*), whereas DNA polymerase *Tte* inserts dideoxy-analog with a considerably lower efficiency as compared to FAB-4-dUTP (lanes *2* and *3*, respectively). K_m value for FAB-4-ddUTP in primer elongation reaction catalyzed by DNA polymerase β is 2.2 μM, which is similar to the K_m value for dTTP (5 μM), whereas these values for DNA polymerase *Tte* are 140 and 2 μM, respectively. Thus, FAB-4-dUTP appears to be a more suitable substrate for DNA polymerase *Tte* than its dideoxy-analog. When Pyr-8-dUTP is added to the reaction mixture, the incorporation of Pyr-8-dUMP occurs, including the misincorporation (lanes *4*, *5*, *9*). In the case of DNA polymerase β, the use of FAB-4-ddUTP minimizes the formation of these products (lane *8*). The data prove that the use of dideoxy nucleotide analogs enables the production of the primers containing a single reactive group at the 3′-end. However, not all DNA polymerases utilize ddNTP equally well. This should be taken into account when working with cellular extracts where most of the cell DNA polymerases are present.

The effect of template structure on DNA polymerase β modification efficiency. Since the efficiency of energy transfer from sensitizer to the reagent depends on the distance between them, we assumed that the DNA polymerase modification efficiency might be increased by using dNTP analog as a sensitizer, owing to the proximity between the 3′-end of the photoreagent-containing primer and a sensitizer within the complementary complex compared to non-complementary. Figure 3 presents the results of DNA polymerase β modification by a photoreactive primer within two template–primer duplexes. After one step primer elongation with photoreactive dTTP analog, the next inserted dNTP or its analog has to be complementary to dCMP in template I and to dAMP in template II. Hence, in the case of template II, Pyr-8-dUTP has to be bound to dNTP-binding site of DNA polymerase in the complex, complementary to the template base, in contrast to template I. The radioactively labeled primer in the complex with templates I and II has been elongated by DNA polymerase β in the presence of photoreagents FAB-4-dUTP or FAB-4-ddUTP, then the reaction mixture was irradiated by UV light of 365-450 nm in the presence or in the absence

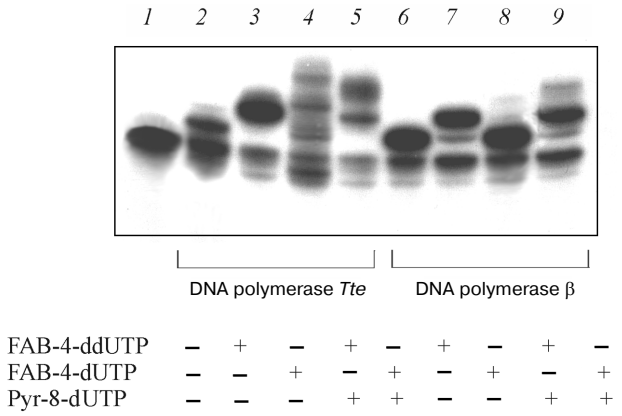


Fig. 2. FAB-4-ddUTP as substrate for DNA polymerase β and DNA polymerase *Tte*. The photoreactive duplex 5′-[^{32}P]-labeled primer–template II was obtained in the presence of FAB-4-dUTP or FAB-4-ddUTP as the figure illustrates. After the incubation for 30 min at 25°C (for DNA polymerase β) or at 70°C (for DNA polymerase *Tte*) Pyr-8-dUTP was added to the reaction mixtures and then incubated again for 30 min at 25°C. The reaction mixtures were separated in 20% polyacrylamide gel with subsequent radioautography.

of Pyr-8-dUTP, used as a sensitizer, or by UV light of >280 nm in the absence of sensitizer. No significant difference in the modification efficiency was detected for the complementary and non-complementary templates (lanes *2* and *4*), which can be explained by minor

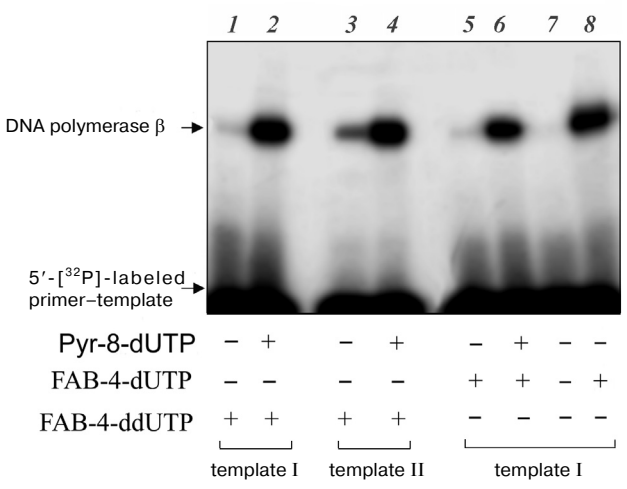


Fig. 3. Effect of template structure on DNA polymerase β modification efficiency. The photoaffinity labeling of DNA polymerase β was performed using either primer–template system I (*1*, *2*, *5*-*8*) or primer–template system II (*3*, *4*) (see "Materials and Methods"). The irradiation by UV light at 365-450 nm was performed for 10 min (*1*-*6*), at >280 nm for 2 min 10 sec (*7*, *8*). The reactive mixtures were separated in 12% polyacrylamide gel with SDS followed by radioautography.

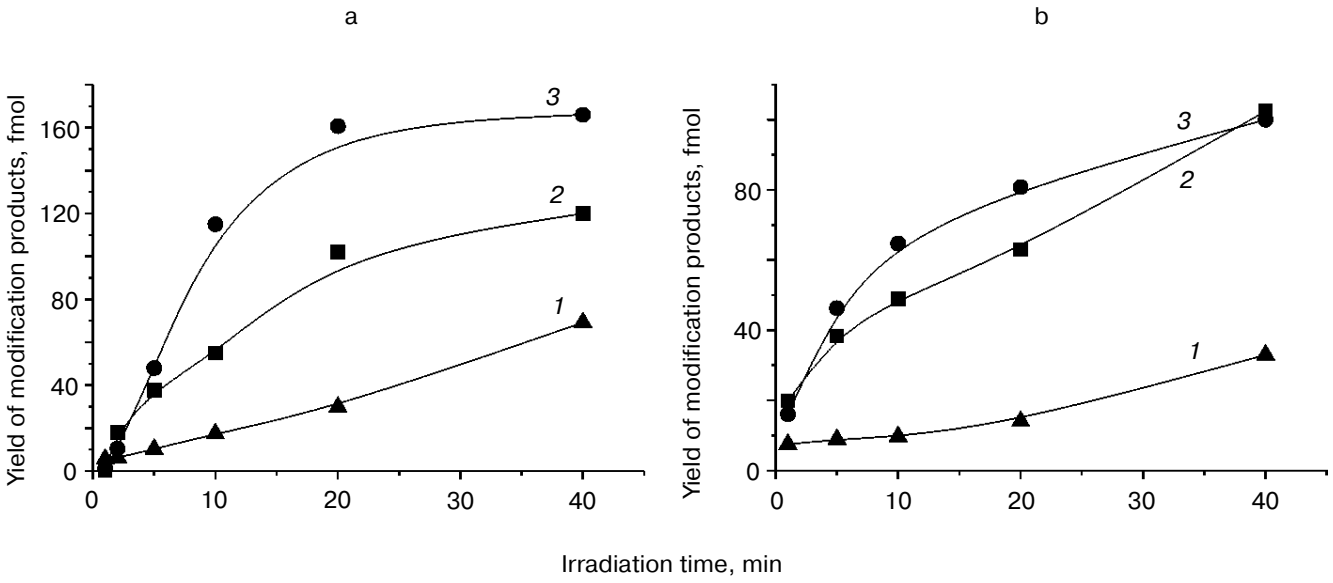


Fig. 4. Effect of reagent and sensitizer structure on DNA polymerase β modification efficiency. The photoreactive duplex 5′-[^{32}P]-labeled primer–template I was obtained in the presence of FAB-4-dUTP (a) or FAB-9-dUTP (b) as described in the "Materials and Methods" section. The reaction mixtures were irradiated with UV light (365-450 nm) in the presence of Pyr-8-dUTP (curve *2*) or Pyr-6-dUTP (curve *3*). Control mixtures did not contain sensitizer (curve *1*). Products of enzyme modification were separated in 12% polyacrylamide gel with SDS, isolated and measured by Cherenkov technique.

distance variations between the incorporated dNTP base and 3′-end primer residue in the case of complementary and non-complementary complexes.

Effect of reagent and sensitizer structure on the DNA polymerase β modification efficiency. The next experiment was focused on the investigation of the influence of linker length connecting FAB group or pyrene residue with affinity moiety of binary system components on the DNA polymerase modification efficiency. Figure 4 presents the kinetic curves of DNA polymerase β affinity modification by photoactive oligonucleotides containing either FAB-4-dUMP (Fig. 4a) or FAB-9-dUMP residue (Fig. 4b) at the 3′-end, whereas both Pyr-8-dUTP and Pyr-6-dUTP are used as sensitizers. As seen from the figure, initial modification rate in the presence of Pyr-6-dUTP is higher for both reagents in comparison with Pyr-8-dUTP (compare curves *3* and *2* on Figs. 4a and 4b), and the highest sensitizing effect is observed for the reagent containing FAB-4-dUMP residue (compare Figs. 4a and 4b). Taking into account the small difference in K_m values for sensitizers (0.1 μM for Pyr-6-dUTP and 0.2 μM for Pyr-8-dUTP) and the fact that their concentrations were much higher than K_m, the conclusion can be made that particularly the length of the linker connecting pyrenyl group with affinity moiety of the reagent influences the enzyme modification efficiency. Similar data were obtained for DNA polymerase *Tte* modification (data not shown). Thus, the combination of the reactive primer with FAB-4-dUMP residue at 3′-end and Pyr-6-dUTP as a sensitizer appears to be the most efficient for DNA polymerase modification. This fact might be explained under the supposition that when these reagents interact with the enzyme, the photoreactive FAB group and photosensitizing pyrenyl group are brought into the closest contact, what facilitates the energy (or electron) transfer and results in the increase of protein modification efficiency.

The overall efficiency of DNA polymerase *Tte* labeling was slightly lower than of DNA polymerase β, which can probably be explained by either the longer distance between the photoreactive groups during the binding to this enzyme, or by different nature of modified acceptor groups. Also, the higher efficiency of DNA polymerase β modification as compared with *Tte* can result from the fact that DNA polymerase β is a distributive enzyme, and the reagent exchange between the solution and enzyme complex occurs much faster than in the case of DNA polymerase *Tte*.

In order to find the Pyr-6-dUTP concentration when the efficiency of DNA polymerases modification is maximal, we varied Pyr-6-dUTP concentration in DNA polymerase β labeling experiments under the conditions of 10-fold excess of photoreagent toward DNA polymerase. The maximum level of modification was observed at Pyr-6-dUTP concentration of 50 μM. The further increase in the concentration has not led to the increase

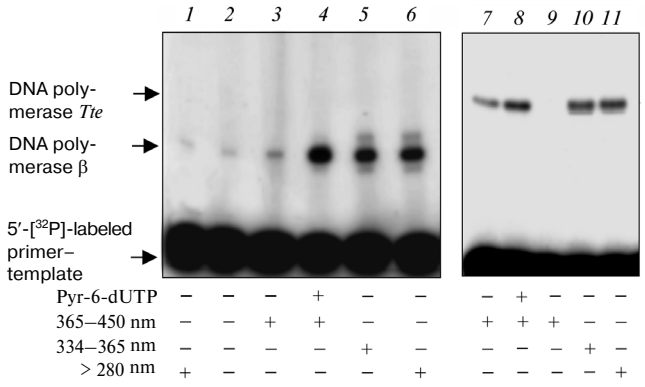


Fig. 5. Photoaffinity labeling of DNA polymerase by the binary system of photoaffinity reagents under the conditions of 10-fold reactive primer excess. The photoreactive primer was synthesized by DNA polymerase β (*1-6*) or by DNA polymerase *Tte* (*7-11*) in the presence of FAB-4-dUTP as described in the "Materials and Methods" section; *1*, *9*) FAB-4-dUTP is absent. The reaction mixtures were irradiated by UV light of different wavelengths in the presence or in the absence of Pyr-6-dUTP. Products of enzyme modification were separated in 12% polyacrylamide gel with SDS and subsequent radioautography.

in DNA polymerase modification efficiency (data not shown).

Photoaffinity labeling of DNA polymerase by the binary system of photoaffinity reagents under conditions of 10-fold reactive primer excess. The comparison of DNA polymerases β and *Tte* modification efficiency under conditions of direct labeling and by using the binary system of photoaffinity reagents at 10-fold reagent excess toward the enzyme was performed. The data are presented in Fig. 5. When determining the intensity of the corresponding bands by the Cherenkov technique (see "Materials and Methods") it was shown that during the light irradiation with λ = 365-450 nm the direct protein labeling does not occur. However, the level of DNA polymerase β and *Tte* modification when utilizing the binary system of photoaffinity reagents under these conditions was sufficiently high (Fig. 5, lanes *4* and *5*, *6*; *8* and *10*, *11*, respectively). This value was 30% for DNA polymerase β and 18% for DNA polymerase *Tte* and remarkably (2 and 1.2 times, respectively) exceeded the level of direct labeling even under harsher irradiation conditions (λ > 280 nm). It should be emphasized that DNA polymerase β labeling efficiency was determined while irradiating the reagent during the half-modification time. Hence, the limit of DNA polymerase β modification efficiency can be 2 times higher and reach the value of 60%.

When the binary system of photoaffinity reagents is employed, not only the increased efficiency but also improved DNA polymerase modification selectivity can be achieved. In the presence of photosensitizer only one product of DNA polymerase β or *Tte* modification is observed (Fig. 5, lanes *4* and *8*), whereas in the absence of photosensitizer there are additional products with both lower and higher gel mobility (lanes *5*, *6* and *10*, *11*). These additional products may indicate the reactive primer cross-linking to various amino acid residues of DNA polymerase, even through nonspecific modification from the solution.

Hence, due to its high efficiency and selectivity the developed binary system of photoaffinity reagents can be applied both for the identification of primer-binding sites in DNA polymerases and for identification of catalytic subunits in DNA polymerases in the cellular and nuclear extracts.

We acknowledge T. M. Ivanova for the synthesis of 2,3,5,6-tetrafluoro-4-azidobenzoyl acid N-hydroxysuccinimidyl ester, V. S. Bogachev for the synthesis of 2′-deoxyuridine-5′-triphosphate and 2′,3′-deoxyuridine-5′-triphosphate, and S. V. Dezhurov for DNA polymerase β half-modification time measurements.

The work was supported by the Russian Foundation for Basic Research (grants No. 00-04-49309, 00-04-22002, 01-04-48861, 01-04-48895, 02-04-48404), "The Project Competition of Young Scientists, Siberian Division of the Russian Academy of Sciences", and by INTAS grant YSF-00-83.

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