Highly efficient modification of DNA polymerase β under conditions of direct and sensitized activation of photoreactive DNAs. Modification of cell extract proteins

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dUTP and dCTP derivatives containing a 4-azido-2,3,5,6-tetrafluorobenzylideneaminooxy group were incorporated into the 3´-end of the DNA primer within complexes with the DNA-matrix as analogs of natural dTTP by virtue of catalytic activity of DNA polymerase β or endogenous DNA polymerases of the cell extract. The photoreactive DNAs synthesized *in situ* were used for affinity modification of DNA polymerase β and DNA-binding proteins of the cell extract. For the photoreactive DNA based on these analogs, the efficiency of formation of covalent adducts with DNA polymerase β under the highest degree of DNA complexation with the enzyme was determined. The yield of covalent DNA adducts with the enzyme was 28—47%, depending on the type of the analog. The effect of the sequence of the DNA template near the localization of the photoreactive group on the redistribution of covalent cross-links between the possible targets was demonstrated. A possibility of increasing the efficiency of DNA polymerase β modification in the presence of a substantial excess of photoreactive DNA using a sensitizer, a dUTP derivative containing a pyrene residue, was studied. When photoreactive DNA containing a 2,3,5,6-tetrafluoro-4-azidobenzoyl (FAB) group was used, about 60% of DNA polymerase β was covalently attached to DNA. Photoreactive dNTP analogs ensuring a high level of protein modification in the cell extract were found.

Key words: DNA polymerase β, photoaffinity modification, sensitized modification, modification efficiency.

Previously,[1—4] we performed a number of comparative studies of the efficiency of formation of photoinduced adducts in primer—template complexes of DNA polymerase β and DNA polymerase of *Thermus thermophilus*. As the photoaffinity reagents, we used DNA primers containing various arylazido groups incorporated into heterocyclic bases at the 3´-end nucleotide. The highest efficiency (~30%) of covalent cross-linking of photolysis products of this type of primers with other components of the complexes was attained with the 4-(4-azido-2,3,5,6-tetrafluorobenzylideneaminooxy)butoxy- (FABO) and 4-azido-2,5-difluoro-3-chloropyridin-6-yl[1] (FAP) groups.[2] A comparative study of the visible-light-sensitized photomodification of the primer—template complex with DNA polymerase β in the presence of ATP derivatives bearing sensitizing groups attached to the γ-phosphate was also carried out.[4] It was found that the total efficiency of cross-linking and the distribution of the cross-links among components of the complexes depend crucially on their structures (the sequence of the primer—template duplex, the structure of the sensitizer, *etc*.) and irradiation conditions. A number of studies[5—7] demonstrated the possibility of conducting sensitized photomodification using 5-substituted dUTP—pyrene derivatives as the sensitizers.

This work aims at further development of the design of affinity photoreagents to study the composition and structure of macromolecular replication and repair protein complexes in reconstructed systems and cell extracts and, in the future, *in vivo*. To this end, we performed a number of studies aimed at increasing the yield of covalent cross-linking of photolysis products of DNA primers to protein components. Taking into account the earlier results, primers containing either a FAP or a FABO group and attached to the bases of 3´-terminal nucleotides were chosen as the affinity photoreagents. We planned to increase the yield of covalent photocross-links with the enzyme by replacing the guanine base, which is highly reactive with respect to the singlet nitrene, in the environment of photoreactive groups by more stable adenine and thymine. We supposed that this would redirect the attacks of the singlet nitrenes generated during photolysis from the

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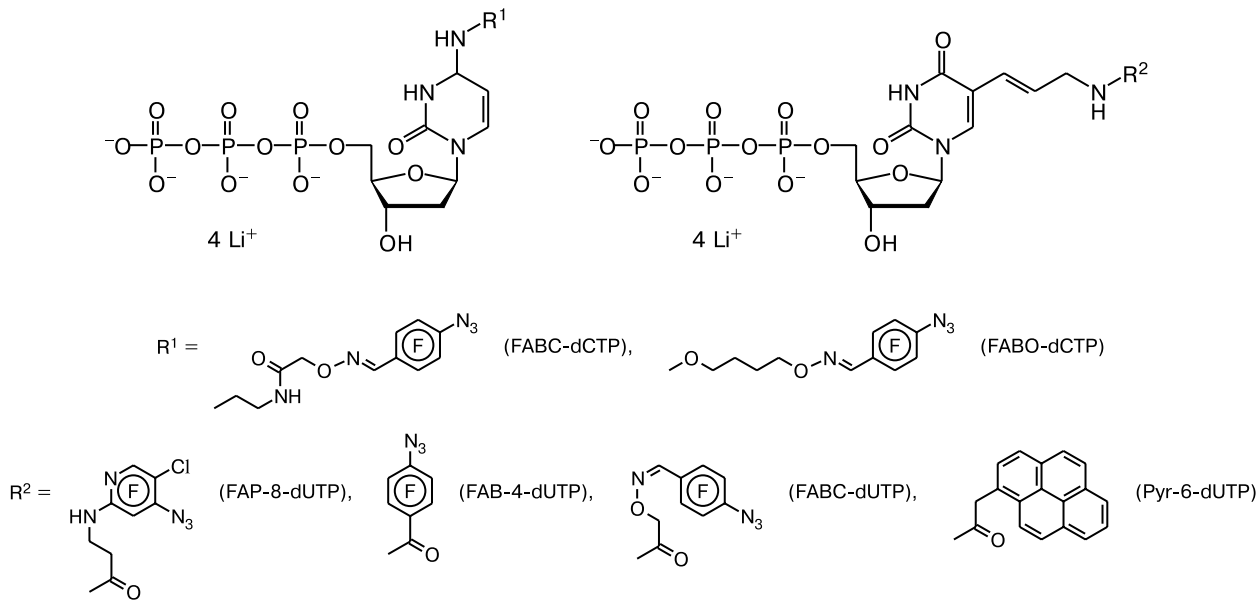


primer and template nitrogen bases to the protein amino acid residues. As in previous publications,[1,2] testing was carried out in model complexes of DNA polymerase β with the primer—template DNA duplex. The affinity photoreagents were prepared *in situ* by elongation of the precursor DNA primer in the presence of photoreactive dTTP analogs; therefore, first we studied the substrate properties of these derivatives. This was followed by comparative estimation of the dependences of the total yield of the photoinduced covalent cross-links and their distribution among components of the complex of DNA polymerase β with photoreactive DNA duplex on the structure of the affinity photoreagent. In addition, we estimated the prospects of these dNTP analogs as substrates in the synthesis of photoreactive DNAs *in situ* in a cell extract with subsequent photoaffinity modification of the DNA-binding proteins of this extract.

Substrate properties of dNTP analogs in the elongation of DNA primers

The substrate properties of the photoreactive dNTP and their close analogs used in this work have already been compared.[2,8,9] For some of them, the kinetic parameters of the DNA-primer elongation were found.[2] In the present study, the attention was focused on the FAP-, (4-azido-2,3,5,6-tetrafluorobenzyleneaminooxy)acetamido- (FABC), and FABO-containing analogs, which can be incorporated under the action of DNA polymerases as dTTP analogs, namely: FAP-8-dUTP, FABC-dUTP, FABC-dCTP, and FABO-dCTP.

For FABC-dUTP, FABC-dCTP, and FABO-dCTP as dTTP analogs, the kinetic constants for incorporation were determined with the use of the PT1 primer template system, while for FABC-dUTP, PT2 was also used (Scheme 1).

The difference between PT1 and PT2 is that they contain bases T and G in the $(n + 2)$nd position of the template, respectively. The determined features of the elongation process observed (Table 1) reasonably supplement the data obtained with the same or related analogs within DNA polymerase β complexes with other template primer complexes.[1,2] In particular, the presence of a bulky substituent results in general deterioration of the substrate properties (simultaneous increase in K_M and decrease in V_{max} as compared with dTTP). Note that this effect is much more pronounced for *N*(4)-substituted dCTP derivatives than for 5-substituted dUTP derivatives. In addition, FABO-dCTP is better incorporated as dTTP than FABC-dCTP (see Table 1) and, as found previously,[2]

Scheme 1

PT1 ↓ T ACA TAT CGG GGA TGG-5′
5′-GGT TAA ATA AAA TAG TAA **T**AA TGT ATA GCC CCT ACC-3′
PT2 ↓ T ACA TAT CGGGGA TGG-5′
5′-GGT TAA ATA AAA TAG TAA **G**AA TGT ATA GCC CCT ACC-3′

Designations: * is the ^{32}P-label, ↓ is the $(n + 1)$st position of the template chain, _ is the $(n + 2)$nd position of the template chain.

Table 1. Kinetic parameters of elongation of DNA primers and modification of DNA polymerase β and the efficiency of formation of the photomodification products

dNTP	Composition of the complexes	Incorporation of dTTP analogs			Photoinduced cross-linking						
		Kinetic parameters		Efficiency W_d (%)	Kinetic parameters			Efficiency (%)			
		V_{max}[a]	$K_M \cdot 10^5$ /mol L^{-1}		Irradiation conditions[b]	k/s^{-1}	$\tau_{1/2}/s$	W_{DNA}	W_X	W_R	$W_{DNA+X+R}$
FABO-dCTP	PT1	1.9±0.2	8.0±1.0	72±6	*A*	0.018±0.003	39±5	5±1	—	47±5	52±6
	PT1/S[c]	—	—	77±4	*B*	0.006±0.001	120±18	—	35±4	14±2	49±6
FABC-dUTP	PT1	5.5±0.5	4.9±0.8	81±4	*A*	0.015±0.002	46±5	13±1	15±4	28±4	56±9
	PT2	3.8±0.2	12.2±1.7	86±1	*A*	0.023±0.005	30±5	21±2	5±1	24±3	50±6
	PT1/S	—	—	79±3	*B*	0.004±0.0005	174±18	—	38±4	17±4	55±8
FABC-dCTP	PT1	0.85±0.03	9.5±1.2	68±5	*A*	—	—	8±1	4±0.3	44±6	56±7.3
FAB-4-dUTP	PT1[d]	—	—	~70	*A*	0.0016±0.0001	429±21	—	—	18	—
	PT1/S	—	—	79±3	*B*	0.003±0.001	246±100	—	38±5	13±2	51±7
FAP-8-dUTP	PT2[d]	—	—	~85	*A*	0.012±0.0015	59±7	—	—	~13	—
	PT1/S	—	—	81±5	*B*	0.006±0.001	126±42	—	22±2	27±2	49±4
dTTP	PT1	7.3±0.7	1.0±0.1	—	—	—	—	—	—	—	—
	PT2	5.1±0.4	2.1±0.3	—	—	—	—	—	—	—	—

[a] In nmol L^{-1} s^{-1}.
[b] *A*: λ = 313—365 nm, $I = 8 \cdot 10^{14}$ quant. s^{-1} cm^{-2}; *B*: λ = 365—450 nm, $I = 5.5 \cdot 10^{14}$ quant. s^{-1} cm^{-2}.
[c] PT1/S, the experiments were carried out in the presence of the Pyr-6-dUTP sensitizer.
[d] The data were taken from Ref. 1 and converted to the units of measurements used in this Table.

much worse than dCTP (the V_{max} value is almost 50 times lower with K_M values being similar). These results are in good agreement with the earlier[10] assumption concerning the possibility of shifting the specific recognition of dCTP substituted at the exocyclic amino group by DNA polymerases from C- to T-type following an increase in the electronegativity of the exocyclic substituent. The efficiency of elongation of the initial primers by the photoreactive nucleotide analogs (W_d) was 68—86% (see Table 1), which is sufficient for subsequent experiments on the affinity photomodification of components of the protein—nucleic acid complexes *in situ* without preliminary separation of the affinity photoreagents obtained from the original primers.

Direct and sensitized photomodification of primer—template DNA polymerase β complexes

The efficiency of covalent cross-linking of the photolysis products of arylazido derivatives of the primers to components of the primer—template DNA polymerase β complexes and other detectable products were estimated by electrophoresis in a polyacrylamide gel (PAAG).[2] The photomodification rate was estimated preliminarily from the time dependence of the yield of primer—enzyme covalent adducts (w_R) under conditions where DNA is predominantly complexed with the DNA polymerase. The results obtained for all affinity photoreagents were approximated by first-order kinetic equations whose kinetic parameters are presented in Table 1. The half-modification times ($\tau_{1/2}$) of DNA polymerase β proved to be relatively low, which made it possible to determine the efficiency of modification of the target after irradiation of the reaction mixtures for 15 min, which is much longer than $\tau_{1/2}$. In addition, the affinity photomodification of the DNA polymerase β primer—template complexes, which contain the FAP and FABO residues, in the presence of pyrene-containing sensitizer Pyr-6-dUTP under conditions selected previously for sensitized photomodification using similar FAB-containing complexes was studied. The principal idea of this approach is as follows[7] (Fig. 1, *a*). The radioactively labeled photoreactive DNA primer complexed with the DNA template synthesized *in situ* is bound in the DNA-binding site of the DNA polymerase, while the dTTP analog containing a pyrene residue (sensitizer) is bound in the dNTP-binding site of the enzyme. The reaction mixture is irradiated under specially selected conditions (λ = 365—450 nm) in which the photoreagent is activated insignificantly. The energy is first absorbed by the photosensitizer and then transferred (probably, through an electron transfer) to the arylazido group of the reagent only within the photoreactive primer—DNA polymerase—photosensitizer ternary complex. This results in covalent binding of the primer to the enzyme. The efficiency of energy transfer decreases with an increase in the distance between the sensitizer and the photoreactive

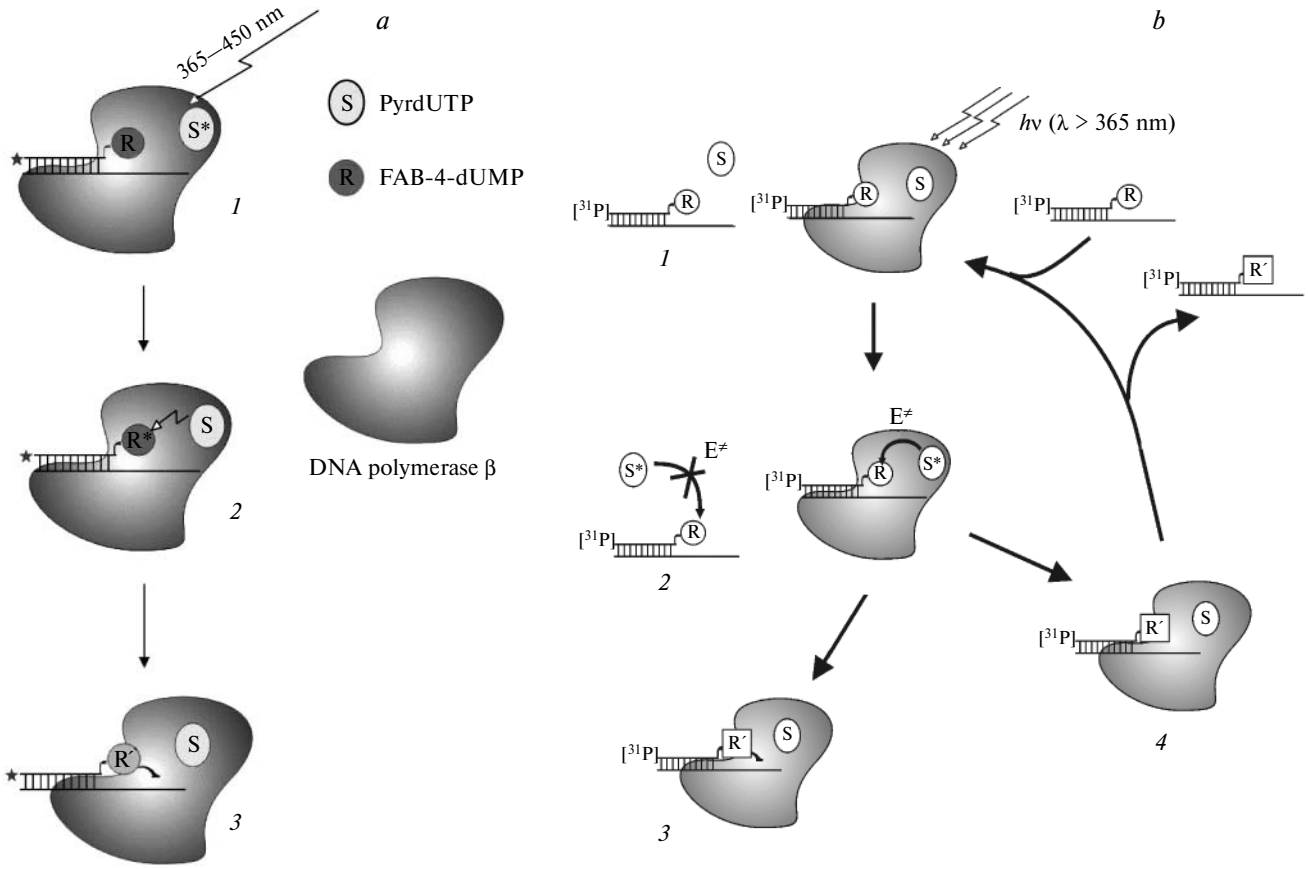
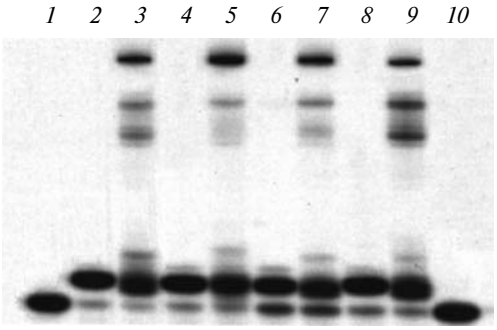


Fig. 1. Sensitized photomodification (*a*) and effective sensitized modification (*b*); *a*: (*1*) photosensitizer excitation; (*2*) energy transfer from the photosensitizer to the photoreagent; (*3*) covalent attachment of the primer to DNA polymerase; *b*: (*1*) reagent and sensitizer binding to the target, sensitizer excitation; (*2*) energy transfer from the photosensitizer to the photoreagent during UV irradiation; the reagent is not photolyzed in the solution; no energy transfer takes place; (*3*) covalent attachment of the primer to the enzyme; (*4*) if the reagent has not been attached to the target after photolysis, there is a probability of exchange with a reactive reagent from the solution during irradiation.

group.[5] In the case of the sensitized modification, the reaction mixtures were irradiated for 30 min, which is also much longer than the corresponding half-modification times (see Table 1).

A comparison of the kinetic parameters of the modification of DNA polymerase β (with excess enzyme and the absence of a sensitizer) by DNA primers bearing the FAB group[1] with the results of this study demonstrates a typical acceleration of the latter process due to the presence of Pyr-6-dUTP despite the milder irradiation conditions (see Table 1). For the photoaffinity reagents based on FABC-dUTP, FABO-dCTP, and FAP-8-dUTP, a reverse relationship of the rate constants was observed. Most probably, in these cases, modification is due to two parallel processes: direct UV activation of the photoreactive DNA primer and the sensitized activation. These data are of obvious interest as regards the influence of dNTP derivatives bound in the active site on the direction of photoinduced reactions in this type of complexes.

Figure 2 shows the autoradiographs of the electrophoretic separation in 20% PAAG of the affinity photomodification products of biopolymers by reactive DNA primers containing 3′-terminal FABC-dUMP, FABO-dCMP, and FABC-dCMP residues (lanes *3*, *5,* and *7*, respectively). It is noteworthy that the product composition was the same both with and without a sensitizer, the corresponding autoradiographs being indistinguishable, except for the intensities corresponding to particular products (not shown). In each case, several groups of products of photoinduced reactions of aryl azide primer derivatives were found, namely, photocross-links with the DNA polymerase β (see Fig. 2, group of bands I) and with the DNA-template (group of bands II), and products with a mobility lower than and similar to that of photoreactive primers (group of bands III and IV, respectively). The group of bands V corresponds to the original DNA primer. The efficiency of formation of each group of products was determined as the fraction of the photoaffinity reagent



FABC-dUTP	–	+	+	–	–	–	–	+	+	+
FABO-dCTP	–	–	–	+	+	–	–	–	–	–
FABC-dCTP	–	–	–	–	–	+	+	–	–	–
*PT1	+	+	+	+	+	+	+	–	–	+
*PT2	–	–	–	–	–	–	–	+	+	–

Fig. 2. Gel autoradiograph after separation of the photomodification products in 20% denaturing PAAG in the presence of 7 *M* urea: (I) modification products of DNA polymerase β, (II) modification products of the DNA template, (III) monomeric products, (IV) elongated DNA primer, (V) initial DNA primer.

consumed for its formation (W_R, W_{DNA}, and W_X, respectively, see Table 1). The group of bands IV appears compact and is similar in mobility to the original photoreagent. Apparently, this is formed by the primers containing arylazido photoreduction products or the products of arylnitrene reactions with water and monomeric components of solutions. Group III is composed, most probably, of the cross-linking products with dNTP derivatives bound in the dNTP-binding site (irradiation was carried out in the presence of ~10 μ*M* photoreactive dNTP analogs or with the addition of 100 μ*M* Pyr-6-dUTP). The assumption that group III includes cross-linking products with dNTP derivatives is in good agreement with the fact that the W_X value sharply increases when the photomodification is carried out in the presence of Pyr-6-dUTP. In this case, Pyr-6-dUTP is used at a concentration of 100 μmol L^{-1}, which nearly saturates the dNTP-binding site, being located in the close proximity of arylnitrene residues and functioning as a potential target.

The general efficiency of covalent cross-linking was evaluated for the sum of group I, II, and III products ($W_{DNA+X+R}$). It can be seen from Table 1 that in the case of PT1, the $W_{DNA+X+R}$ values were rather similar (~50—56%) for all photoaffinity reagents containing FABO groups both in the absence and in the presence of Pyr-6-dUTP. Together with the results of earlier studies, these data reveal a number of factors influencing the $W_{DNA+X+R}$ value and the distribution of the photoinduced cross-links of the components of DNA polymerase β complexes with the photoreactive DNA duplex. As noted above, the FAP and FABO groups were selected due to the fact that they ensured higher yields of photoinduced cross-links of photoreactive DNA primers to DNA polymerases than other arylazido groups. The distribution of covalent photocross-links among the protein molecules and the nucleotide components might be affected by different reactivities of arylnitrenes generated in the photolysis of aryl azides. The photochemical properties of aryl azides have been studied using the pyridine ylide method.[11,12] It was shown that the yield of the singlet nitrene products in the photolysis of aryl azides decreases in the series 2,3,5,6-tetrafluoro-4-azidobenzoic acid > > 2,3,5,6-tetrafluoro-4-azidobenzaldehyde > *p*-azidotetrafluorobenzaldehyde hydrazone ≫ nonfluorinated aryl azides. When the complementary-addressed photomodification of oligodeoxyribonucleotides was carried out under conditions optimal for photocross-linking of the complementary oligonucleotides (the photoreactive groups were located in the close vicinity of the guanine bases of the DNA-targets), the yields of the photoinduced cross-links of the DNA chains decreased in the same sequence.[12] This decrease in the reactivities of arylnitrenes with respect to DNA bases could hardly affect the efficiency of photocross-linking with more nucleophilic amino acid residues of DNA polymerase, *i.e.*, a slight decrease in the reactivity of arylnitrenes could be optimal for redistribution of the photocross-links from DNA to DNA polymerase β and could result in a higher yield of covalent adducts with DNA polymerase compared to that reported previously.[2] A similar, although less pronounced trend has been observed in a comparative study of the photomodification in the primer template complexes of the DNA polymerase of *Thermus thermophilus*.[1] Nevertheless, these effects cannot be attributed unambiguously to the chemical nature of the generated arylnitrenes alone, since the aryl azide linker groups differed in the length and the rigidity. The latter characteristics could also influence the position of these groups in the complexes and largely dictate the photocross-linking pattern.

As noted above, here we investigated the effect of another chemical parameter, namely, the replacement of the guanine base highly reactive with respect to singlet nitrene by less reactive bases (adenine and thymine) in the DNA-template sequence in the vicinity of the photoreactive group of the 3′-terminal nucleotide of the DNA primer. In particular, the efficiency of modification of various targets using a 36-mer DNA-template (PT1) was studied with respect to the FABC-dUTP analog (see Fig. 2, lanes *7*, *8*). The results imply (see Table 1) that the use of PT2 containing dGMP in the (n + 2)nd position of the template chain results in higher W_{DNA} (21%) than the use of DNA-template PT1 containing dTMP in the same position (W_{DNA} ≈ 13%). This is consistent with earlier data[13] on higher nucleophilicity of guanine compared to other nucleosides. The W_R value decreases when PT2 is used, indicating that the process is competitive. A threefold increase in W_X (from 5 to 15%) and a substantial

decrease in W_{DNA} (from 21 to 13%) were observed. When the photomodification was conducted in the presence of Pyr-6-dUTP, no photocross-linking with the template was detected, while the W_X value increased to 38% and W_R decreased to 17%. This suggests that only redistribution of covalent cross-links between the targets takes place, but no significant change in the total fraction of photocross-links in the products of arylazide photolysis. In other words, the decrease in the number of cross-links with the template caused by its lower reactivity with respect to nitrenes brings about switching of the reaction to the analog bound in the dNTP-binding site. This is possible due to the fact that for photoreactive analog concentrations used for the elongation *in situ* ($\sim 10^{-5}$ mol L^{-1}), a substantial portion of the enzyme may form complexes of the type photoreactive primer—template—enzyme—dNTP analog. The dTTP analogs fixed in the dNTP-binding site of the enzyme could participate in photoinduced reactions as both targets and photoreagents, thus becoming covalently attached to the labeled primer. The replacement of the highly reactive base G by T could favor both pathways of formation of group III products (see Table 1). With this in mind, it appears quite reasonable that the displacement of the photoreactive analog by the Pyr-6-dUTP derivative can induce a sharp additional increase in the yield of group III products (from 15 to 38%) due to disappearance of photocross-linking products with the template and substantial reduction of the photocross-linking with the enzyme. This increase in W_X as compared with that for the photoreactive dNTP may be due to the fact that the pyrenyl group is a rather good target for the formation of covalent adducts in the reactions with singlet nitrenes, and the activation method knowingly ensures the needed proximity of the components at the instant when singlet nitrene is generated.

The presence of Pyr-6-dUTP entails, in all cases, an abrupt re-direction of the photoinduced reactions toward group III products, which result most likely from the photocross-linking of primers and Pyr-6-dUTP; this also appears quite reasonable from the standpoint of elongation mechanism of the DNA primer.

In conclusion, it is important to mention that photoreactive DNAs based on the FABO-dCTP and FABC-dCTP analogs ($W_R \approx 47$ and 44%, respectively) proved to be most efficient for DNA polymerase modification. The photoreactive group of these analogs was attached to the exocyclic amino group of dCTP. Previously,[2] we have used FABO-dCTP and FABC-dCTP as dCTP analogs. The efficiency of modification of DNA polymerase β reached 25 and 3% for FABO-dCTP and FABC-dCTP, respectively. The increase in the modification efficiency of DNA polymerase β observed in this study is attributable to the use of a different primer—template complex, which results in a more favorable orientation of the reactive species for modification of the amino acid acceptor. The resulting W_R values are more than twice as great as those found in the previous study[1] using diverse photoreactive dUTP derivatives. The major distinguishing feature of the analogs we studied and, apparently, the main reason for the increase in the yield of covalent adducts is the presence of the electron-withdrawing —CH=N—O— group conjugated with aryl azide.

The photoreactive DNA that was obtained in the presence of FABC-dUTP containing a chemically similar group but attached at position 5 of the dTTP base residue was much less efficient in the modification of DNA polymerase β ($W_R \approx 28\%$). This attests to a pronounced influence of the position of the substituent in the heterocyclic base on the efficiency of enzyme modification.

Highly efficient sensitized modification of DNA polymerase β

The possibility of increasing the efficiency of DNA polymerase modification using a binary system of photoaffinity reagents was first noted by Lebedeva *et al.*[5] The association—dissociation processes in the DNA polymerase complex with DNA may result in the exchange of the photolyzed reagent, that has not formed a covalent cross-link with the enzyme, with the intact photoaffinity reagent from the solution. As a consequence, subsequent irradiation can additionally increase the yield of the photocross-linking products with the enzyme (see Fig. 1, *b*). The kinetic dependences of the accumulation of covalent adducts with the enzyme on the irradiation time (Fig. 3, *a* and *b*) in the presence of a sevenfold excess of the photoaffinity reagent (containing a FAB or a FAP group) with respect to DNA polymerase β clearly indicate that sensitized modification takes place only in the former case.

To study in detail the potential of this approach, we determined the efficiency of modification of biopolymers in the presence of excess enzyme, *i.e.*, under conditions used to determine the efficiency of covalent attachment of photoreactive DNA in the absence of a sensitizer. We considered analogs (FABO-dCTP, FABC-dUTP, FAP-8-dUTP, and FAB-4-dUTP) whose introduction into DNA ensures the highest efficiency of direct modification of DNA polymerase β, according to the results of this work and in comparison with FAP-8-dUTP and FAB-4-dUTP described previously.[1] The photomodification of the primer—template complexes formed by DNA polymerase β and DNA polymerase of *Thermus thermophilus* using PT2, elongated by FAB-4-dUTP and FAP-8-dUTP, and a number of other photoreactive dUTP analogs have been analyzed in sufficient detail in the previous study.[1] With allowance for the data outlined above, we estimated the efficiency of photocross-linking with the enzyme during sensitized photomodification under

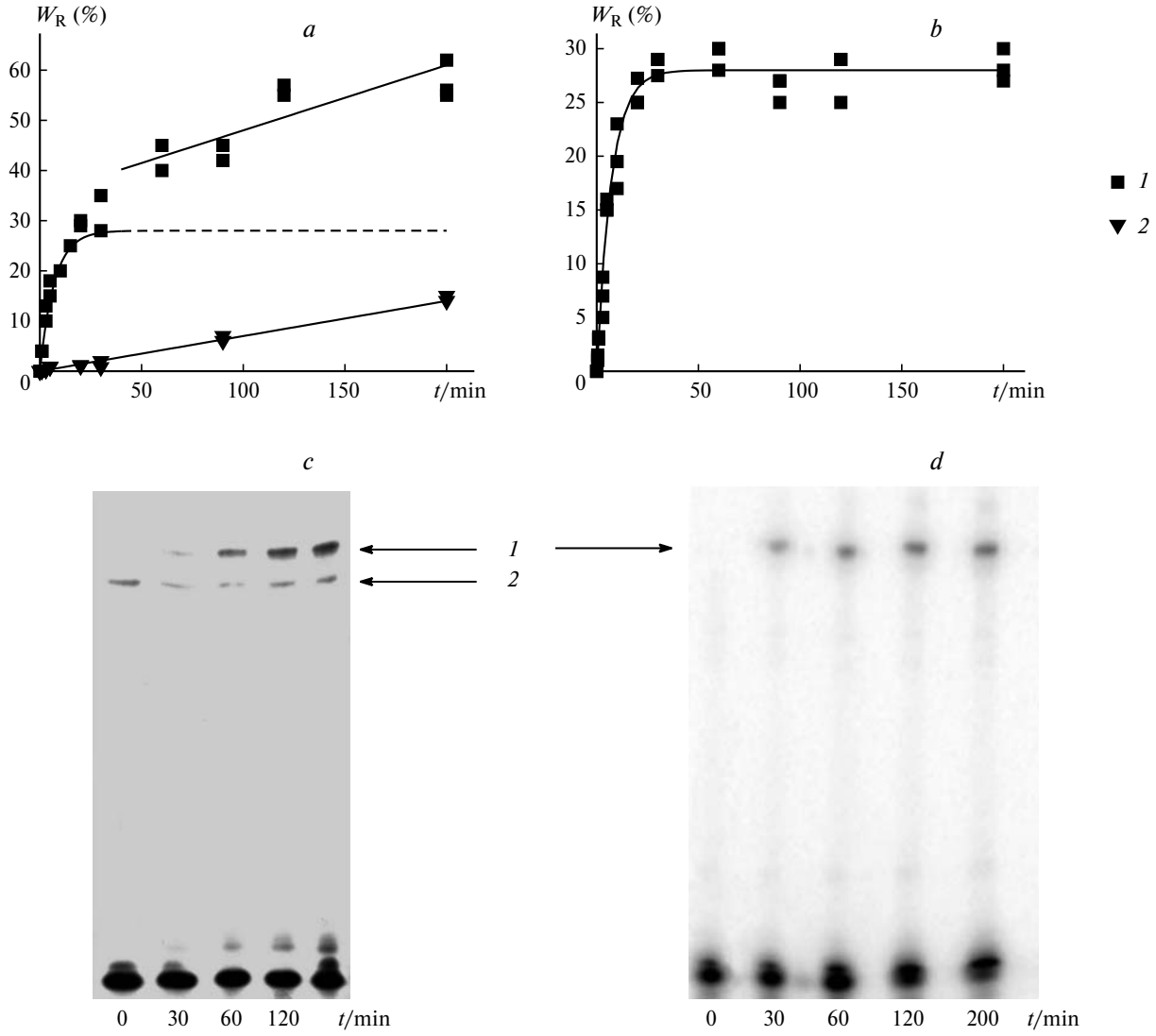


Fig. 3. Yields of modification products of DNA polymerase β by the DNA primer obtained in the presence of FAB-4-dUTP (*a*) and FAP-8-dUTP (*b*) *vs.* time under conditions of sensitized modification (UV irradiation with λ = 365—450 nm) in the presence (*1*) and in the absence (*2*) of the Pyr-6-dUTP sensitizer and identification of the products of Pyr-6-dUTP sensitized photomodification of DNA polymerase β by the DNA primer obtained in the presence FAB-4-dUTP: (*c*) the gel after product separation was silver stained, (*d*) gel autoradiograph; (*1*) modified DNA polymerase β, (*2*) DNA polymerase β. The electrophoretic separation of the products was carried out in 10% denaturing PAAG in the presence of 0.1% sodium dodecyl sulfate. The dashed line in Fig. *a* designates the standard first-order kinetic curve.

conditions that currently appear more appropriate for attaining high yields of photocross-links with the enzyme, namely, using the PT1 primer—template system and Pyr-6-dUTP as the sensitizer. As noted above, all of the photoreagents studied, except for that obtained in the presence of FAB-4-dUTP, retarded the formation of covalent adducts in comparisom with that observed in the direct modification (see Table 1). Meanwhile, the highest yield of covalent adducts with DNA polymerase β (W_R = 27%) was attained using FAP-8-dUTP. Thus, binary systems based on these dUTP derivatives can be considered as initial systems for the development of a highly efficient protein-specific sensitized photomodification procedure.

Despite the fact that in the absence of a sensitizer, the FAB-4-dUTP-based photoreagent did not exhibit so high modification efficiency as FAP-8-dUTP, the tentative experiments in which the photoreactive DNA was present in an excess with respect to the enzyme (7 : 1) demonstrated a highly efficient (Pyr-6-dUTP-sensitized) modification of DNA polymerase β. On long-term (200 min) irradiation of the reaction mixtures (see Fig. 3, *a*, *c*, *d*) in the presence of FAB-4-dUTP, about 60% of DNA polymerase β was cross-linked to the photoreactive primer.

The high level of formation of covalent adducts of DNA polymerase β with DNA was additionally confirmed by silver staining of the gels after separation of the reaction mixtures (see Fig. 3, *c*). It should be noted that the index of modification efficiency W_R is now represented by the ratio of DNA polymerase β bound covalently to the primer to its original amount. Thus, the W_R values were calculated on the basis of the initial concentrations of the reagent and the enzyme and the fraction of the covalently attached DNA primer.

The high modification efficiency with FAB-4-dUTP is due to the fact that the modification kinetics differs from the usual first-order kinetics. It can be seen from Fig. 3 that after irradiation for 20—30 min, the kinetic plot of the formation of the reaction products does not reach a plateau but has one more linear section, the amount of adducts of photoreactive DNA with DNA polymerase β increasing with time. However, this was not observed when FAP-8-dUTP was used (see Fig. 3, *b*).

The results altogether suggest that in the case of FAB-4-dUTP, the process follows the previously proposed scheme[5] according to which no noticeable photolysis of the reagent takes place in the solution, and the efficiency of modification of DNA polymerase β increases on long-term photolysis owing to the DNA exchange. The lower modification efficiency in the case of FAP-8-dUTP is also consistent with this scheme. The spectroscopic characteristics of FAB-4-dUTP (λ_{max} = 255 nm)[14] and FAP-8-dUTP (λ_{max} = 300 nm)[3] suggest that on irradiation with light at 365—450 nm in the presence of FAP-containing reagents, the difference between the rates of photolysis in solution and in the photoreactive DNA—DNA polymerase—photosensitizer ternary complex would be much lower than for the FAB-containing reagents. This would result in a pronounced photolysis of the free reagent outside the complex and a decrease in the efficiency of modification of DNA polymerase due to both nonproductive consumption of the reagent and the competition of the photolyzed and intact primer—template complexes for binding to the enzyme. Since the spectroscopic characteristics of other analogs are similar to those of FAP-8-dUTP,[10] there are grounds for assuming that with Pyr-6-dUTP as the sensitizer under these irradiation conditions, an increase in the modification efficiency associated with the use of the sensitizer is hardly possible.

Photoaffinity modification of the cell extract proteins

The photoaffinity modification with photoreactive dNTP is used rather widely to study protein—nucleic acid interactions in supramolecular DNA replication and repair complexes.[5,15—19] In particular, it was shown[5,17] that sensitized photoaffinity modification allows selective enhancement of the modification of DNA polymerase β.

This approach, in combination with additional techniques, can be used to identify new proteins in cell extracts that specifically interact with photoreactive DNA simulating intermediates of DNA replication and repair (see, for example, Ref. 15). High yields of the protein—nucleic acid covalent adducts are very significant for this purpose if subsequent identification of the protein modification products, for example, by MALDI mass spectrometry is envisaged. In these studies, photoreactive DNAs were obtained *in situ* by incorporation of photoreactive dNMP residues by endogenous DNA polymerases of extracts whose concentrations are relatively low. Therefore, the efficiency of the synthesis of photoreactive DNAs in extracts depends on the substrate properties of the analogs used. The efficiency of formation of protein—nucleic acid covalent adducts, together with the photochemical characteristics of the analogs used largely depends on the degree of conversion of the initial DNA into a photoreactive one, because both types of DNA can compete for binding to proteins. In cell extracts, the low catalytic efficiency of dNTP analogs may prove to be a critical parameter. To estimate the prospects of using dNTP analogs in cell extracts, the efficiency of *in situ* incorporation of three dTTP analogs (FABC-dUTP, FABO-dCTP, and FABC-dCTP) into DNAs under the action of endogenous DNA polymerases of the HeLa cell extract (see below) was studied. The photoreactive DNA synthesized in the extract were used to modify DNA-binding proteins of the cell extract and the efficiency of protein modification was estimated.

Two analogs, namely FABC-dUTP and FABO-dCTP, ensured a comparative efficiency of protein modification (Fig. 4, *a*, lanes *1*, *2*, *4*), whereas with FABC-dCTP, almost no modification products were detected (see Fig. 4, *a*, lane *3*). This analog possesses the poorest substrate properties (see Table 1). This substance was found to exhibit the lowest catalytic efficiency $F = V_{max}/K_M$. Hence, at low concentrations of the DNA polymerase in the cell extract, the elongation of the primer could be incomplete. Indeed, analysis of the products of primer elongation by cell extract DNA polymerases shows that the efficiency of incorporation of FABC-dCTP is very low (see Fig. 4, *b*, lanes *7*, *8*) compared to FABC-dUTP or FABO-dCTP. Poor substrate properties of FABC-dCTP make this derivative almost inapplicable for the use in cell extracts as the dTTP analog. In a model system with purified DNA polymerase β under conditions ensuring total primer elongation with the enzyme present in an excess with respect to DNA, FABC-dCTP ensured an efficient modification of the enzyme. About 44% of photoreactive DNA containing FABC-dCMP was attached to DNA polymerase. The derivative FABC-dUTP, which manifested lower modification efficiency than the

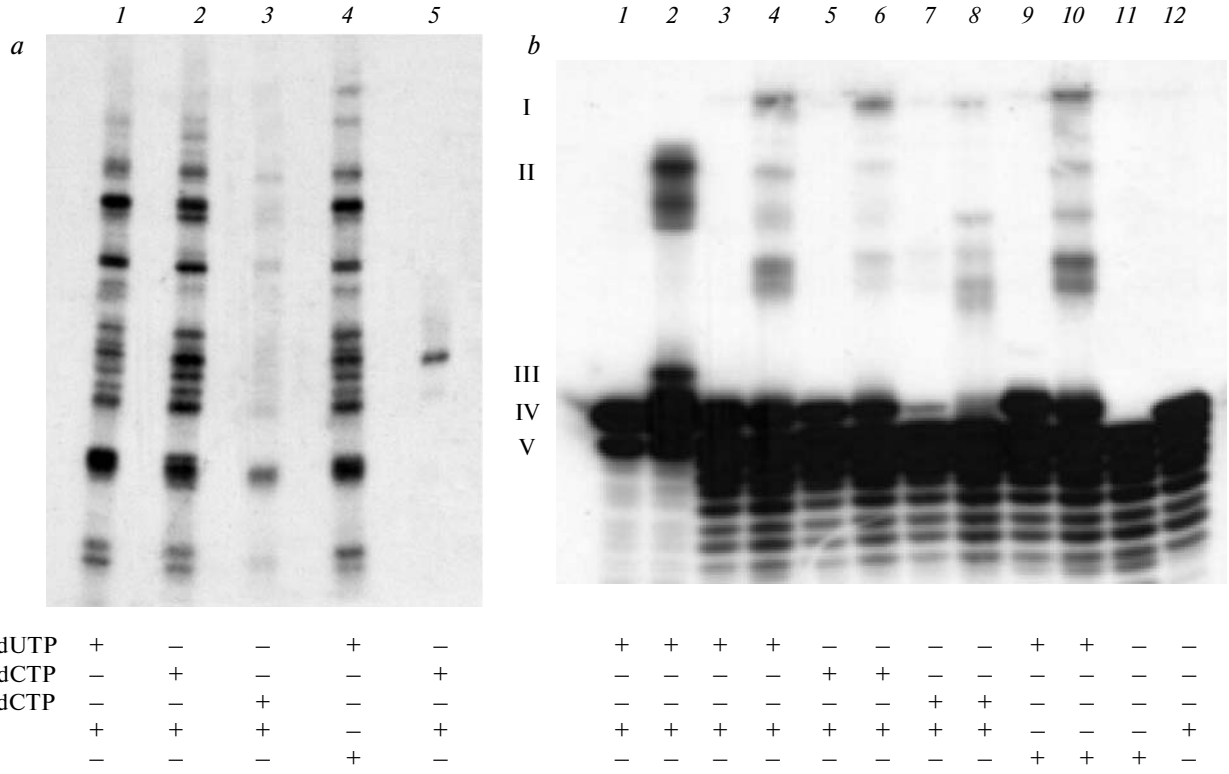


Fig. 4. Gel autoradiograph after electrophoretic separation of the photomodification products: (*a*) the separation was carried out in 10% denaturing PAAG in the presence of 0.1% sodium dodecyl sulfate; lane *5* presents the control run on the modification of DNA polymerase β; (*b*) separation in 20% denaturing PAAG in the presence of 7 *M* urea; (I) products of modification of cell extract proteins, (II) products of DNA modification, (III) monomeric modification product, (IV) elongated DNA primer, (V) initial DNA primer; (*1*, *2*) DNA polymerase β, (*3*—*10*) cell extract.

two other analogs in a model system but had the best substrate properties of the three analogs studied, was effective for the use in the cell extract.

It is noteworthy that the ratio and the set of protein modification products are somewhat different for different analogs, which is most probably due to different orientations of the reacting photoreactive group and acceptors in particular complexes. Further development of the photoaffinity modification technique and study of the factors influencing the modification selectivity would allow choosing the reagents optimal for modification of particular target proteins.

Experimental

The following materials were used: the 36-mer template (*1*) 5′-GGTTAAATAAAATAGTAATAATGTATAGCCCCT ACC-3′, 36-mer template (*2*) 5′-GGTTAAATAAAATAG TAAGAATGTATAGCCCCTACC-3′, and 16-mer primer 5′-GGTAGGGGCTATACAT-3′ oligonucleotides (prepared by the group of Oligonucleotide Synthesis of the Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the RAS); T4-polynucleotide kinase (SibEnzyme); [γ-^{32}P]-ATP with a specific activity of 80 MBq mmol^{-1} (Biosan); EDTA, TEMED, ammonium persulfate, the Xylene Cyanole and Bromophenol Blue dyes (Sigma); X-ray film (Fuji); *N*,*N*′-methylenebisacrylamide (BioRad); acrylamide, formamide (ICN); $MgCl_2$, NaCl (special purity grade); 2-mercaptoethanol (Fluka); Rainbow molecular mass markers (Amersham); tris(hydroxymethyl)aminomethane (Tris) (Sigma); and DE-81 DEAE-paper (Whatman). The rat recombinant DNA polymerase β was isolated as described previously.[2] The HeLa cells were kindly provided by M. A. Zenkova (the Laboratory of Biochemistry of Nucleic Acids, Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the RAS). The extract from HeLa cells was prepared by a reported procedure.[20] The reactive dNTP analogs, FABC-dUTP, Pyr-6-dUTP, and FAP-8-dUTP, were synthesized as described previously;[3,21,22] FABO-dCTP and FABC-dCTP were prepared by a known method.[10] The UV-irradiation was carried out by passing the light from a DRK-120 high-pressure mercury lamp: a VIO-1 illuminator (Lomo, St. Petersburg), a 110 mm distance: (*A*) through a UFS-6 light filter at 313—365 nm with the incident light intensity $I = 8 \cdot 10^{14}$ quant. s^{-1} cm^{-2} and (*B*) through a combination of BS-7 and FS-1 light filters at λ = 365—450 nm with the incident light intensity $I = 5.5 \cdot 10^{14}$ quant. s^{-1} cm^{-2}.

^{32}P-labeling of the 5′-end of the primer was carried out by a previously reported procedure.[23] The labeled oligonucleotides were purified as described previously.[24]

Determination of the steady-state kinetic parameters for the incorporation of the first nucleotide into the primer 3′-end by DNA polymerase β for photoreactive dNTP analogs. The incor-

poration of the nucleotide into the 3′-end of the primer was carried out in a reaction mixture (60 μL) containing the enzyme (20 n*M*), various concentrations of a dNTP analog ($1 \cdot 10^{-5}$—$2.2 \cdot 10^{-4}$ mol L^{-1}) or a dTTP analog ($1 \cdot 10^{-6}$—$4 \cdot 10^{-5}$ mol L^{-1}), 1 μ*M* 5′-[^{32}P]-labeled primer, 1.5 μ*M* DNA-template (*1*) or (*2*), and standard components: 50 *mM* tris-HCl, pH 8.6 (at 25 °C), 50 m*M* NaCl, 10 m*M* $MgCl_2$, 2.5% glycerol, and BSA (0.2 mg mL^{-1}). The incubation was carried out at 37 °C. At regular time intervals (0—60 min), 5-μL aliquots were withdrawn. The reaction was terminated by adding a solution containing 0.02% Bromophenol Blue, 90% formamide, and 50 μ*M* EDTA and the samples were heated for 2 min on a water bath at 95 °C. The reaction products were analyzed by electrophoresis in 20% denaturing PAAG in the presence of 7 *M* urea.[22] The positions of the ^{32}P-labeled oligonucleotides were determined by autoradiography. Bands corresponding to the elongation products and the initial primer were cut out of the gel. The sample radioactivity was determined according to Cherenkov. The K_M and V_{max} values were found from the dependences of the initial reaction rates on the dNTP concentration.

Synthesis of photoreactive DNA primers *in situ* and determination of the degree of primer completion. The reaction mixtures (10 μL) contained a 10 μ*M* solution of a dNTP analog, 0.7 μ*M* [^{32}P]-labeled primer complexed with the DNA-template (*1*) or (*2*), and 3 μ*M* DNA polymerase β, and also the standard components mentioned in the previous section. The photoreactive DNA primer was synthesized for 30 min at 25 °C. The degree of primer completion (W_d) was determined by analysis of reaction mixtures as described in the previous section. The W_d values were calculated as the ratio of the amount of elongated primer to the total amount of oligonucleotide.

Photoaffinity modification of DNA polymerase β and DNA-template by photoreactive primers. Determination of the fraction of the DNA primer covalently attached to DNA polymerase β. The photoaffinity modification of DNA polymerase β and DNA-template by photoreactive primers was carried out immediately after elongation of the primer, as described in the previous section. The reaction mixtures (20 μL) were irradiated for 15 min under conditions of experiment *A*. After irradiation, two 10-μL aliquots were withdrawn from the reaction mixtures. One was subjected to electrophoretic separation in 20% PAAG under denaturing conditions for the analysis of nucleic acids;[25] the second aliquot was analyzed by electrophopresis according to Laemmli to separate the protein components.[26] The radioactivity of the reaction products was determined as described earlier. In the analysis of the second aliquot, the apparent efficiency of modification of DNA polymerase β (w_R) was determined as the fraction of the primer attached covalently to DNA polymerase β divided by the total radioactivity of the initial DNA.

Kinetics of the photoaffinity modification of DNA polymerase β. To verify the fulfillment of the kinetics of the photoaffinity modification of DNA polymerase β, the rate constants for the modification (k) and the half-modification times ($\tau_{1/2}$) were determined. During the irradiation of the reaction mixture (110 μL) as described in the previous section, 10-μL aliquots were withdrawn at regular intervals (0—900 s). The products of covalent addition of the primer to DNA polymerase β were separated from free DNA and template modification products by electrophoresis in 10% PAAG,[25] cut out of the gel, and processed according to Cherenkov. The rate constants for modification (k) were determined from the time dependence of w_R. The resulting plot was approximated by a first-order kinetic equation. The calculation was carried out using the Origin program. The half-modification time was calculated from the formula $\tau_{1/2} = (\ln 2)/k$.

Calculation of the efficiency of modification of DNA polymerase β (W_R), DNA-template (W_{DNA}), and the monomeric product (W_S). The efficiency of modification of DNA polymerase β was calculated from the formula $W_R = 100\% \cdot w_R/W_d$ (for definitions of W_d and w_R, see the previous sections). For more accurate calculation of the efficiency of modification of biopolymers, the correction $x = w_R \cdot (A_2 + A_3 + A_4 + A_5)/A_1$ was applied to take into account the loss of the modified protein that takes place during the separation of modification products by the above-described method.[25] The A_1, A_2, A_3, A_4, and A_5 values are the amounts of radioactive modification products I, II, III, IV, and V (see Fig. 2). This correction is the ratio of the efficiencies of protein modification determined by two methods.[25,26] Thus, the recalculated amount of the modified protein (A_1') equals $x \cdot A_1$. The corresponding modification efficiencies of DNA and the monomeric target were calculated as follows:

$$W_{DNA} = 100\% \cdot A_2/(A_4 + A_3 + A_2 + A_1'),$$

$$W_S = 100\% \cdot A_3/(A_4 + A_3 + A_2 + A_1').$$

Synthesis of photoreactive DNA by cell extract enzymes and photoaffinity modification of cell extract proteins. The incorporation of the photoreactive dNMP residues by cell extract enzymes was carried out in 12-μL reaction mixtures containing 3 μL of the extract, a dNTP analog in concentrations corresponding to $4K_M$, 0.7 μ*M* 5′-[^{32}P]-labeled primer—template complex (PT1 or PT2), 50 m*M* tris-HCl, pH 8.6 (25 °C), 10 m*M* $MgCl_2$, and 50 m*M* KCl. The mixtures were incubated for 10 min at 37 °C. The irradiation, separation of the reaction products, and visualization were carried out as described in the preceding sections.

Sensitized modification of DNA polymerase β and DNA-template by photoreactive primers in the presence of Pyr-6-dUTP. Determination of the fraction of the DNA primer covalently attached to DNA polymerase β and DNA-template. The sensitized modification of DNA polymerase β and DNA-template by photoreactive primers was carried out immediately after completion of the primer elongation, as described above. Pyr-6-dUTP was added to reaction mixtures (150 μL) up to a concentration of $1 \cdot 10^{-4}$ mol L^{-1} and irradiated under conditions of run *B*. During the irradiation, two 10 μL aliquots were withdrawn at regular intervals (0—30 min). One aliquot was subjected to electrophoretic separation in 20% PAAG under denaturing conditions to analyze nucleic acids;[25] the second one was analyzed by electrophopresis according to Laemmli to separate the protein components.[26] The subsequent processing of the data was carried out as described above. The modification efficiency was determined over a period $t \gg \tau_{1/2}$ (30 min).

In addition, the same experiments were carried out with excess photoaffinity reagent. The corresponding concentrations of DNA polymerase β and the primer—template complex were $1 \cdot 10^{-6}$ and $7 \cdot 10^{-6}$ mol L^{-1}. The irradiation time was 0—200 min. The gels thus obtained were additionally stained with silver, as described earlier.[27]

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