Rose Bengal Mediated Inhibition of DNA Polymerases: Mechanism of Inhibition of Avian Myeloblastosis Virus Reverse Transcriptase under Photooxidative Conditions†

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ABSTRACT: DNA polymerases from prokaryotic, eukaryotic, and oncornaviral sources are irreversibly inactivated upon exposure to Rose Bengal in the presence of light (photooxidation conditions). Inactivation of these enzymes under dark (nonoxidative) conditions is totally reversible [Srivastava, S. K., & Modak, M. J. (1982) *Biochemistry 21*, 4633–4639]. The primary effect of photooxidation on the enzyme–Rose Bengal complex was found to be the loss of template-primer binding ability within 5 min of exposure to light. The presence of template-primer but not the substrate deoxynucleotides consistently provided partial protection against Rose Bengal mediated photooxidative inactivation. Preformed enzyme–template-primer complexes were not dissociated by Rose Bengal under these conditions. However, prolonged exposure (25–30 min) of this complex to light in the presence of Rose Bengal led to complete inactivation of catalytic activity without affecting the ability of enzyme to bind to template-primer. These results clearly indicate that oxidative inactivation of avian myeloblastosis virus reverse transcriptase in the presence of Rose Bengal involves a domain within the enzyme that contains the template-primer binding site as well as an additional site which is required for the expression of both the polymerization and nuclease activities of the reverse transcriptase.

The enzymatic synthesis of DNA is a complex process that involves a series of interdependent reactions containing multiple components (Kornberg, 1980). The mechanism of catalysis and structure–function relationships in this class of enzymes is therefore not fully understood. In our attempts to clarify the mechanics of template-dependent polymerization of deoxynucleoside triphosphates (dNTPs),[1] we have used a variety of site-specific inhibitors of DNA polymerases and have investigated their mechanisms of inhibition (Modak, 1976a,b; Modak et al., 1980, 1982; Srivastava & Modak, 1980a,b). In some instances, we have identified the amino acid residues situated at the active site region of the enzyme that reacted with the inhibitor. Using Rauscher leukemia virus reverse transcriptase and AMV RT, we and others have shown that pyridoxal 5′-phosphate is a substrate binding site-directed reagent that reacts with lysine residues situated at that site (Modak, 1976a,b; Papas et al., 1977; Modak & Dumaswala, 1981). Simultaneously, we have also been investigating the function and/or participation of various known structural features of DNA polymerases, particularly reverse transcriptase, in the various catalytic reactions. We reported that both intrinsic zinc and sulfhydryl groups present in the enzyme protein are not required for the expression of ribonuclease H activity as well as the pyrophosphorolytic activity of AMV RT (Srivastava & Modak, 1980a,b; Modak & Srivastava, 1979). The present study is an extension of our long-term efforts to identify and define the various structural domains in DNA polymerases that are crucial for the expression of catalytic activity. In this context, we have examined the effect of a fluorescent dye, Rose Bengal (RB) on the enzymatic synthesis of DNA catalyzed by a variety of DNA polymerases under both oxidative and nonoxidative conditions. The choice of RB as a possible domain-specific inhibitor was based on the fact that a nucleotide-polymerizing enzyme, RNA polymerase from *Escherichia coli*, has been shown to be extremely sensitive to exposure to this dye (Ishihama & Hurwitz, 1969; Wu, C. W., & Wu, F. Y. H., 1973; Wu, F. Y. H., & Wu, C. W., 1973), and fluorescence studies of enzyme–dye interaction indicated that a hydrophobic region of RNA polymerase was a target of RB action (Wu, F. Y. H., & Wu, C. W., 1973). We have used AMV reverse transcriptase as a model enzyme in these studies and have reported the mechanism of its inactivation under nonoxidative conditions (Srivastava & Modak, 1982). The present paper details the results of oxidative inactivation of this enzyme by RB and the nature of the two functionally distinct sites involved in that inactivation.

Materials and Methods

Materials. All the radioactive dNTPs were obtained from New England Nuclear, Inc. Unlabeled triphosphates and template-primers were the products of P-L Biochemicals, Inc. The molar ratio (based on total nucleotide) of template-primer was 1:1 unless otherwise indicated. Phage fd DNA was purchased from Miles Laboratories; poly(dT) was from Collaborative Research, Inc.; Globin mRNA was obtained from Searle. Rose Bengal was procured from Sigma Chemical Co. Nitrocellulose filters (13-mm diameter) were obtained from Schleicher & Schuell (BA 85, 45 μm). Purified AMV RT was made available by the Division of Cancer Cause and Prevention, National Cancer Institute, through Dr. Joseph Beard. The DNA polymerases from viral or cellular sources were purified as described earlier (Modak & Marcus, 1977b; Modak, 1978; Srivastava & Modak, 1980a). fd DNA·[^{3}H]-RNA hybrid for RNase H assays was prepared as described before (Srivastava & Modak, 1980b).

DNA Polymerase Assays. Assays were carried out in a final volume of 100 μL and contained the following components: 20 mM Hepes (pH 7.8), 1 mM dithiothreitol, 10 μg of bovine serum albumin, 20 μM of appropriate [^{3}H]dNTP adjusted to

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[1] Abbreviations: AMV, avian myeloblastosis virus; dNTP, deoxynucleoside triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RB, Rose Bengal; RNase H, ribonuclease H; RT, reverse transcriptase; DTT, dithiothreitol; E-TP, enzyme–template-primer complex; DTNB, dithiobis(2-nitrobenzoic acid).

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a final specific activity of 1000 cpm/pmol, and 0.5 μg of desired template-primer. In addition to the above-mentioned standard components, different enzymes required different salt and divalent cation concentrations for synthesis directed by various template-primers. These conditions for AMV RT are as follows: 50 mM KCl and 5 mM $MgCl_2$ with poly(rC)·$(dG)_{12-18}$ and poly(dC)·$(dG)_{12-18}$; 100 mM KCl and 10 mM $MgCl_2$ with poly(rA)·$(dT)_{12-18}$; 100 mM KCl and 0.5 mM $MnCl_2$ with poly(dA)·$(dT)_{12-18}$. *E. coli* DNA polymerase I assays were performed with 100 mM KCl and 10 mM $MgCl_2$ with poly(dA)·$(dT)_{12-18}$ as a template-primer, while calf thymus DNA polymerase β was assayed in the presence of 100 mM KCl and 0.5 mM $MnCl_2$ with poly(rA)·$(dT)_{12-18}$ as template-primer.

Assays of terminal deoxynucleotidyltransferase were carried out with 0.5 μg of $(dA)_{12-18}$ as a primer, 1 mM $MnCl_2$ and 20 μM [^{3}H]dGTP as substrate (Modak, 1978, 1979).

Incubations were carried out at 37 °C for 30 min unless otherwise indicated, and the reactions were terminated by the addition of 5% (w/v) trichloroacetic acid (Cl_3CCOOH) containing 10 mM pyrophosphate. The acid-insoluble precipitate was collected on Whatman GF/B filters, washed extensively with Cl_3CCOOH, water, and finally with ethanol, dried, and counted in toluene-based scintillation fluid.

RNase H Assay. AMV RT associated RNase H assays were performed with either poly(dT)·[^{3}H](rA) or fd DNA·[^{3}H]RNA as substrate. Poly(dT)·[^{3}H](rA) was synthesized by using poly(dT) as a template with [^{3}H]ATP as substrate in a reaction catalyzed by *E. coli* RNA polymerase. Reaction conditions and the isolation of the product were identical with those described previously for the fd DNA·[^{3}H]RNA hybrid (Modak & Srivastava, 1979).

Conditions for RB-Mediated Photooxidation of DNA Polymerases. The preincubation mixture for photooxidation (10–20 μL) contained enzyme alone or together with desired concentrations of template-primer and/or dNTPs in the presence of appropriate concentrations of RB. Samples were exposed, at 4 °C, to a 200-W GE electric bulb at a distance of 10 cm. The control samples contained corresponding concentrations of RB but were kept on ice in the dark.

Binding of Enzyme–Template-Primer Complexes to Nitrocellulose Filters. The selective binding of template-primer–enzyme complexes (but not free template-primer) to nitrocellulose filters permits the evaluation of the ability of an enzyme to bind template-primer. Standardization of this assay for the determination of the template-primer binding activity of AMV RT has been previously described (Srivastava & Modak, 1982). All filter binding assays were performed in triplicate, and appropriate standards were always included to monitor the reproducibility of the technique.

Results

Sensitivity of Various DNA Polymerases to RB. A typical dose–response pattern of the effect of RB-mediated photooxidation on the activity of various DNA polymerases is presented in Figure 1. The individual enzymes in this experiment were preincubated with various concentrations of RB and the mixture exposed to visible light (a 200-W incandescent electric bulb) at a distance of 10 cm for a period of 5 min. Temperature during the operation was maintained at 4 °C or below. Duplicate tubes that were not exposed to light served as a control. An additional control in the form of enzyme exposed to light in the absence of RB was also included. It is clear from the results that addition of RB (at the concentrations used) in the absence of light or exposure to light alone has no adverse effect on the activity of any of the DNA polymerases (Figure 1). However, all the enzymes, namely, *E. coli* DNA polymerase I, calf thymus DNA polymerase β, AMV RT and its associated RNase H, and terminal deoxynucleotidyltransferase, were severely inhibited when exposed to light in the presence of RB (Figure 1). The shapes of the inhibition curves (Figure 1) and the I_{50} values for the various DNA polymerases (2–4 μM) are remarkably similar, suggesting a common mode of photooxidative inactivation of these enzymes. For all subsequent experiments, we have used AMV RT as a model enzyme.

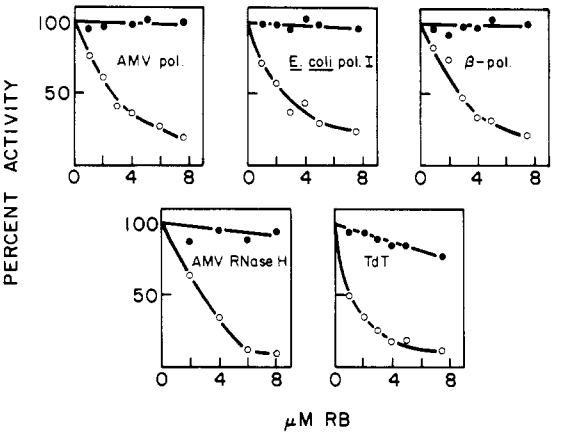

FIGURE 1: Dose–response pattern of the effect of Rose Bengal mediated inactivation of DNA polymerases under photooxidative conditions. A standard photooxidation mixture in a final volume of 10 μL contained 20 mM Hepes buffer (pH 7.8), 1 mM dithiothreitol, 2 μL of desired enzyme, and the indicated amount of RB. Photooxidation of samples was carried out for 5 min as described under Materials and Methods. A duplicate set of samples that was not exposed to light served as the control. At the end of photooxidation all the samples received 90 μL of appropriate reaction mixture that contained the desired template-primer, substrate, and divalent cation (see Materials and Methods). One hundred percent polymerization activities of the various enzymes ranged from 90 to 500 pmol of [^{3}H]dNTP incorporation into acid-insoluble material. One hundred percent activity for AMV RT associated RNase H corresponded to solubilization of 60 pmol of nucleotide from poly(dT)·[^{3}H](rA) substrate. The open circles represent photooxidized enzyme, and the closed circles indicate nonoxidized control samples.

Properties of RB-Mediated Inhibition of AMV RT. Rose Bengal inhibits AMV RT under both oxidative and nonoxidative conditions. However, there are important differences in the requirements to sustain the inhibitory effects of RB under the two conditions. The final concentration of RB in the reaction mixture is the most critical factor for the degree of inhibition under nonoxidative conditions, while the concentration of RB present during the exposure of enzyme to light (rather than in the reaction mixture) is the critical factor for the photooxidative type of inhibition. Thus, preincubation of enzyme with 10 μM RB under dark conditions followed by 10-fold dilution with standard reaction mixture (final effective concentration of RB = 1 μM) showed no inhibition of enzyme activity. However, exposure of the same preincubation mixture containing enzyme and RB to visible light resulted in the total loss of enzyme activity. The presence of high concentrations of inhibitor are required for sustained, yet reversible, inhibition of enzyme activity under nonoxidative conditions (see Table I). A detailed account of the mechanism of RB inhibition of AMV RT under nonoxidative conditions has appeared (Srivastava & Modak, 1982). A typical time course of the photooxidative inactivation of AMV RT as a function of the exposure time of enzyme–RB complex to visible light is presented in Figure 2. It is clear from the figure that photooxidative inactivation is not an instantaneous process and that an exposure of approximately 2 min is required for 50%

Table I: Inhibitory Effect of Rose Bengal on AMV RT DNA Polymerase Activity under Oxidative and Nonoxidative Conditions[a]

preincubation concn of RB (μM)	condition	final concn of RB in reaction mixture (μM)	act. (%)
none	oxidative and nonoxidative		100
10	oxidative	1	<1
none	nonoxidative	10	15
10	nonoxidative	1	98
100	nonoxidative	10	20

[a] Preincubation mixtures in a final volume of 10 μL contained 6 ng of AMV RT and the desired concentration of RB. Photooxidation was carried out as described under Materials and Methods, while nonoxidized samples were covered with aluminum foil (to protect them from light) and were kept on ice. Preincubation mixtures were then diluted with 90 μL of standard reaction mixture, and the DNA polymerase activity was of AMV RT determined by using poly(rC)·$(dG)_{12-18}$ as a template-primer. One hundred percent activity corresponds to 220 pmol of [^{3}H]dGMP incorporation into acid-insoluble material.

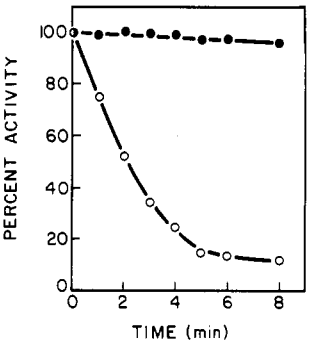

FIGURE 2: Rose Bengal mediated photooxidative inactivation of AMV RT as a function of oxidation time. A standard photooxidation mixture containing 5 μM RB was exposed to light, and 10-μL aliquots of that reaction mixture were withdrawn at the desired times for determination of DNA polymerase activity (○). Similarly derived aliquots from samples containing 5 μM RB but which were not exposed to light (●) were used for control activity measurements. One hundred percent activity corresponds to 220 pmol of dGTP incorporation in a poly-(rC)·$(dG)_{12-18}$ directed reaction.

inactivation of enzyme activity.

Effect of Template, Primer, and dNTPs on the RB-Mediated Photooxidative Inactivation of AMV RT. Since, during the catalytic reactions, DNA polymerase must bind to both template-primer and substrate dNTPs, it was important to determine if the presence of one or more of these components would have any protective effect against the photooxidative inactivation of AMV RT by RB.

(*a*) *Effect of Addition of Template-Primer.* Three synthetic template-primers, namely, poly(rA)·$(dT)_{12-18}$, poly(rC)·$(dG)_{12-18}$, and poly(dC)·$(dG)_{12-18}$ as well as globin mRNA·oligo$(dT)_{10}$ and [^{3}H]RNA·fd DNA hybrid, were individually added to photooxidation mixtures together with enzyme and RB. The activity of photooxidized enzyme was then determined with appropriate substrates. Results presented in Table II clearly show that all of the template-primers provided only partial (40–50%) but consistent protection from RB inactivation. The ribonuclease H activity associated with AMV RT was also protected to the same degree when RNA·DNA hybrid was present during photooxidation (Table II). The requirement for both template and primer for an optimal protective effect was evident from the observations that addition of either polynucleotide template or oligomeric primer provided significantly less protection. The protective effect shown by the addition of template-primer is presumably due to its binding to these components which would then protect the involved binding region from photooxidation. Indeed, if 0.5 M KCl is added to the enzyme–template-primer complex (a condition which dissociates this complex) during the photooxidation, no protective effect of the template-primer is observed (Table III).

(*b*) *Effect of the Addition of Substrate and Nonsubstrate dNTPs.* Addition of any one of the four dNTPs, in the absence of a template-primer, did not prevent the inactivation of AMV RT by RB mediated photooxidation (Table II). However, the presence of a template-primer together with dNTP complementary to the template strand consistently increased the resistance of AMV RT to photooxidation. The increase in the resistance amounted to approximately 10–20% over that afforded by template-primer alone. The concentration of dNTP, complementary to template nucleotide, needed to show the additional protective effect was rather high (1–2 mM, which is about 50-fold its K_m); however, identical concentrations of

Table II: Effect of the Presence of Template-Primer and dNTPs on the RB-Mediated Photooxidative Inactivation of AMV RT[a]

present in preincubation mixture		pmol of dNTP incorporation/15 min		inhibition (%)
template, primer, or template-primer (2 μg)	dNTP (2 mM)	control (RB)	RB + light exposure	
		425	<1	99
	dATP, dCTP, dGTP, or dTTP	410–550	3–15	≈95
poly(rC)·$(dG)_{12-18}$		465	195	58
poly(rC)		440	88	80
$(dG)_{12-18}$		410	62	85
poly(rC)·$(dG)_{12-18}$	dGTP	590	345	42
poly(rC)·$(dG)_{12-18}$	dTTP	450	180	60
poly(rC)	dGTP	480	95	80
poly(rA)·$(dT)_{12-18}$		520	208	60
poly(rA) or $(dT)_{12-18}$		505	95	81
poly(rA)·$(dT)_{12-18}$	dTTP	605	375	38
poly(rA)·$(dT)_{12-18}$	dGTP	410	130	68
poly(rA)	dTTP	520	120	77
poly(dC)·$(dG)_{12-18}$		420	210	50
globin mRNA·$(dT)_{10}$		177	87	50
fd DNA·RNA hybrid		24	12	50

[a] The preincubation mixture in a final volume of 20 μL contained 15 ng of AMV RT together with the desired concentration of template-primer and/or dNTP, 1 mM DTT, 50 mM KCl, and 5 mM $MgCl_2$. RB was added at a final concentration of 5 μM to this reaction mixture in duplicate. One set was then exposed to light for a period of 5 min (see Materials and Methods) while the other set was stored on ice in the dark. The enzyme activity of all the samples was then determined by the addition of an appropriate quantity of required components (final volume 100 μL) as described under Materials and Methods.

Table III: Effect of High Salt Concentrations on the Protective Effect of Template-Primer against Photooxidative Inactivation of AMV RT[a]

preincubation condition		activity (pmol/30 min)		
template-primer	KCl concn (mM)	control	+RB	inhibition (%)
none	50	185	5	97
none	500	188	7	96
poly(rC)·$(dG)_{12-18}$	50	159	65	59
poly(rC)·$(dG)_{12-18}$	500	145	5	97
poly(dC)·$(dG)_{12-18}$	50	270	120	55
poly(dC)·$(dG)_{12-18}$	500	320	30	90

[a] The preincubation mixture in a final volume of 10 μL containing 6 ng of AMV RT. 1 μg of desired template primer, 1 mM DTT, 5 mM $MgCl_2$, and either 50 or 500 mM KCl was exposed to light in the presence and absence of 5 μM RB. Enzyme activity was then measured by 10-fold dilution of preincubation mix with standard reaction mixture containing appropriate components. The final salt concentration in the reaction mixture was adjusted to 50 mM whenever necessary.

Table IV: Effect of RB-Mediated Photooxidation of AMV RT on the Template Binding Function[a]

oxidation condition	nitrocellulose filter binding (cpm)	template binding activity (%)
no oxidation (control)	15 000	100
enzyme + 10 μM RB	600	4
enzyme + template-primer complex + RB	13 700	92

[a] The ability of AMV RT to bind to template-primer was determined by selective retention of the complex on nitrocellulose filters. The conditions are described under Materials and Methods. Fifty nanograms of enzyme and 300 ng of poly(rC)·[^{3}H](dG) were used in each experiment. Results are expressed as the average of binding assays carried out in triplicate.

noncomplementary dNTPs were without any effect (Table II). Since high concentrations of dNTPs in a reverse transcriptase reaction have been shown to produce DNA products with significantly larger length than that produced at lower substrate concentrations (Haseltine et al., 1976), it was interesting to determine if both template and hydrogen-bonded primer were essential for the additional protective effect exhibited by complementary dNTP addition. Further analysis revealed that primer, hydrogen-bonded to template, was absolutely necessary in order to observe an increase in the protective effect by dNTPs against RB-mediated photooxidation of AMV RT (Table II). However, it was intriguing that the presence of divalent cation (obligatory for catalysis) was not required to exert the additional protective effect by dNTPs.

Effect of RB-Mediated Photooxidation of AMV RT on the Template Binding Activity. Direct determination of the template-primer binding activity of photooxidatively inactivated and control enzyme was carried out by using a millipore filter binding technique previously described (Srivastava & Modak, 1982). Results clearly indicated that photooxidized enzyme had lost its ability to bind to template primer. However, a preformed enzyme–template-primer complex did not dissociate when exposed to photooxidation (Table IV). The effect of RB described above was confirmed by an alternate method which was designed to measure the formation of catalytically active template-primer–enzyme complex during the polymerization reaction. For this purpose, a standard reaction mixture that contained poly(rC)·$(dG)_{12-18}$ as a template-primer and [^{3}H]dGTP as a substrate was incubated for a period of 3 min. Catalysis was then terminated by the addition of 10 mM EDTA, and the amount of newly synthesized (radioactive) template-primer bound to enzyme protein was determined by its selective retention on nitrocellulose filters. The bound or retained complex is considered to represent catalytically active complex. This complex could be completely dissociated by treatment with 0.5 M KCl and could be subsequently reassociated by merely diluting the reaction mixture containing salt by 5-fold. When RB is added, however, together with 0.5 M salt and photooxidation of the reaction mixture is carried out, no reassociation of the enzyme–template-primer complex upon dilution is observed (data not shown). These results clearly point out that addition of template-primer prior to photooxidation fully protects the enzyme with respect to its template binding activity. Yet it does not provide complete protection of catalytic activity. Therefore, it appeared that an additional site on AMV RT is also involved in RB-mediated photooxidation of AMV RT.

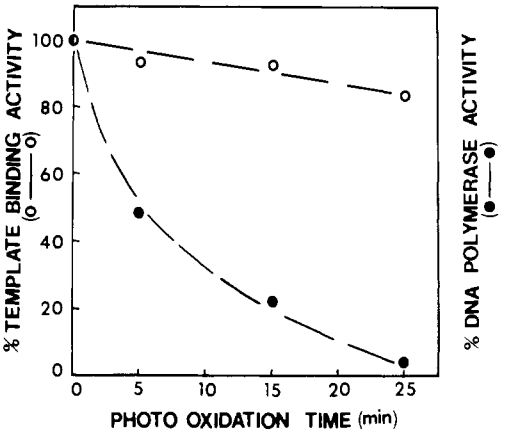

FIGURE 3: Effect of Rose Bengal on template binding and DNA polymerase activity of preformed template-primer–AMV RT complexes as a function of the time of photooxidation. The photooxidation mixture in a final volume of 50 μL contained 300 ng of AMV RT and 1 μg of poly(rC)·[^{3}H](dG) together with other standard components. After a 3-min preincubation at 25 °C which permitted optimal template-primer binding to enzyme, RB was added to a final conentration of 5 μM. Reaction mixtures were instantly chilled in ice and were exposed to light for various periods of time. At desired time intervals, a 5-μL aliquot of oxidized sample was removed for polymerization activity determination, and a 10-μL aliquot of the reaction mixture was diluted 3-fold with nitrocellulose filter binding assay buffer and then processed to determine the extent of template-primer binding as described under Materials and Methods. Enzyme similarly treated but not exposed to light served as a control. One hundred percent template binding activity was equal to 15 000 cpm equivalent retention of poly(rC)·[^{3}H](dG)–enzyme complex on a Millipore filter, while 100% DNA polymerase activity of enzyme aliquots was equivalent to 900 of pmol [^{3}H]dGMP incorporation with poly(rC)·$(dG)_{12-18}$ as template-primer.

Additional Target of RB-Mediated Photooxidation of AMV RT. In order to show that a site on AMV RT in addition to that involved in template-primer binding activity is indeed affected by the photooxidative process, we exposed a preformed enzyme–template-primer complex to photooxidation in the presence of RB for an extended period of time. Determination of both template binding activity and catalytic activity during this time was carried out. It was noted that complete inactivation of the enzyme present in the enzyme–template-primer complex occurred in 25 min, although during this time period no loss of the template-primer binding ability of the enzyme occurred (Figure 3). Addition of neither substrates nor PP_i prevented photooxidative inactivation, indicating that the target of photooxidation in the presence of template-primer was not the substrate binding site (data not shown).

Inactivation of Ribonuclease H Activity Associated with AMV RT by RB-Mediated Photooxidation. AMV RT associated RNase H activity utilizes the same template binding site, functionally, that is utilized in polymerase activity (Modak & Marcus, 1977a; Marcus et al., 1978). Its inactivation by RB-mediated photooxidation (Figure 1) therefore appears to be due to the impairment of the same primary target, namely, the template binding activity of AMV RT. The inclusion of RNA·DNA hybrid during the photooxidation has been found to provide partial protection (40–50%) similar to that found for polymerase activity by inclusion of template-primer (Table II). Complete inactivation of RNase H activity, similar to polymerase activity, has also been noted (data not shown) by exposure of the RNA·DNA hybrid–enzyme complex to light in the presence of RB for an extended period of time (25–30 min). Thus, the target site present in AMV RT when complexed to template-primer seems to be important for the expression of both nucleolytic and polymerization reactions.

Possible Targets of RB-Mediated Photooxidation on AMV RT. Several amino acid residues, e.g., histidine, cysteine, tyrosine, tryptophan, and methionine, have been implicated as possible targets of RB-mediated photooxidation in a variety of enzymes which have been found to be sensitive to RB (Weil & Buchert, 1953; Weil et al., 1951; Westhead, 1972). The major target of this dye in most cases has been reported to be specific histidine residue(s), and hence RB has been used as a histidine-specific reagent (Hoffee et al., 1967; Martinez-Carrion et al., 1967, 1970). The photooxidative histidine destruction in sensitive enzymes by RB exhibits a characteristic pH dependence where the maximal ionization of the imidazolyl ring occurs (Westhead, 1972; Tso & Zalkin, 1981). When inactivation of AMV RT by RB-mediated photooxidation was measured as a function of pH (ranging from 6.2 to 8.5 with increments of 0.2 pH unit), no classical optimum for inactivation was noted (data not shown), implying that histidine residues may not be the targets of photooxidation in this enzyme. Cysteine residue(s) present in AMV RT were considered to be another favorable target for photooxidation since this enzyme has been shown to require reduced sulfhydryl residues for the expression of activity (Gorecki & Panet, 1978; Parnaik & Das, 1981). The photooxidation of enzyme pretreated with dithiobis(2-nitrobenzoic acid) (DTNB), followed by the removal of bound DTNB through addition of excess dithiothreitol, did not prevent the inactivation of enzyme. Similarly, the presence of a 200-fold molar excess of dithiothreitol over RB during the photooxidation process had no protective effect. These results clearly indicate that sulfhydryl group bearing cysteins were not the targets of RB-mediated photooxidation in AMV RT. We then examined the effect of 2 mM each of histidine, cysteine, tyrosine, tryptophan, and methionine during the photooxidation process. None of these amino acids either individually or together exerted any protective effect, suggesting that radicals generated from unbound RB, upon excitation with light, were not responsible for (random) oxidation of amino acid residues in AMV RT. It is therefore most probable that the residue(s) in the vicinity of enzyme-bound RB is (are) the target(s) of photooxidation (Table V).

Our attempts to identify the target amino acid residues by means of gross amino acid composition studies of photooxidized and control enzymes were unsuccessful since no significant differences in the amino acid composition of the treated and untreated enzyme could be discerned. The amino acid composition analysis of separated α and β subunits also failed to reveal any differences. These results suggest that no extensive oxidation of any of the amino acid residues had occurred via RB-mediated photooxidation.

Table V: Effect of DTNB, DTT, and Amino Acids on the Photooxidative Inactivation of AMV RT[a]

preincubation conditions	activity (pmol/30 min) control (−RB)	activity (pmol/30 min) photooxidized (+RB)
enzyme alone	215	24
enzyme + DTT	210	22
enzyme + DTNB	195	20
enzyme + 1 mM each cysteine, tyrosine, tryptophan, histidine and methionine	188–210	21–24

[a] Six nanograms of AMV RT was pretreated (5 min at 4 °C) with either 1 mM DTNB, 25 mM DTT, or a mixture of the indicated amino acids. The reaction mixtures were then exposed to light in the presence and absence of 5 μM RB for 5 min. At the end of photooxidation, 25 mM DTT was added to samples containing DTNB, and the enzyme activity in all of the samples was determined as described in footnote *a* of Table I.

Discussion

The present study clearly demonstrates that DNA polymerases from a wide variety of sources are sensitive to RB-mediated photooxidative inactivation (Figure 1). The inactivation of DNA polymerases is irreversible in contrast to reversible inactivation observed under nonoxidative (dark) conditions (Srivastava & Modak, 1982; Table I). By use of AMV RT as a model enzyme, both polymerase and its associated ribonuclease H activities were found to be irreversibly inactivated upon RB-mediated photooxidation of the enzyme. The photooxidation of AMV RT appeared to be directed toward its template binding site, since the inclusion of various template-primers as well as a DNA·RNA hybrid during the photooxidation but not substrate dNTPs showed partial yet consistent protection of both polymerase and RNase H activity (Table II). This observation conforms to earlier reports from this laboratory that expression of RNase H and template binding function of AMV RT may utilize the same site (Modak & Marcus, 1977a,b; Marcus et al., 1978; Modak & Srivastava, 1979). We therefore expected that the protective effect afforded by the presence of template-primers is due to complex formation between enzyme and template-primer (E-TP). Further support for this notion was obtained by our finding that addition of 0.5 M KCl to the E-TP complex during photooxidation, which dissociates enzyme from template-primer, renders it sensitive to RB-mediated inactivation (Table III). Indeed, when the template binding ability of AMV RT, prior to and after photooxidation was measured, it was noted that photooxidized enzyme had completely lost its ability to form E-TP complexes as judged by filter binding assays (Table IV). As expected, a preformed E-TP complex exhibited considerable resistance to RB-mediated photooxidation. Thus, the loss of template binding ability of AMV RT upon photooxidation is primarily responsible for the inactivation of both polymerase and RNase H activities of AMV RT.

Although addition of any one of the four substrate dNTPs did not prevent the enzyme inactivation due to RB-mediated photooxidation of AMV RT, the partial protective effect of template-primer was further increased (by 10–20%) if high concentrations ($\simeq$2 mM) of substrate dNTP were also present. The enhanced protective effect was clearly not seen with noncomplementary dNTPs. Furthermore, the presence of both template and hydrogen-bonded primer was essential for the substrate effect. The fact that high concentrations of substrate dNTP are required and the presence of divalent cation is

unnecessary for the protective effect implies that the protective effect of dNTP is not rendered via classical substrate binding (which occurs via metal–dNTP complex) phenomena. The exact nature of the protective effect of substrate dNTP is not clear, although the protection of a secondary site (see below) which is the target of photooxidation in the presence of template-primer is one possibility. In any event, complete inactivation of AMV RT via photooxidation occurs within 5 min with concomitant loss of template binding activity and therefore oxidation of residue(s) situated in this functional region (domain) may be regarded as a primary target of RB-mediated photooxidation of AMV RT.

The failure to completely protect AMV RT from oxidative inactivation by inclusion of template-primer, however, strongly suggested that an additional site/target for RB-mediated oxidation may exist in AMV RT. Indeed, when catalytic activity as well as the template binding function of AMV RT in the presence of template-primer was examined as a function of exposure time, a complete loss of catalytic activity (both polymerization and nuclease function) was observed without any significant loss of template binding activity by the enzyme. Thus, a site on AMV RT which is essential for the expression of both polymerase and RNase H activity has been identified as an additional target of photooxidation. The effect of RB on substrate binding by this enzyme cannot be directly tested since no substrate binding occurs in the absence of template-primer (Modak & Gillerman-Cox, 1982). However, studies on terminal deoxynucleotidyltransferase in our laboratory have shown that RB does not affect the substrate binding site on that enzyme (M. J. Modak et al., unpublished data). Since this putative additional site on AMV RT, which is the target of RB, is apparently not involved in substrate binding and yet is in close proximity to the template binding site (by being amenable to photooxidation), it may be speculated that this site/region is responsible for the translocation of enzyme required in both catalytic functions.

Thus, the present study has clearly demonstrated that the primary target of RB on AMV RT, under both oxidative and nonoxidative conditions, is the template binding domain. However, under oxidative conditions, an additional target for RB-mediated photooxidation on AMV RT has been identified. It may be pointed out here that in the case of *E. coli* RNA polymerase, Wu and Wu (Wu, C. W., & Wu, F. Y. H., 1973; Wu, F. Y. H., & Wu, C. W., 1973) concluded that inhibition of that enzyme by RB under both oxidative and nonoxidative conditions involved reactivity of RB toward a single site situated in the hydrophobic region of that enzyme. Identification of an additional site in AMV RT under oxidative conditions thus appears to be the major difference between the two classes of enzymes with respect to their RB sensitivity.

In order to learn about the possible amino acid targets of RB-mediated photooxidation on AMV RT, we examined the inactivation patterns of AMV RT under conditions that might implicate a specific amino acid residue such as cysteine or histidine, of which the latter is known to be a target of RB-mediated photooxidation in other systems (Westhead, 1972). The addition of reducing agents such as dithiothreitol protect neither enzyme activity nor reverse inactivation caused by exposure to RB. Similarly, when inactivation by RB as a function of pH was carried out, no pH optimum which might be suggestive of histidine (Gorecki & Panet, 1978) was found. Therefore, in the absence of quantitative amino acid analysis, no definitive assignment of a target residue within AMV RT for RB photooxidation could be made at this time. However, the above studies as well as our earlier report (Srivastava & Modak, 1982) have shown that RB-mediated oxidation of DNA polymerases may be useful in the physical localization and characterization of known and as yet undefined functional domains within these enzymes.

Registry No. RT, 9068-38-6; RB, 11121-48-5.

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