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Novikoff hepatoma deoxyribonucleic acid polymerase. Sensitivity of the β-polymerase to sulfhydryl blocking agents[1]

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ABSTRACT

Unlike other β-class eukaryotic DNA polymerases, the enzyme purified from the Novikoff hepatoma is inhibited by both sulfhydryl blocking agents N-ethylmaleimide (NEM) and p-hydroxymercuribenzoate (pHMB). The degree of sensitivity varies depending on the enzyme purity, pH of the reaction, and the presence of sulfhydryl reducing agents. Novikoff β-polymerase activity is unaffected by the presence of 2-mercaptoethanol (2-Me) or dithiothreitol (DTT); however, the combination of 2-mercaptoethanol and NEM or pHMB acts to reverse the inhibition of the sulfhydryl blocking agent. The reversal of inhibition involves more than just a titration of NEM with 2-mercaptoethanol since a) the combination of these two reagents actually stimulates the DNA polymerase, and b) dithiothreitol did not reverse the inhibition. Binding of the polymerase to DNA did not affect the enzyme sensitivity to NEM.

INTRODUCTION

Sulfhydryl blocking agents have served as useful tools in the identification, isolation and characterization of proteins involved in prokaryotic DNA synthesis. Modification of the cysteine residue by sulfhydryl blocking agents in *Escherichia coli* DNA polymerase I causes no loss of polymerase or 3'⟶5' exonuclease activity (1,2). However, both *E. coli* DNA polymerase II and III are inactivated by treatment with sulfhydryl inhibitors (3,4). The DNA polymerase system from *B. subtilis* is characterized by the same response, with polymerase I being resistant whereas II and III are sensitive (5). N-ethylmaleimide treatment of azide-poisoned *B. subtilis* inhibits DNA replication while a repair-type synthesis is unaffected (6).

At a recent international eukaryotic DNA polymerase conference (7) and in current reviews (8,9), several criteria have

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been proposed as distinguishing features to differentiate the α and β classes of DNA polymerases. The α-polymerases have a high molecular weight, are inhibited by high ionic strength, have a neutral pH optimum, a pI in the acid range and are strongly inhibited by sulfhydryl blocking agents. The β-polymerases, in contrast, have a low molecular weight (<50,000), are not inhibited by high ionic strength, have an alkaline pH optimum, a pI in the alkaline range and are resistant to sulfhydryl blocking agents. Bollum has suggested that NEM-sensitivity is the most useful analytical difference between these two groups of enzymes since mammalian α-polymerases are totally inhibited by 1 mM NEM while β-polymerases are not affected by 10 mM NEM (9).

We have previously described a homogeneous DNA polymerase, isolated from Novikoff hepatoma cells, which fits all of the criteria of a β-polymerase except that it is sensitive to NEM (10). Because of this anomalous behavior compared with other β-polymerases, we have examined the effects of sulfhydryl blocking agents in more detail. In the present study we have analyzed the effects of sulfhydryl reducing agents, DNA, pH and stage of enzyme purity on the sulfhydryl blocking agent-sensitivity of the Novikoff enzyme. For comparison, we have also examined the sensitivity of a β-polymerase purified from guinea pig liver. The results indicate that caution must be used in identifying β-polymerases solely on the basis of sulfhydryl sensitivity and in differentiating polymerase activities in tissue extracts containing both enzymes.

MATERIALS AND METHODS

Reagents and Chemicals

All chemicals were of analytical or reagent grade. Unlabeled deoxyribonucleoside triphosphates, calf thymus DNA and 2-mercaptoethanol were purchased from Sigma. BSA and ^{3}H-dTTP were obtained from Schwarz-Mann. Dithiothreitol and gylcylglycine were purchased from Calbiochem. N-ethylmaleimide was obtained from Eastman Organic Chemicals and p-hydroxymercuribenzoate from K & K Laboratories.

Enzyme Preparations

Novikoff hepatoma DNA polymerase-β, Fraction II (45-75% Ammonium Sulfate step), Fraction V (Hydroxyapatite step), and homogeneous Fraction VI (DNA-cellulose step) were purified as de-

scribed elsewhere (10,11). The guinea pig liver DNA polymerase-β, Fraction II (50-80% Ammonium Sulfate step), Fraction III (DEAE-Sephadex step), and Fraction VI (DNA-cellulose step) were prepared, using similar methods as for the Novikoff β-polymerase (12). Homogeneous E. coli DNA polymerase I (13) and sea urchin nuclear DNA polymerase (14) were purified by published methods. Since the Novikoff hepatoma and guinea pig β-polymerases were purified in the presence of 5mM 2-mercaptoethanol, sulfhydryl reducing agents were removed from all enzyme samples by dialysis prior to DNA polymerase assays in the experiments reported below.

DNA Polymerase Assay

Standard reactions were carried out in disposable 12 x 75 mm glass tubes in 125 µl volumes containing 25mM Tris-HCl pH 8.4, 10mM magnesium chloride, 0.5mM EDTA, 50mM NaCl, 0.015mM each of dATP, dCTP, dGTP and ^{3}H-dTTP (specific activity 325 mCi/mmol), 15% (w/v) glycerol, 100 µg/ml activated calf thymus DNA and 0.1-0.3 units of DNA polymerase. A unit of DNA polymerase activity is defined as the incorporation of 1nmol of total nucleotide per hour at 37°. Incubations were carried out in stoppered tubes for 60 min at 37°. After incubation, 100 µl samples were pipetted onto filter paper discs which were dropped into cold 10% TCA. The discs were processed for liquid scintillation counting as described previously (15) and cold acid-insoluble radioactivity was measured in a Packard 3313 tricarb or Beckman 3155 liquid scintillation spectrometer using 0.4% 2,5-bis-2-(5-tertbutylbenzoxazolyl)-thiophene (BBOT) in toluene as the scintillator at an efficiency of 16-33% (15).

DNA Polymerase Assays Containing Sulfhydryl Blocking Agents

N-ethylmaleimide was dissolved in water, and p-hydroxymercuribenzoate was dissolved in 0.5M glycylglycine buffer pH 8.0; both were used immediately or stored for no more than a day or two in the dark at -20°. Reactions containing sulfhydryl blocking agents were carried out after preincubating the DNA polymerase samples with the blocking agent for 15 min. at 4°. After preincubation, the reaction mixture, which also contained the same concentration of sulfhydryl inhibitor, was added to the enzyme sample and incubated as usual at 37° for 1 hr. Sulfhydryl blocking agents were added to the reaction mixtures just prior to the incubation step. An identical polymerase reaction, containing equal

amounts of sulfhydryl blocking agent buffer but no sulfhydryl blocking agents, served as a control. All other samples were compared to this level of incorporation and expressed as per cent of control activity.

Reactions Containing Sulfhydryl Reducing and Blocking Agents

Reactions containing sulfhydryl reducing and blocking agents were prepared as described above except that reaction mixtures and polymerase samples contained either 5mM 2-mercaptoethanol or 0.5mM dithiothreitol. The reducing agents were added back to enzyme samples and preincubated with sulfhydryl blocking agents prior to the DNA polymerase assay. The control did not contain 2-mercaptoethanol but was diluted equally with water.

Formation of Polymerase - DNA Complex
Prior to N-ethylmaleimide Exposure

A reaction mixture was prepared containing all components except ^{3}H-dTTP. The reaction mixture was then added to Fraction VI Novikoff β-polymerase samples and preincubated for 15 min at 4°. N-ethylmaleimide was then added, and the sample was incubated for an additional 15 min at 4°. ^{3}H-dTTP was added and incubated at 37° for DNA polymerase activity. Control samples were treated identically but contained no N-ethylmaleimide.

N-ethylmaleimide Reaction at Different pH's

Identical samples of Fraction VI Novikoff DNA polymerase were dialyzed into either 25mM potassium phosphate buffer pH 6.0 or 7.0, Tris-HCl pH 8.0, or glycine-NaOH pH 9.0 or 10.0. Reaction mixtures were prepared containing 25mM of the above buffers as well. Samples and reaction mixtures were adjusted to 10mM N-ethylmaleimide, preincubated and assayed as above. A control polymerase sample at each pH was treated indentically as the test sample except that the sulfhydryl blocking agent was omitted from the reaction, in order to establish the control level of activity at each pH.

RESULTS

Effect of Sulfhydryl Blocking Agents

In the absence of sulfhydryl protecting agents, the homogeneous Novikoff DNA polymerase-β (Fraction VI) is inhibited by both NEM and pHMB under optimal assay conditions for the enzyme (Fig. 1 & 2) (10). Escherichia coli DNA polymerase I, which is insen-

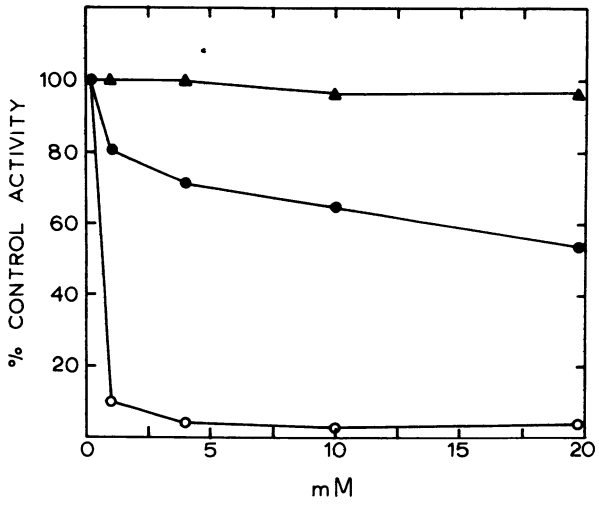

Figure 1. N-ethylmaleimide-sensitivity of Novikoff hepatoma DNA polymerase-β. Reaction conditions were as described in Methods except that 2-mercaptoethanol was omitted and the samples preincubated for 15 min at the NEM concentration shown in the figure, and the equivalent amount of NEM was present during incubation. Each assay tube contained approximately 1ng of Fraction VI Novikoff DNA polymerase (●——●), and gave 26.0 pmol of nucleotide incorporated in the absence of NEM (control). For comparison, the NEM-sensitivity of E. coli DNA polymerase I (▲——▲) and sea urchine DNA polymerase (○——○) are shown.

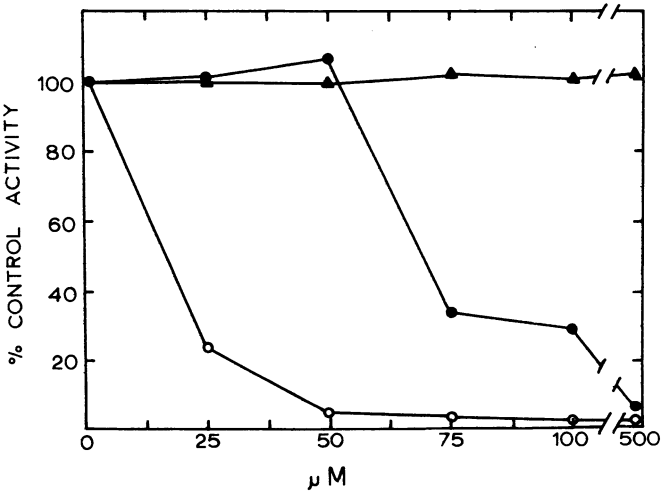

Figure 2. p-Hydroxymercuribenzoate sensitivity of Novikoff hepatoma DNA polymerase-β. Reaction conditions were as given in the legend to figure 1 except pHMB was substituted for NEM. Novikoff hepatoma β-polymerase (●——●), E. coli DNA polymerase I (▲——▲), sea urchine DNA polymerase (○——○).

sitive (1, 2), and sea urchin DNA polymerase, which is sensitive (16) to sulfhydryl blocking agents, were used as controls and exhibited the expected response (Fig. 1 & 2). The Novikoff β-polymerase is inhibited by NEM at all concentrations tested. Inhibitions of 20%, 35% and 55% at 1 mM, 10 mM and 20 mM NEM, respectively (Fig. 1) were observed. However, total inhibition of the β-polymerase was not obtained even at the highest concentration tested, 50 mM, where 35% of the activity still remained (data not shown). At 50 μM pHMB, the Novikoff polymerase is unaffected, but higher concentrations are inhibitory (Fig. 2). At 500 μM pHMB all activity is abolished. These data argue that the homogeneous Novikoff β-polymerase is sensitive to sulfhydryl blocking agents and not that the observations are an artifact or due to some contaminant.

Interaction of Sulfhydryl Protecting and Sulfhydryl Blocking Agents

The Novikoff β-polymerase has no requirement for and is not stimulated by 2-mercaptoethanol up to 5 mM or dithiothreitol up to 0.5 mM (data not shown). When the Novikoff β-polymerase (Fraction VI) containing 5 mM 2-mercaptoethanol was preincubated and assayed with NEM or pHMB, the inhibition of activity was reversed (Fig. 3 & 4). The β-polymerase containing the sulfhydryl reducing agents showed 115%, 91% and 68% of control activity at 1 mM, 10 mM and 20 mM NEM, respectively (Fig. 3). The results show that at low NEM concentrations (1-4 mM), the polymerase activity is actually stimulated above control activity by the addition of both 2-mercaptoethanol and N-ethylmaleimide to the reaction. The stimulation was also observed for 2-mercaptoethanol in the presence of p-hydroxymercuribenzoate (Fig. 4) at all concentrations tested (25-500 μM). The sulfhydryl reducing agent dithiothreitol did not show a reversal of NEM inhibition for the Novikoff enzyme, but rather increased the inhibitory action of N-ethylmaleimide on the β-polymerase (Fig. 3).

The results also suggest that the reduction in sensitivity to NEM involves more than just reducing the effective N-ethylmaleimide concentration by titration with 5mM 2-mercaptoethanol, since 1) the reduction of inhibition is greater than the inhibition observed for 5mM N-ethylmaleimide (the effective concentration of

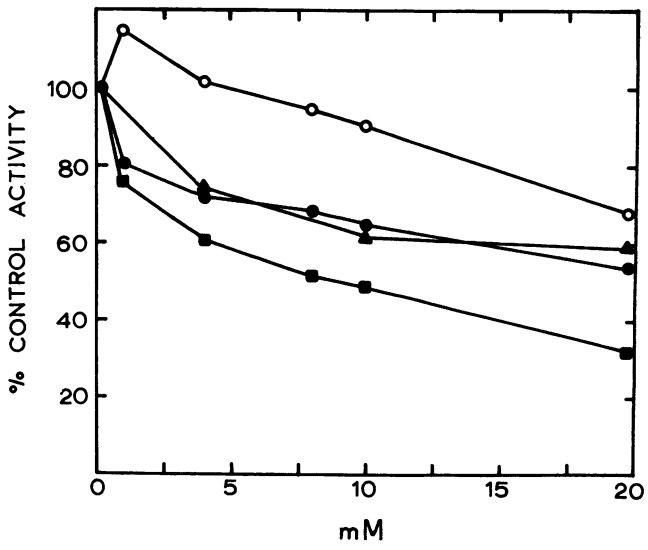

Figure 3. Effect of sulfhydryl reducing agents and DNA on the NEM-sensitivity of Novikoff hepatoma DNA polymerase-β. Preincubation and reaction conditions with NEM were as given in the legend to figure 1 except for the following additions: None (●—●); 0.5mM dithiothreitol added (■—■); 5.0mM 2-mercaptoethanol added (○—○); or DNA included during preincubation (▲—▲).

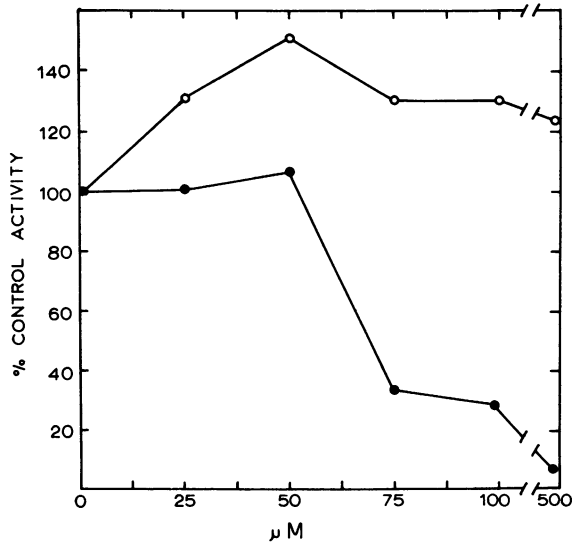

Figure 4. Effect of 2-mercaptoethanol on the pHMB-sensitivity of Novikoff hepatoma DNA polymerase-β. Conditions were as described in Methods except either 5mM 2-mercaptoethanol was included (○—○) or absent (●—●) during preincubation and assay in the presence of pHMB.

NEM when 10mM NEM is titrated with 5mM 2-mercaptoethanol) and 2) the guinea pig liver β-polymerase, Fraction II, which is not inhibited by N-ethylmaleimide and not stimulated by 2-mercaptoethanol, is nevertheless stimulated by the combination of the two reagents added simultaneously (Table I). Dithiothreitol was ob-

TABLE I. Effect of NEM and NEM plus sulfhydryl reducing agents on Novikoff hepatoma and guinea pig liver β-polymerases

	pmoles nucleotide incorporated	percent of control activity
Fraction VI Novikoff β-polymerase		
(control)	37.0	100
+ 10 mM NEM	24.0	64.9
+ 10 mM NEM + 5mM 2-Me*	33.6	90.8
+ 10 mM NEM + 0.5mM DTT*	18.1	48.9
Fraction II Guinea Pig Liver β-polymerase		
(control)	32.0	100
+ 10 mM NEM	31.7	99.1
+ 10 mM NEM + 5mM 2-Me	50.2	157
+ 10 mM NEM + 0.5mM DTT	36.8	115

*2-Me, 2-mercaptoethanol; DTT, dithiothreitol

served to increase the inhibition of N-ethylmaleimide in highly purified guinea pig β-polymerase fractions but either stimulated or had no effect on less pure samples (Table I).

Effect of DNA on Inhibition by N-ethylmaleimide

Samples of Novikoff polymerase, Fraction VI, which were preincubated in the presence of all reaction components except ^{3}H-dTTP prior to the additional of N-ethylmaleimide and ^{3}H-dTTP, showed no change in sensitivity (Fig. 3). The data suggests that the N-ethylmaleimide inhibited site is not protected by binding the Novikoff β-polymerase to DNA.

Effect of pH on N-ethylmaleimide Inhibition

The inhibition of the homogeneous Novikoff β-polymerase is differentially affected by pH and buffer conditions. The effect of 10mM NEM on the β-polymerase was observed and compared to the activity of samples assayed under the same conditions without N-ethylmaleimide. The results (Table II) indicate that the Novikoff β-polymerase is less sensitive to 10mM N-ethylmaleimide in potassium phosphate buffer pH 6.0 or 7.0 than at other pHs tested. The β-polymerase was observed to be only slightly inhibited (3%) by N-ethylmaleimide at pH 6.0. Greatest inhibition is observed at the higher pHs (pH 9-10).

TABLE II. Effect of pH on the degree of inhibition of Novikoff β-polymerase by 10 mM NEM

Buffer	pH	percent of control activity
Potassium Phosphate	6.0	97.4
	7.0	88.0
Tris - HCl	8.0	64.9
Glycine - NaOH	9.0	52.0
	10.0	50.7

Control activity was measured at each pH using 0.1 units of Fraction VI Novikoff hepatoma DNA polymerase-β.

Sensitivity With Stage of Purification

The state of purification of the Novikoff β-polymerase shows an effect on the sensitivity of the enzyme to N-ethylmaleimide. Fraction II, the crude ammonium sulfate sample, is more sensitive to NEM than are samples tested after later steps of purification (Table III). We have recently shown that an accessory factor,

TABLE III. Effect of state of purity on the NEM-sensitivity of Novikoff hepatoma and guinea pig liver β-polymerases

Fraction and Step	percent of control activity +10mM NEM	+20mM NEM
Novikoff hepatoma β-polymerase		
II, Ammonium Sulfate	40.2	34.4
V, Hydroxyapatite	60.9	52.3
VI, DNA-Cellulose	65.0	54.0
Guinea pig liver β-polymerase		
II, Ammonium Sulfate	99.1	91.0
III, DEAE-Sephadex	53.3	49.0
IV, DNA-Cellulose	68.7	66.3

Control activity was measured at each stage of purity using 0.1 units of Novikoff hepatoma or guinea pig liver DNA polymerase-β in the absence of N-ethylmaleimide.

Novikoff Factor IV, is purified away from a protein complex of β-polymerase and Factor IV by DNA-cellulose chromatography to yield the homogeneous β-polymerase (Fraction VI) (17). However, the sensitivity to N-ethylmaleimide of Fraction V and Fraction VI is not significantly altered by the removal of Factor IV (Table III). Another mammalian β-polymerase, isolated from guinea pig liver, also shows differential sensitivity to N-ethylmaleimide during purification. The guinea pig liver β-polymerase Fraction II is not affected by 10mM N-ethylmaleimide and is only slightly sensitive to 20mM NEM (Table III). Upon purification of the guinea pig

enzyme, the resistance to N-ethylmaleimide is altered. Fraction III is quite sensitive to N-ethylmaleimide, but the highly purified enzyme, Fraction VI, was slightly less sensitive (Table III). These data indicate that highly purified fractions of both are inhibited by the sulfhydryl blocking agent. However, the degree of sensitivity of both enzymes changes with the state of purity.

DISCUSSION

The homogeneous Novikoff hepatoma DNA polymerase has been extensively characterized (10) and fulfills all of the critera for a β-polymerase (7-9) except for its sensitivity to the sulfhydryl blocking agents. Bollum, in a recent review (9), has pointed out that while most β-polymerases are sensitive to pHMB, they are resistant to 10 mM NEM and that this resistance provides a convenient analytical tool for distinguishing α from β-polymerases. Although most β-polymerases are resistant to NEM, the Novikoff enzyme reported here as well as a few other β-polymerases are sensitive to this sulfhydryl blocking agent (18-19). Thus, caution must be exercised in using NEM alone to identify a particular enzyme as α or β-polymerase and to quantitate α and β-polymerase activities in cell extracts on the basis of such activity. Our results illustrating the effect of the stage of purity on NEM-sensitivity underscore this, for the Novikoff enzyme becomes less sensitive but the guinea pig liver enzyme becomes more sensitive to this agent upon purification. Similar changes in NEM-sensitivity with purification have been observed by Srivastava with human DNA polymerases (20).

The pH of the buffer influences the sensitivity of the Novikoff enzyme to NEM, with the greatest inhibitory effect noted at at pH 9-10. DNA, on the other hand, had no effect, suggesting that either the NEM-sensitive SH-group is not in the DNA-binding site or that binding of the enzyme to DNA does not sequester this SH-group from NEM. In contrast, Haines, Holmes, and Johnston (21) have reported that DNA provided some protection for the rat liver nuclear DNA polymerase to NEM-inhibition.

It is interesting to note that the simultaneous presence of 2-merceptoethanol and NEM or pHMB (Figs. 3 and 4) stimulated the Novikoff polymerase. A similar phenomenon was first reported by Bohn, Matsukage & Wilson (22,23) with a murine β-polymerase. How-

ever, in contrast to the Novikoff polymerase, the mouse enzyme is stimulated by a combination of dithrothreitol and pHMB, with 2-mercaptoethanol being ineffective. Guinea pig β-polymerase also shows stimulation by a combination of NEM plus 2-mercaptoethanol or dithiothreitol. The mechanism of stimulation seen when both sulfhydryl blocking and sulfhydryl reducing agents are used together remains unknown.

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