Influence of template inactivators on the binding of DNA polymerase to DNA

Werner E.G. Müller, Jürgen Obermeier, Atsuko Totsuka and Rudolf K. Zahn

**Institute für Physiologische Chemie der Johannes Gutenberg Universität,
65 Mainz (Rhein), Johann Joachim Becher Weg 13, Germany)**

Received 1 November 1973

ABSTRACT

The agents daunomycin, ethidium bromide, distamycin A and cytochrome c inhibit DNA dependent DNA polymerase I (*E. coli*) reaction competitively to DNA. The influence of these template inactivators on the binding of DNA polymerase to native as well as denatured DNA has been determined by affinity chromatography. Cytochrome c blocks the binding of the enzyme to double-stranded and to single-stranded DNA Sepharose. In contrast to these results daunomycin, ethidium bromide or distamycin A reduce the binding affinity only with denatured DNA Sepharose as matrix. These data are discussed with respect to the modification by template inactivators of the affinity of DNA to the different binding sites of the DNA polymerase.

INTRODUCTION

The DNA dependent DNA polymerase I (E.C. 2.7.7.7) isolated from *E. coli* contains one binding site for all four deoxynucleoside triphosphates¹ and several sites for DNA². The binding of DNA polymerase I to DNA has been shown² to be dependent on the DNA structure ; thus the enzyme will not bind to unbroken sequences of duplex DNA. Binding of enzyme occurs along single-stranded chains, to nicks and to ends. In the case of single-stranded DNA, the polymerase binds at the template binding site, while with helical DNA where binding at nicks and at ends of molecules occurs, both the template binding site as well as the primer terminus binding site are involved³. Inhibition experiments have shown that the polymerization reaction mediated by the enzyme can be blocked by DNA template inactivators and substrate analogues competitively^{4, 5}. In the case of the template inactivators, the inhibition of DNA synthesis may be due either to prevention of polymerase to bind along the template or to blocking of the movement of the DNA template relative to the enzyme.

In this paper we describe the influence of some template inactivators on the binding of DNA polymerase I (*E. coli*) to DNA. The experiments have been carried

out by affinity chromatography using single-stranded as well as native DNA Sepharose and an exonuclease-free DNA polymerase I preparation⁶.

MATERIALS

Source of the materials: unlabelled deoxyribonucleoside triphosphates and DNA dependent DNA polymerase I (E. coli; specific activity: 3,200 Units/mg) obtained by limited proteolytic action from highly purified enzyme fractions (fraction VII;⁷) according to Klenow et al.⁶ from Boehringer, Mannheim (Germany); [³H] dATP (spec. act.: 3.4 Ci/mmol) from The Radiochemical Centre, Amersham (England); Sepharose 4 B from Pharmacia, Uppsala (Sweden); ethidium bromide from Serva, Heidelberg (Germany); dialysis tubing (diameter 7 mm) from Visking Co., Chicago (USA); N-cyclohexyl-N'-[β -(N-methylmorpholine)-ethyl]-carbodiimide-p-toluene-sulphonate from E. Merck, Darmstadt (Germany); cytochrome c and DNA from herring sperm and from *Holothuria forskali* sperm, prepared according to Zahn et al.⁸ from H. Mack, Illertissen (Germany).

METHODS

Preparation of DNA Sepharose. The procedure described by Rickwood⁹ was applied except that N-cyclohexyl-N'-[β -(N-methylmorpholine)-ethyl]-carbodiimide-p-toluenesulfonate and Sepharose 4 B were used. Native as well as denatured (heated 10 min at 100°C and chilled) DNA was used for covalent coupling to the Sepharose. About 10% of the original DNA will usually be covalently linked to Sepharose (240 μ g per packed ml).

DNA Sepharose chromatography. DNA Sepharose was equilibrated with TEM buffer (20 mM Tris-HCl, 5 mM EDTA, 6 mM MgCl₂; pH 7.4) containing 0.1 M NaCl. A column, 1 x 8 cm, was prepared with this slurry. The enzyme (10 Units/0.5 ml) was applied and washed with 2 column volumes TEM buffer + 0.1 M NaCl. Then the enzyme was eluted with TEM buffer + 2 M NaCl. The column was run at 5 ml/h; 1.7 ml fractions were collected. In experiments set up for the determination of the influence of several template inactivators on the binding properties of the polymerase, the loading buffer as well as the elution buffer were supplemented with the inactivators. While prewashing with the loading buffer, the DNA matrix

was kept saturated with respect to the template inactivator used. This was controlled spectrophotometrically.

Polymerase assay. The DNA dependent DNA polymerase activity in the column fractions was assayed as described¹⁰. Fractions eluted from the DNA Sepharose column with buffers containing DNA template inactivating agents were dialyzed in the case of daunomycin, distamycin A and ethidium bromide prior to the determination of enzyme activity with dialysis tubing against TEM buffer + 0.1 M NaCl. In the case of cytochrome c an ultrafiltration cell (Millipore Corp., Bedford, Mass.) equipped with "Pellicon" membrane 1D030 with TEM buffer + 0.1 M NaCl on its outside was used. The activity of DNA polymerase bound to the DNA Sepharose matrix, 0.2 g wet centrifugation sediment, was assayed in 0.2 ml of a reaction mixture without adding DNA¹⁰. After incubation for 30 min at 37°C the reaction was stopped by addition of 1 Vol 5% TCA. The acid-insoluble radioactivity was determined after washing the precipitate (by centrifugation 3 min; 2,000 x g) twice with 5% TCA followed by desiccation with ethanol (3 x 10 min) and ether (5 min). The dry residue was dissolved in NCS tissue solubilizer (Nuclear Chicago; Amersham/Searle, Arlington, Ill., USA) and counted as described¹¹.

One unit is the enzyme activity which produces an incorporation of 10 nmoles of total nucleotides into an acid precipitable fraction during 30 min at 37°C, using poly d[A-T] as template and primer¹².

RESULTS

DNA dependent DNA polymerase I (*E. coli*) binds to native as well as to denatured DNA Sepharose and can be recovered by raising the NaCl concentration in TEM buffer from 0.1 to 2 M (Fig. 1; Fig. 2; Table 1). Under the conditions described in this paper, about 12% of the enzyme activity, are not adsorbed to native DNA Sepharose (Fig. 1; Table 1); using denatured DNA Sepharose about 6% of the enzyme elute with the loading buffer (Fig. 2; Table 1). The recovery of enzymatic activity was 78 to 92%. The unspecific adsorption of the enzyme to DNA-free Sepharose can be neglected (Table 1).

As shown in Table 1, daunomycin, ethidium bromide and distamycin A do not prevent heavy binding of the enzyme by native DNA Sepharose, whereas cytochrome c drastically reduces the binding of the enzyme (Table 1; Fig. 1).

Fig. 1: Effect of cytochrome c on the binding of DNA polymerase to native DNA Sepharose columns. The column, prewashed with ten volumes of TEM buffer containing 0.1 M NaCl and either 0 or 100 $\mu\text{g/ml}$ cytochrome c was loaded with DNA polymerase. The conditions for loading and elution of the column were as described under methods; after dialysis the eluted fractions were assayed for DNA polymerase activity. The enzyme activity in the fractions of the effluent from DNA Sepharose columns, treated with 0 (o—o) or with 100 $\mu\text{g/ml}$ ($\bullet$ — $\bullet$) cytochrome c are shown.

Fig. 2: Influence of distamycin A on the binding of DNA polymerase by denatured DNA Sepharose. The procedures of prewashing, loading and elution were performed as described in legend to Fig. 1. In the experiment with distamycin the buffers contained 20 μ g distamycin/ml. After dialysis the fractions were assayed for enzyme activity; pattern without (o--o) and with distamycin (●—●).

Table 1: Influence of four template inactivators on binding of DNA polymerase by Sepharose with native herring DNA attached to it (n-DNA Sepharose) and Sepharose with denatured DNA (d-DNA Sepharose). Experimental details are given under Methods and in the legends to Fig. 1 and Fig. 2. The concentrations of the different template inactivators in the buffers used were: daunomycin: 20 $\mu\text{g/ml}$, ethidium bromide: 20 $\mu\text{g/ml}$, distamycin A: 20 $\mu\text{g/ml}$ and cytochrome c: 100 $\mu\text{g/ml}$.

Matrix	Substance added	Enzyme units applied	DNA polymerase activity		Ratio
			Units adsorbed	Units eluted	
Sepharose	none	12.70	0.05	10.65	0.01
n-DNA Sepharose	none	12.70	9.42	1.48	6.36
	daunomycin	12.70	9.28	1.32	7.03
	ethidium bromide	12.70	9.13	1.97	4.63
	distamycin A	12.70	9.36	2.34	4.00
	cytochrome c	12.70	2.24	8.46	0.26
d-DNA Sepharose	none	12.70	10.39	0.81	12.83
	daunomycin	12.70	2.17	8.13	0.27
	ethidium bromide	12.70	3.54	7.36	0.48
	distamycin A	12.70	2.93	8.07	0.36
	cytochrome c	12.70	2.36	7.64	0.31

Contrary to the results with native DNA Sepharose, the template inactivators daunomycin, ethidium bromide and distamycin A strongly reduce the binding affinity of the DNA polymerase to the denatured DNA coupled to the Sepharose matrix (Table 1; Fig. 2). Cytochrome c prevents the binding of the enzyme by denatured DNA Sepharose to about the same amount as determined in assays using native DNA Sepharose.

To our knowledge the inhibition types of the template inactivators daunomycin, ethidium bromide and cytochrome c have not been determined. The kinetics, shown in Fig. 3, clearly demonstrate that the inhibition types in all three cases are competitive with respect to DNA. The inhibitor constants for the three substances in the reaction with DNA polymerase I (*E. coli*) using native DNA are given in the legend to Fig. 3. In enzyme assays with denatured DNA the enzymatic polymerization is also inhibited competitively to DNA; the inhibitor constant is for: Daunomycin 4.83 ± 0.91 , for ethidium bromide 2.70 ± 0.35 and for cytochrome c $77.5 \pm 13.6 \mu\text{g/ml}$.

Table 2: Activity of DNA polymerase bound to native or denatured DNA Sepharose. DNA polymerase was bound to the DNA-matrix using TEM buffer containing 0.1 M NaCl. After washing twice with TEM buffer containing 0.1 M NaCl by centrifugation (3 min; $2,000 \times g$), the wet sediment containing the DNA-Sepharose bound DNA polymerase was assayed as described under methods. 1 Unit was bound per mg native as well as denatured DNA.

Addition of template inactivator ($\mu\text{g/ml}$)	Acid-insoluble radioactivity (cpm)	
	native DNA-Sepharose	denatured DNA-Sepharose
None; control	2,600	7,800
Daunomycin (40)	270	360
Ethidium bromide (40)	210	210
Distamycin A (40)	330	490
Cytochrome c (200)	170	250

In another set of experiments the properties of native DNA as well as of denatured DNA, immobilized on Sepharose gels serving as template, have been checked. The results are summarized in Table 2. Both native DNA and denatured DNA coupled to Sepharose are fairly good templates for the DNA polymerase. Compared with the enzyme activity determined with free DNA in the reaction mixture the enzyme activity with immobilized denatured DNA is about 1/10; in assays with immobilized native DNA the activity is diminished about 18 times. The results presented in Table 2 show that the template inactivators daunomycin, ethidium bromide, distamycin A and cytochrome c drastically reduce the activity of the polymerase using bound DNA as template.

DISCUSSION

DNA polymerase has been shown to be bound at low ionic strength to native as well as to single stranded DNA, immobilized by coupling to cellulose and to agarose respectively^{13,14}. As shown in this study, single-stranded and native DNA Sepharose can be used to bind DNA polymerase I (*E. coli*). The influence of template inactivators on the affinity of the enzyme to DNA has been evaluated. Previous experiments for the determination of the influence of template inactivators on the polymerase affinity to DNA have been carried out by sedimenting DNA enzyme mixtures through sucrose density gradients¹⁵. However this method has serious limitations due to the influence of high sucrose concentrations on the binding affinity of the enzyme and to its low sensitivity². With the affinity chromatography technique, the binding properties of the polymerase can be studied under conditions which are more optimal for enzymatic activity as shown in a separate set of experiments. The DNA Sepharose matrix obviously can serve as template for enzymatic DNA synthesis (Table 2). In order to rule out the possibility that the 5' → 3' exonuclease of the highly purified DNA polymerase may modify the DNA coupled to the Sepharose matrix by degradation to mono- and oligonucleotides, the large enzyme fragment (mol wt 76,000) produced by limited proteolytic cleavage of the polypeptide chain¹⁶, has been used in the present studies. The 3' → 5' exonuclease, closely associated with the DNA polymerase in the large fragment¹⁷, was shown to be directed specifically against mispaired primer termini¹⁸. Because the occurrence of mispaired termini in the DNA used in this study is unlikely, this nuclease activity has been disregarded.

Fig. 3: Inhibition of DNA polymerase by daunomycin, ethidium bromide and cytochrome c. Plot according to Lineweaver et al.²⁵. Abscissa: $[1/s]$, reciprocal values of the template concentration in ml/mg native DNA. Ordinate: $[1/v]$ reciprocal values of the initial reaction velocity in $10^9 \times 30$ min per moles $[^3H]$ dATP incorporated. To the reaction mixture 1 Unit of DNA polymerase was added. Linear regressions: $\bullet-\bullet$, control; $o--o$ with 45 $\mu\text{g/ml}$ cytochrome c; $x-\cdots x$ 0.5 $\mu\text{g/ml}$ daunomycin and $\Delta-\cdots\Delta$ 0.15 $\mu\text{g/ml}$ ethidium bromide. The inhibitor constants are: cytochrome c: $90.2 \pm 15.9 \mu\text{g/ml}$; daunomycin: $0.27 \pm 0.04 \mu\text{g/ml}$ and ethidium bromide: $0.13 \pm 0.02 \mu\text{g/ml}$.

In this study four template inactivators are under consideration. The daunomycin had been shown before¹⁹ to intercalate between adjacent base pairs (A + T as well as G + C) in native DNA; with denatured DNA, daunomycin binds much less and only through ionic forces between a positively charged amino group of daunosamine and the phosphate groups of the DNA²⁰. Ethidium bromide has DNA binding sites similar to daunomycin; it intercalates with native DNA and interacts with denatured DNA electrostatically²¹. Distamycin A forms complexes with native as well as with denatured DNA mainly by electrostatic attraction between some of its basic groups and negative phosphate sites of DNA²². Cytochrome c, a basic protein²³, forms stable complexes with the phosphate groups in the DNA at low to moderate ionic strength.

The influence of different DNA polymerase template inactivators on the massive binding of DNA polymerase to DNA Sepharose is dependent on the physical nature of the DNA attached to the matrix. With native DNA Sepharose, the intercalating agents daunomycin and ethidium bromide as well as distamycin A do not alter the binding capacity. These findings are in contrast to the results obtained with denatured DNA Sepharose, where the agents drastically reduce the binding capacity of the DNA polymerase. Since DNA polymerase is bound to denatured DNA exclusively at its template site² we suggest that daunomycin, ethidium bromide and distamycin A in denatured DNA block the recognition sites for template binding in the enzyme. The three agents do not seem to exert an effect on the primer terminus site of the DNA, since the binding of the polymerase to native DNA was found to be independent from the presence of these agents. The nicks in native DNA serving as primer terminus sites may be caused during the isolation procedure of DNA and during the procedure of binding native DNA to Sepharose.

Distamycin A²⁴ as well as daunomycin and ethidium bromide, as shown in this paper, inhibit the DNA synthesis in a competitive way, with native as well as with denatured DNA. Consequently the inhibition observed, in the case of denatured DNA dependent reaction, is caused by a interference with the formation of the enzyme-DNA complex, while in the native DNA dependent reaction, the binding of the polymerase to DNA is not affected by the three inhibitors. The inhibition in this case may be attributed to an inhibition of the DNA chain movement during the polymerizing activity of enzyme. Cytochrome c is shown to be an inhibitor for the enzyme-DNA complex formation with native as well as with denatured DNA. When

bound to the phosphate sites of the DNA cytochrome c will cover besides the template sites of the DNA the primer terminus sites, too.

ACKNOWLEDGEMENTS

We thank the H. Mack, Illertissen (Germany) for generous gifts of highly purified sperm DNA and cytochrome c. We also thank Dr. M. Ghione, Farmitalia, Milano (Italy) for donating Daunomycin and Distamycin.

REFERENCES

- 1 Englund, P.T., Huberman, J.A., Jovin, T.M. and Kornberg, A. (1969) J. biol. Chem. 244, 3038-3044
- 2 Englund, P.T., Kelly, R.B. and Kornberg, A. (1969) J. biol. Chem. 244, 3045-3052
- 3 Huberman, J.A. and Kornberg, A. (1970) J. biol. Chem. 245, 5326-5334
- 4 Hurwitz, J., Furth, J.J., Malamy, M. and Alexander, M. (1962) Proc. nat. Acad. Sci., Wash. 48, 1222-1230
- 5 Atkinson, M.R., Deutscher, M.P., Kornberg, A., Russel, A.F. and Moffatt, J.G. (1969) Biochemistry 8, 4897-4904
- 6 Klenow, H. and Henningsen, I. (1970) Proc. nat. Acad. Sci., Wash. 65, 168-175
- 7 Jovin, T.M., Englund, P.T. and Bertsch, L.L. (1969) J. biol. Chem. 244, 2996-3008
- 8 Zahn, R.K., Tiesler, E., Kleinschmidt, A.K. and Lang, D. (1962) Biochem. Z. 336, 281-298
- 9 Rickwood, D. (1972) Biochim. Biophys. Acta 269, 47-50
- 10 Müller, W.E.G., Heicke, B. and Zahn, R.K. (1971) Biochim. Biophys. Acta 240, 506-514
- 11 Hellung-Larsen, P. (1971) Anal. Biochem. 39, 454-461
- 12 Richardson, C.C., Schildkraut, C.L., Aposhian, H.V. and Kornberg, A. (1964) J. biol. Chem. 239, 222-232
- 13 Alberts, B.M., Amodio, F.J., Jenkins, M., Gutmann, E.D. and Ferris, F.L. (1968) Cold Spr. Hrb. Symp. quant. Biol., Vol. 33, pp. 289-305. Cold Spring Harbor, New York
- 14 Schaller, H., Nüsslein, C., Bonhoeffer, F.J., Kurz, C. and Nietzsche, I. (1972) Eur. J. Biochem. 26, 474-481
- 15 Puschendorf, B., Petersen, E., Wolf, H., Werchau, H. and Grunicke, H. (1968) Biochem. Biophys. Res. Commun. 43, 617-624
- 16 Setlow, P. and Kornberg, A. (1972) J. biol. Chem. 247, 232-240
- 17 Setlow, P., Brutlag, D. and Kornberg, A. (1972) J. biol. Chem. 247, 224-231

- 18 Brutlag, D. and Kornberg, A. (1972) J. biol. Chem. 247, 241-248
- 19 Marco, A. di (1967) in Antibiotics, Vol. 1, pp. 190-210, Springer Verlag, Berlin
- 20 Zunino, F., Gambetta, R., Marco, A. di and Zaccara, A. (1972) Biochim. Biophys. Acta 277, 489-498
- 21 Pecq, J.B. le (1971) in Methods of Biochemical Analysis, Vol. 20, pp. 41-86, Interscience Publishers, New York
- 22 Zimmer, C., Luck, G., Thrum, H. and Pitra, C. (1972) Eur. J. Biochem. 26, 81-89
- 23 Leaf, G., Gillies, N.E. and Pirrie, R. (1958) Biochem. J. 69, 605-611
- 24 Müller, W.E.G., Obermeier, J., Maidhof, A. and Zahn, R.K., submitted
- 25 Lineweaver, H. and Burk, D. (1934) J. amer. chem. Soc. 56, 408-412