THE INHIBITION OF DNA-DEPENDENT RNA POLYMERASE OF *E. COLI* BY SHOWDOMYCIN

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1. Introduction

In the course of a search for inhibitors of RNA polymerase (EC 2.7.7.6) from *E. coli*, it was found that several sulphydryl reagents were able to inactivate the enzyme. Since *N*-ethylmaleimide was among the compounds found to be inhibitory, it was decided to examine the effectiveness of the naturally occurring maleimide antibiotic, showdomycin. This is an antibiotic produced by *Streptomyces showdoensis* which inhibits the growth of several bacteria [1]. It has the structure 3-β-D-ribofuranosylmaleimide [2] (fig. 1).

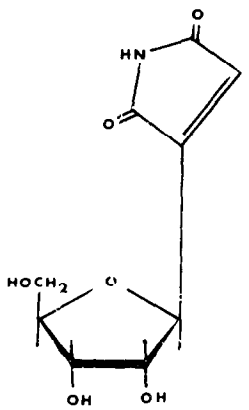

Fig. 1. Showdomycin.

The results indicate that showdomycin is an effective inhibitor, and that DNA, and to a lesser extent the metals used in the assay, Mg^{2+} and Mn^{2+}, are able to protect the enzyme against inactivation.

2. Materials and methods

2.1. *Materials*

Showdomycin was obtained from Charles Druce Ltd., London, W.1; NEM* from Hopkin and Williams, Chadwell Heath, Essex. *E. coli* MRE 600 was obtained from the Microbiological Research Establishment, Porton Down, Wiltshire.

2.2. *Enzyme isolation*

The polymerase was isolated from *E. coli* MRE 600 by modification of the general procedure of Furth, Hurwitz and Anders [3]. All buffers contained 0.5 mM EDTA and 1 mM β-mercaptoethanol. After breaking 100 g of cells with glass beads and centrifuging at 78,000 *g*, the supernatant was titrated with 0.5% protamine sulphate solution to give 90% precipitation of the enzyme. Inactive protein was extracted from the protamine precipitate with 0.03 M ammonium sulphate in 0.01 M tris-HCl, pH 7.5, followed by elution of the enzyme with 0.15 M ammonium sulphate in 0.01 M imidazole-HCl, pH 6.5. The protamine eluate was then fractionated with saturated ammonium sulphate, and the 37%–52% precipitate retained. Finally, after separation of this fraction on DEAE-cellulose with a linear gradient of 0.1 M–0.5 M KCl in 0.01 M tris-HCl, pH 7.5, the peak containing enzyme was concentrated by ammonium sulphate precipitation and run on Sephadex G-100 in 1.3 M ammonium sul-

* *Abbreviations*
NEM, *N*-ethylmaleimide
NTP, nucleoside triphosphate
UMP, uridine monophosphate
DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)

phate in 0.01 M tris-HCl, pH 7.5, to remove a lower molecular weight inhibitor. Enzyme was stored in a buffer containing 0.2 M ammonium sulphate, 40% glycerol, 0.05 M tris-HCl and 0.5 mM each EDTA and dithiothreitol, pH 7.5.

As isolated the enzyme had a specific activity of approximately 2000 units/mg protein. (1 unit incorporates 1 nmole of ^{14}C-AMP per hour into an acid-insoluble form). The 280/260 ratio was 1.5–1.6. The enzyme was completely DNA-dependent.

2.3. *Enzyme assay*

The enzyme assay contained the following in a volume of 0.25 ml: 50 mM tris-HCl, pH 7.5; 8 mM $MgCl_2$; 2 mM $MnCl_2$; 0.4 mM each CTP, GTP, UTP and 8-^{14}C-ATP (10^6 cpm/μmole); 40 μg calf thymus DNA; and 2–10 μg enzyme protein. The omission of mercaptoethanol had little effect on the amount of ^{14}C-AMP incorporation. After incubation at 37°C for 10 min, the reaction was stopped with 5 μmoles EDTA followed by 3 ml of ice-cold 0.5 N perchloric acid containing 0.125 M sodium pyrophosphate, and the radioactivity collected on 0.45 μm Millipore filters which were then washed successively with 0.5 N perchloric acid, ethanol and toluene, dried, and counted by liquid scintillation.

2.4. *Inhibition experiments*

The enzyme (about 2 mg) in storage buffer was diluted 2- to 5-fold with dialysis buffer (0.01 M tris-HCl, 15% glycerol, 0.5 mM EDTA) and precipitated by addition of 1.2 volumes of saturated ammonium sulphate. It was then dissolved in dialysis buffer and dialysed against 100 volumes of the same buffer for 18 to 22 hr. About 30 to 50% of the activity was lost in this procedure; bubbling solutions with oxygen-free nitrogen did not prevent this loss. For inhibition, 0.02–0.05 ml portions of dialysed enzyme were mixed with 2 volumes of dialysis buffer and 1 volume of inhibitor dissolved in the same buffer. After incubation at 37°C, aliquots were removed at intervals and assayed in the standard system. In each experiment, a control tube, containing enzyme but no inhibitor was similarly incubated and samples of it assayed.

Protein concentration was determined from the extinction at 280 nm using an $E^{1\%}_{1\,cm}$ of 6.5 as described by Richardson [4].

3. Results

Fig. 2 shows the effect of preincubating RNA polymerase in the presence of various concentrations of showdomycin or NEM. In the experiment shown, after 30 min preincubation, 50% inactivation was obtained with showdomycin at a concentration of 2.5 mM whereas only 0.3 mM NEM was required to produce the same result. It was also found that when showdomycin was added directly to the assay mixture in a final concentration of 1.6 mM it had no inhibitory effect.

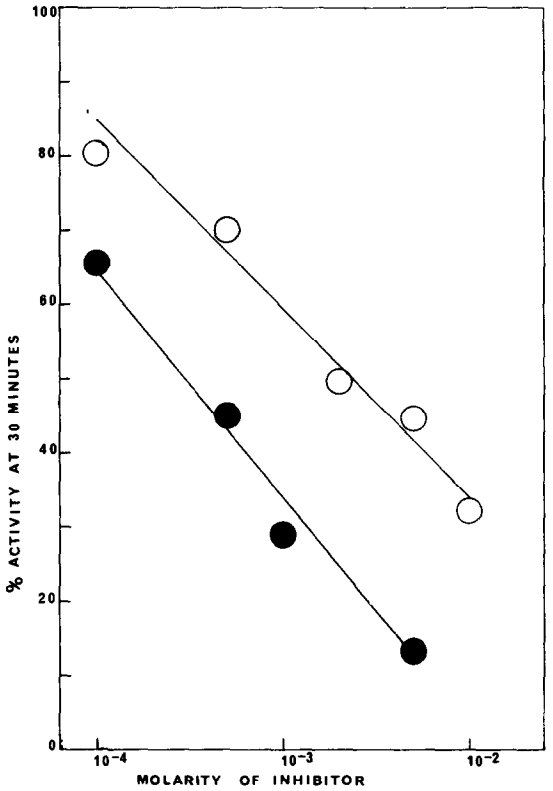

Fig. 2. Effect of preincubating RNA polymerase in the presence of various concentrations of showdomycin and NEM. Incubation mixtures contained 36 units of enzyme, showdomycin or NEM in varying concentrations, and dialysis buffer in a total volume of 80 μl. After 30 minutes incubation at 37°C, 25 μl aliquots were removed for assay in the standard assay mixture. The control was incubated in the same way, but contained only enzyme and buffer. 100% activity remaining at 30 minutes, based on this control, was equal to an incorporation of 1.27 nmoles of ^{14}C-AMP in the 10 minute assay.
○—○ showdomycin ●—● NEM.

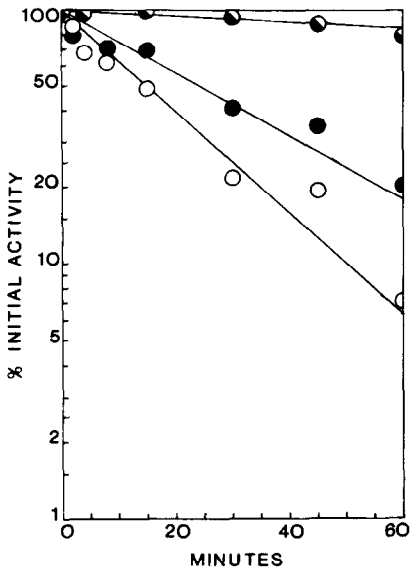

Fig. 3. Inactivation of RNA polymerase by showdomycin as a function of preincubation time.
Incubation mixtures contained either 156 or 312 units of enzyme, showdomycin at a final concentration of 2 mM, and dialysis buffer, in a total volume of 0.4 ml. Incubations were carried out at 37°C. At various times after the addition of enzyme, 20 μl aliquots were removed for assay in the standard assay mixture. The control contained only enzyme and buffer. Initial activity was 1.75 nmoles ^{14}C-AMP incorporated for 312 units of enzyme, 0.895 nmoles for 156 units of enzyme.
●—● 156 units enzyme ○—○ 312 units enzyme ◐—◐ control.

Table 1
Protection of RNA polymerase by components of the standard assay mixture against inactivation by showdomycin and NEM.

Addition	Amount of component added	% Activity remaining after 30 min	
		Showdomycin	NEM
None		100 (2.35 nmoles)	100 (3.28 nmoles)
Inhibitor		51	67
Inhibitor + DNA	7 μg	57	71
	14 μg	67	71
	28 μg	108	81
	70 μg	110	100
Inhibitor + metals	2 mM Mg^{2+} + 0.5 mM Mn^{2+}	69	86
	4 mM Mg^{2+} + 1 mM Mn^{2+}	69	84
	8 mM Mg^{2+} + 2 mM Mn^{2+}	75	83
	20 mM Mg^{2+} + 5 mM Mn^{2+}	82	103
Inhibitor + NTP's	0.25 mM each	52	71
	0.50 mM each	59	69
	0.75 mM each	59	68
	1.00 mM each	59	67

Incubation mixtures contained either showdomycin, 2 mM, or NEM, 0.2 mM, except in the control; enzyme, 56 units in the showdomycin experiment, 79 units in the NEM experiment; and the components listed, in a final volume of 0.2 ml. The mixtures were incubated 5 min at 37°C prior to the addition of inhibitor. After a further 30 min at 37°C, 50 μl aliquots were removed for assay. The assay mixtures were adjusted so that the final concentration of each component was brought to its normal level by the addition of the aliquot.

Fig. 3 shows that when the enzyme is preincubated with showdomycin at a concentration of 2 mM, there is an exponential loss of activity with time, the rate varying with enzyme concentration.

Since loss of activity is dependent on preincubation of the polymerase with showdomycin in the absence of substrate, the antibiotic is acting directly on the enzyme. Further experiments were therefore undertaken to determine which of the components of the assay mixture were capable of protecting against the inactivation of the enzyme by showdomycin. The results are shown in table 1. For comparison a similar set of experiments was conducted using NEM.

NTP's alone afforded little protection against inactivation by either showdomycin or NEM. On the other hand, increasing concentrations of metals alone provided some protection against showdomycin, although against NEM protection was complete. However, in three separate experiments a mixture of NTP's and metals in the proportions used in the assay gave no better protection against showdomycin than NTP's alone. Only DNA provided complete protection against both showdomycin and NEM. The antibiotic therefore attacks the free enzyme but not the enzyme-DNA complex. The only other antibiotic knwon to inhibit RNA polymerase by direct attack on the enzyme is rifampicin [5]. When RNA polymerase was incubated alone or with each of the separate assay components for the same period of time, loss of activity was less than 10%.

It was ascertained that showdomycin and NEM solutions in dialysis buffer were stable during the time in which inhibition experiments were performed. After incubation for 90 min at 37°C, showdomycin and NEM had lost none of their effectiveness in activating RNA polymerase.

4. Discussion

Although it is possible that *in vivo* showdomycin may be converted to a phosphorylated form, extracts of Ehrlich ascites cells (against which showdomycin exhibits antitumour activity) are incapable of phosphorylating the antibiotic [6]. In fact, the same authors found that showdomycin is an effective inhibitor of UMP phosphorylation at concentrations of 1.5 to 2.5 mM.

Fig. 2 makes it clear that NEM is a more effective inhibitor of RNA polymerase than is showdomycin. This could be due either to differences in the reactivity of the two maleimides or to steric hindrance by the ribose group in showdomycin. Although both showdomycin and NEM are unable to attack the enzyme-DNA complex, it is interesting that the metals, which are not essential for binding the enzyme to DNA [7], do afford some protection against inactivation. However, addition of the NTP's to the metals eliminates the protection which they offer, probably by chelation.

Since maleimides will react with both amino and sulphydryl groups [8], the nature of the alkylation remains to be studied. In other experiments we have found that DTNB, as well as *p*-chloromercuribenzoate, iodoacetamide and bromopyruvate will also inactivate RNA polymerase of *E. coli*.

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