DEGREE OF INHIBITION OF EUKARYOTIC DNA-DEPENDENT RNA POLYMERASES BY THURINGIENSIN

Květa Horská and Karel Šebesta

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received January 25th, 1980

It was shown in experiments with purified DNA-dependent RNA polymerases from calf thymus that the inhibition of these enzymes caused by thuringiensin is competitive with respect to ATP. Enzyme AI is more sensitive to the inhibitor ($K_i = 0{\cdot}34$ μM) than enzyme *B* ($K_i = 1{\cdot}49$ μM). This finding eliminates the discrepancies with respect to this subject which exist in literature.

Thuringiensin, a naturally occurring inhibitor of DNA-dependent RNA polymerase, was exhaustively studied in this Laboratory in experiments with *Escherichia coli* DNA-dependent RNA polymerase. We were able to show that this inhibitor behaves as an ATP analog and competes with the latter for the ATP binding site on the enzyme [1-3]. Klier and coworkers demonstrated that the DNA-dependent RNA polymerase from *Bacillus thuringiensis* is inhibited in a similar manner[4].

Thuringiensin has been shown to act as an efficient inhibitor of RNA biosynthesis in *in vivo* experiments with eukaryotic systems[5-7]. Numerous authors have shown that the inhibition of DNA-dependent RNA polymerases occurs even in these systems. The results of the individual studies, however, were not uniform as regards the intensity of the effect on the individual types of eukaryotic RNA polymerases. The situation was complicated by the fact that isolated enzyme preparations were used in several studies only[8,9]. Many other experiments were undertaken with isolated cell nuclei as a source of enzyme[9-12].

In an effort to cast light on this problem we studied the inhibition of highly purified calf thymus RNA polymerases AI and B by thuringiensin. A part of this work was carried out by one of us (K.H.) at the Institut de Chimie Biologique, Strasbourg (France).

EXPERIMENTAL

Material. Highly purified calf thymus RNA polymerases AI and B, highly polymerized calf thymus DNA (Worthington, U.S.A.) as well as [^{3}H]-UTP (13·6 Ci/mmol, ammonium salt, Amersham, UK), were generous gifts by Professor Pierre Chambon (Institut de Chimie Biologi-

que, Strassbourg, France). Enzyme AI was represented by fraction SE (ref.[14]) and enzymes B (mixture of B I and B II) were the GG fraction (ref.[15,16]). Aliquots of labeled UTP were diluted with the unlabeled nucleotide, lyophilized overnight and redissolved in water so as to obtain the desired specific activity. Dithiothreitol was purchased from Koch-Light Laboratories (UK). Unlabelled nucleoside triphosphates were from Calbiochem (U.S.A.) $MnSO_4.H_2O$ was supplied by Merck (FRG).

Instafluor and Soluene 300 were purchased from Packard (The Netherlands), Omnifluor was from NEN Chemicals (FRG). Whatman GF/C glass microfibre filters (diameter 24 mm) were used.

RNA *polymerase assay*: The assay mixture contained in a final volume of 0·125 ml: 10 μmol of Tris-HCl, pH 7·9 (80 mM); 0·5 μmol of Mn^{2+} (4 mM); four nucleoside triphosphates: GTP, CTP 0·125 μmol (1 mM), ATP 2·5—25 nmol (20—200 μM), [^{3}H]-UTP 15 nmol, 5 μCi (0·12 mM); 13 μg of calf thymus DNA. In addition the mixture contained 5 μmol (40 mM) (enzyme AI) and 12·5 μmol (100 mM) (enzymes B) of ammonium sulfate, respectively. RNA synthesis was initiated by the addition of the enzyme usually in 0·030 ml of buffer containing 1·5 μmol of Tris-HCl, pH 7·9 (50 mM), 3 nmol of EDTA (0·1 mM), 3 nmol of dithiothreitol (0·1 mM), 50% glycerol; dithiothreitol was added immediately before the experiment. The samples were incubated

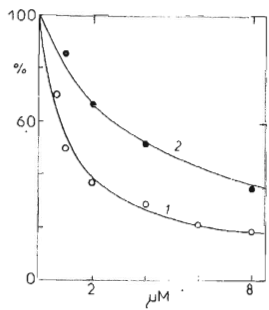

FIG. 1

Inhibition of RNA Polymerases AI and B by Thuringiensin

For composition of reaction mixture and conditions of incubation see Experimental. Final concentration in the assay: CTP, GTP 1 mM; [^{3}H]-UTP 0·12 mM; ATP 0·05 mM. Amounts of RNA polymerases AI *1* and B *2* in the assay: 5 μg and 0·6 μg of protein, respectively. Enzyme activity in %.

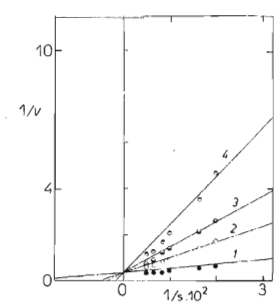

FIG. 2

Double Reciprocal Plot of RNA Polymerase AI Activity at Different Thuringiensin/ATP Ratios

For composition of reaction mixture and conditions of incubation see Experimental. Final concentration in the assay: CTP, GTP 1 mM; [^{3}H]-UTP 0·12 mM; ATP 0·05—0·20 mM; enzyme 5 μg of protein. Values for 0, 1, 2 and 4 μM concentration of thuriginensin (curves *1*, *2*, *3*, *4* respectively).

10 min at 37°C, then chilled in ice and precipitated by 3 ml of 10% trichloroacetic acid containing 9 μmol (30 mM) of sodium pyrophosphate. After 30 min the precipitates were collected on Whatman GF/C glass microfibre filters and washed 7 times with 6 ml of cold 3% trichloroacetic acid. After drying (120°C, 15 min) the pellets were solubilized for 30 min in 0·2 ml of Soluene 300. The radioactivity was measured 60 min after the addition of the scintillation counting solution (5 ml of 0·4% Omnifluor in toluene or 5 ml of Instafluor) in Isocarb liquid scintillation counter. All assays were made in duplicate. Values of the blank assays (not incubated) were substracted.

RESULTS AND DISCUSSION

The inhibitory effect of thuringiensin on DNA-dependent RNA polymerases AI and B, isolated from calf thymus, was studied. Thuringiensin inhibits both enzymes (Fig. 1). Similarly to prokaryotic polymerase, the inhibition is specific since it can be

TABLE I

Effect of Elevated Concentration of ATP, GTP and CTP, respectively, on the Inhibition of DNA-Dependent RNA Polymerase AI by Thuringiensin

Normal concentration of unlabeled nucleoside triphosphates: 0·5 mM; elevated concentration of ATP, GTP and CTP, respectively, 1 mM. For concentration of the remaining components of reaction mixture and conditions of incubation see Experimental. Concentration of thuringiensin 6 μM.

Concentration of nucleoside triphosphates	Enzyme activity, c.p.m. (%)	
	without thuringiensin	with thuringiensin
Normal	2 857 (100)	1 208 (42·3)
Elevated ATP	2 862 (100)	2 805 (98·2)
Elevated GTP	2 607 (100)	1 120 (43·0)
Elevated CTP	2 447 (100)	915 (37·4)

TABLE II

K_I and K_M of DNA-Dependent RNA Polymerases of Different Origin

Enzyme	K_M[a], μM	K_i, μM
Calf thymus AI	50·00	0·45
Calf thymus B	80·00	1·49
E. coli	[b]	30·00[c]

[a] For ATP; [b] not determined; [c] ref.[1,19].

reverted with both enzymes by a mere increase of ATP concentration (Table I). It was shown in experiments with varying ATP concentration (50–200 μM) in the reaction mixture that both enzymes, like the prokaryotic polymerases are inhibited by thuringiensin in a manner competitive with ATP (Fig. 2 and 3). The K_M- and K_i-values for both enzymes were calculated from the Lineweaver–Burk plot (Table II).

Hence, these results solve the present discrepancies in recorded data[8,9] on the inhibition of isolated eukaryotic DNA-dependent RNA polymerases A and B by thuringiensin. In the papers mentioned the isolation of the enzymes was effected by ammonium sulfate precipitation according to Roeder and Rutter[17,18] up to fraction 4, followed by chromatography on DEAE-Sephadex. The active fractions were collected in two peaks representing polymerases A and B and used in inhibition studies either as such or after a brief dialysis against the buffer. The methods[14,16] used for the isolation of RNA polymerases AI and B in this study afford enzymes of a higher degree of purity: The methods involve further purification steps such as column chromatography on phosphocellulose and Sepharose (in the case of enzyme AI) and column chromatography on phosphocellulose and hydroxyappatite, as well as centrifugation in glycerol gradients (in the case of enzyme B).

The following conclusions can be made from the results obtained: Like Beebee and coworkers[8], we also observed that RNA polymerase AI is inhibited to a higher degree than enzyme B by thuringiensin. This conclusion is in accordance with information obtained in studies using cell nuclei in which the individual RNA polymerases were distinguished, even though incompletely, by the addition of Mg^{2+}- and Mn^{2+}-ions (ref.[9,13]).

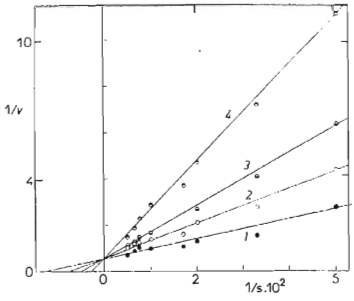

FIG. 3

Double Reciprocal Plot of RNA Polymerase B Activity at Different Thuringiensin/ATP Ratios

For composition of reaction mixture and conditions of incubation see Experimental. Final concentration in the assay: CTP, GTP 1 mM; [^{3}H]-UTP 0·12 mM; ATP 0·02—0·20 mM; enzyme 0·6 μg of protein. Values for 0, 1, 2 and 6 μM concentration of thuringiensin (curves *1*, *2*, *3*, *4* respectively).

A comparison of the inhibition constants determined for the individual eukaryotic RNA polymerase and for the *E. coli* RNA polymerase (ref.[1,19]) shows that the differences between the individual eukaryotic enzymes in the sensitivity to thuringiensin are far smaller than the differences between the eukaryotic enzymes on the one hand and the prokaryotic enzyme on the other (Table II). The results can be interpreted by postulating that – provided the structure of the active center of prokaryotic and eukaryotic polymerases is similar – the active center of the eukaryotic polymerases can better accommodate thuringiensin. Attention deserve in this respect the results obtained with the same inhibitor with DNA-dependent RNA polymerase from phage T7 pointing to a principally different structure of the active center of this enzyme[20].

We are greatly indebted to Professor P. Chambon, Strasbourg, for enabling one of us (K. H.) *to perform a part of these experiments in his Laboratory. The technical assistance of Mrs D. Veverková and of Mr J. Hanzlik is gratefully acknowledged.*

REFERENCES

1. Šebesta K., Horská K.: Biochim. Biophys. Acta *209*, 357 (1970).
2. Šebesta K., Sternbach H.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *8*, 233 (1970).
3. Johnson D. E.: Can. J. Microbiol. *27*, 537 (1978).
4. Klier A., Lecadet M.-M., deBarjac H.: C. R. Acad. Sci., Ser. *D 277*, 2805 (1973).
5. Mackedonski V. V., Nikolaev N., Šebesta K., Hadjiolov A. A.: Biochim. Biophys. Acta *272*, 56 (1972).
6. Mackedonski V. V., Hadjiolov A. A., Šebesta K.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *21*, 211 (1972).
7. Mackedonski V. V.: Biochim. Biophys. Acta *390*, 319 (1975).
8. Beebee T. J. C., Korner A., Bond R. P. M.: Biochem. J. *127*, 619 (1972).
9. Smuckler E. A., Hadjiolov A. A.: Biochem. J. *129*, 153 (1972).
10. Beebee T. J. C., Bond R. P. M.: Biochem. J. *136*, 1 (1973).
11. Beebee T. J. C., Bond R. P. M.: Biochem. J. *136*, 9 (1973).
12. Mackedonski V. V., Hadjiolov A. A.: C. R. Bulg. Acad. Sci. *27*, 1117 (1974).
13. Čihák A., Horská K., Šebesta K.: This Journal *40*, 2912 (1975).
14. Gissinger F., Chambon P.: Eur. J. Biochem. *28*, 277 (1972).
15. Kedinger C., Chambon P.: Eur. J. Biochem. *28*, 283 (1972).
16. Wasylyk B., Chambon P.: Eur. J. Biochem. *98*, 317 (1979).
17. Roeder R. G., Rutter W. J.: Nature (London) *224*, 234 (1969).
18. Roeder R. G., Rutter W. J.: Proc. Nat. Acad. Sci. U.S.A. *65*, 675 (1970).
19. Kalvoda L., Horská K., Šebesta K.: This Journal, in press.
20. Küpper H. A., McAllister W. T., Bautz E. K. F.: Eur. J. Biochem. *38*, 581 (1973).

Translated by V. Kostka.