EFFECT OF THURINGIENSIN ON THE INITIATION OF *in vitro* RNA SYNTHESIS

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Thuringiensin, a structural analog of ATP, which inhibits the elongation phase of RNA synthesis catalyzed by DNA-dependent RNA polymerase, also inhibits the initiation phase of this reaction. During the latter thuringiensin is not incorporated into the first site of the chain synthesized but inhibits the formation of pApU from 5′-AMP and UTP. If ATP and UTP are used as substrates the amount of the dinucleotide, a product of abortive synthesis, increases at low thuringiensin concentrations. Both elongation and abortive synthesis are inhibited at higher concentrations of the inhibitor.

RNA synthesis catalyzed by DNA-dependent RNA polymerase involves several steps. First, the preinitiation complex of the enzyme and DNA is formed, next comes initiation, *i.e.* the formation of the first internucleotide bond, followed by elongation of the polynucleotide chain; the whole reaction sequence is completed by termination[1,2]. Important for a deeper insight into initiation was the finding that a continuous synthesis of the complete RNA transcript need not necessarily follow immediately after the formation of the first phosphodiester bond[3,4]. Hence, continuous elongation of the chain does not take place during this initial phase of RNA synthesis (so-called abortive synthesis), yet a part of the product synthesized, predominantly the dinucleotide and other oligonucleotides, are cleaved off from the enzyme–template complex and accumulate in the reaction mixture. The size of the abortive products varies with the template used[5–12]. Only after a transcript of 3–11 nucleotides has been synthesized the ternary enzyme–template–RNA complex is sufficiently stable to allow a continuous elongation. Systematic studies on abortive synthesis were impossible until the mid-seventies because of the detection techniques (precipitation and chromatographic) then available which did not permit all reaction products to be separated and identified quantitatively[13,14]. This information was obtained only after high resolution polyacrylamide gel electrophoresis of the reaction mixtures had been introduced[5]. The mechanism of abortive initiation, however, has not been explained until now[15].

Studies on the mechanism of action of thuringiensin, a naturally occurring inhibitor of prokaryotic[16–18] and eukaryotic DNA-dependent RNA polymerases[19] have been

in progress in our Laboratory for a number of years. The present paper reports on the effect of thuringiensin on the process of initiation and abortive synthesis.

EXPERIMENTAL

Material. [α^{23}P-UTP] ($1{\cdot}72 \cdot 10^{13}$ Bq) was from Amersham (England). DNA-dependent RNA polymerase (the holoenzyme) was prepared from *Escherichia coli* K12 by the modified method of Burgess[20] involving chromatography on heparin-Sepharose 4B as described by Sternbach[21]. The enzyme solution in 50% glycerol containing 10 mg of protein per ml was stored at −20°C. Poly[d(A-T)$_n$] was supplied by Miles (England).

Conditions of transcription. All the reaction mixtures described in this paper had the following standard salt content: 40 mmol l^{-1} Tris-HCl, pH 7·9, 80 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, and 1 mmol l^{-1} dithiothreitol. The reaction mixture (15 µl) also contained 1 µg of poly[d(A−T)$_n$] and 30 µg of the enzyme protein. The quantity of substrates and inhibitors present in the individual reaction mixtures is shown in the legends to the figures. Following preincubation of the templates and RNA polymerase in the presence of the salts for 10 min at 37°C the enzymatic reaction itself was triggered by the addition of ATP, labeled UTP ($3{\cdot}7 \cdot 10^5$ Bq) or, alternatively, of the inhibitor. The reaction was terminated 15 min later by adding the reaction mixture to the lyophilisate of 15 µl of the so-called "reaction-stop mixture" containing 9 mol l^{-1} urea and 0·05 mol l^{-1} EDTA. Bromophenol blue (0·1%) and xylene cyanol (0·1%) were added to the samples which were then analyzed by gel electrophoresis.

Conditions of electrophoresis and its evaluation. The reaction products were analyzed in 24% polyacrylamide gels (0·04 × 13 × 40 cm) at room temperature according to Maxam and Gilbert[22]. The gels contained methylenebis (acrylamide) and acrylamide at a ratio of 1 : 29, 7 mmol l^{-1} urea, 50 mmol l^{-1} Tris-borate, pH 8, and 1 mmol l^{-1} EDTA. Before the application of samples the gels were subjected to preelectrophoresis at 1 000 V for 30 min. The electrophoresis itself was allowed to proceed at 500−1 000 V until the spot of bromophenol blue had moved approximately 17 cm from the origin. After completion of the electrophoretic run the gels were evaluated by autoradiography. Selected product lanes were cut out of the gel for quantitative evaluation after autoradiography and scanned according to Cerenkov.

RESULTS AND DISCUSSION

Studies on the inhibitory effect of thuringiensin on DNA-dependent RNA polymerase of both bacterial and animal origin have shown that the inhibitor can enter the elongation binding site of the enzyme[16−19]. However, the mechanism of the effect of the inhibitor on the initiation phase of RNA synthesis remained unclear. According to previous data a number of adenosine derivatives, some of which resemble to a limited degree[23−28], the naturally occurring substrates can enter the initiation site and form the first phosphodiester bond. It was therefore reasonable to expect that thuringiensin containing an adenosine moiety would act in a similar manner. It has been shown though that the 5′−3′ phosphodiester bond between the UMP residue and thuringiensin (Fig. 1,* lane *c*) is not formed if thuringiensin and labeled UTP are used as substrates for the RNA polymerase reaction with poly [d(A−T)$_n$]

* See insert on the p. 288.

as template. We obtained the same negative result when dephosphorylated thuringiensin was used (Fig. 1, lane *d*). The synthesis of pApU under identical experimental conditions with 5'-AMP and labeled UTP as substrates served as a control for the evaluation of the reactions products in polyacrylamide gels (Fig. 1, lane *a*).

In further experiments we investigated whether thuringiensin affects the synthesis of dinucleoside phosphate pApU from 5'-AMP and labeled UTP as substrates. When equimolar concentrations of 5'-AMP and thuringiensin are used a 90% inhibition of the synthesis of the dinucleoside phosphate can be observed (Fig. 1, lane *b*). This finding can be interpreted by postulating that thuringiensin replaces AMP in the initiation site even though it is not capable to form a phosphodiester bond with UTP as the second substrate.

Investigating the inhibition of pApU formation we considered interesting to find out whether and in what manner thuringiensin affects the subsequent abortive synthesis, *i.e.* the formation of short oligonucleotides which parallel the first elongation phase. The results obtained with ATP and labeled UTP as substrates of the RNA polymerase reaction in the presence of varying thuringiensin concentrations (Table I) show that

TABLE I

Electrophoretic resolution of products of RNA polymerase reaction as function of thuringiensin concentration in reaction mixture. The concentration of thuringiensin is given in μmol l^{-1}, the quantity of the products is expressed by per cent of total radioactivity incorporated

Position in gel[a]	Product	Thuringiensin, μmol l^{-1}			
		—	0·77	7·7	77
1	High molecular weight product[b]	44·3	32·7	32·5	16·6
2		27·3	26·4	26·8	29·6
3		7·8	9·1	8·9	14·3
4		3·1	3·3	4·0	6·9
5		1·2	1·5	1·6	3·0
6		0·4	0·5	0·6	1·0
7		0·2	0·3	0·3	0·5
8		0·3	0·4	0·3	0·5
9		0·2	0·3	0·2	0·3
10	Heptanucleotide[c]	0·2	0·5	0·3	0·4
11	Hexanucleotide[c]	0·3	0·5	0·4	0·4
12	Pentanucleotide[c]	0·3	0·5	0·4	0·5
13	Tetranucleotide[c]	0·8	1·2	1·1	1·3
14	Trinucleotide[c]	2·2	2·7	2·5	3·6
15	Dinucleotide	11·5	19·6	20·0	21·4

[a] See Fig. 1, slot *g*; [b] in the region or origin; [c] assigned tentatively.

even at a 0·77 μmol l^{-1} inhibitor concentration (*i.e.* at a 350-fold excess of the substrate over the inhibitor) a shift in the distribution of the individual products in the reaction mixture occurs. The previously reported relative decrease of the amount of high molecular weight chains, which remain at the origin during polyacrylamide gel electrophoresis[16], is due to a slow-down of the synthesis during the elongation phase caused by the presence of thuringiensin and above all, to its effect on the synthesis of the main product of abortive synthesis, *i.e.* dinucleotide pppApU. The relative amount of this dinucleotide in the reaction mixtures increases practically to the double with the increasing concentration of thuringiensin (Table I). This effect cannot be accounted for by inhibition of the elongation phase and is most likely caused by an increase in the number of dinucleotide molecules released from the complex during the abortive phase of the synthesis. Hence, thuringiensin probably increases the instability of the ternary complex during this phase, most likely by entering the binding site for ATP. Whereas during the elongation phase thuringiensin is competitively displaced from this site by ATP and the synthesis of the chain continues, during the initial phase of the synthesis the initial dinucleotide is most likely released, thus enhancing the effect which is typical of this phase even in the absence of the inhibitor. A similar effect can be observed, yet to a smaller extent, also with the trinucleotide and tetranucleotide whose formation is also regarded as abortive synthesis. The labilization of the ternary complex by thuringiensin manifests itself in the same manner at all three inhibitor concentrations used as shown in Table I. If the excess of thuringiensin over the substrate, ATP, is ten-fold a practically complete inhibition of the synthesis occurs; this is similar to the inhibition of pApU formation by equimolar concentrations of the inhibitor and 5′-AMP (Fig. 1, lane *e*). The reason why the inhibition of synthesis of the dinucleotide from ATP and UTP takes place at a higher inhibitor excess only can be explained by the much higher affinity of ATP for the binding site for the first nucleotide as reported by Scheit[27]. The synthesis of pApU is namely about 14 times lower than the synthesis of pppApU at equal concentration of 5′-AMP and 5′-ATP.

Our results show that thuringiensin acts also on the initiation phase of the synthesis by inhibiting its process at higher concentrations. At lower concentrations the entry of thuringiensin in the binding site for elongation enhances the abortive synthesis of the initial dinucleotide.

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REFERENCES

1. Chamberlin M. J.: *The Enzymes*, Vol. XV, p. 61.
2. Chamberlin M. J. in the book: RNA *Polymerase* (R. Losick M. Chamberlin, Eds), p. 17. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1976.

3. Johnston D. E., McClure W. R. in the book: RNA *Polymerase* (R. Losick, M. Chamberlin, Eds), p. 413. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1976.
4. McClure W. R., Cech C. L.: J. Biol. Chem. *253*, 8949 (1978).
5. Carpousis A. J., Gralla J. D.: Biochemistry *19*, 3245 (1980).
6. Hansen U. M., McClure W. R.: J. Biol. Chem. *255*, 9564 (1980).
7. Gralla J. D., Carpousis A. J., Stefano J. E.: Biochemistry *19*, 5864 (1980).
8. Grachev M. A., Zaychikov E. F.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *115*, 23 (1980).
9. Nierman W. C., Chamberlin M. J.: J. Biol. Chem. *255*, 4495 (1984).
10. Kinsella L., Hsu Ch.-Y.J., Schulz W., Dennis D.: Biochemistry *21*, 2719 (1982).
11. Munson L. M., Reznikoff W. S.: Biochemistry *20*, 2081 (1981).
12. Schulz W., Zillig W.: Nucleic Acids Res. *9*, 6889 (1981).
13. McClure W. R., Cech C. L., Johnston D. E.: J. Biol. Chem. *253*, 8941 (1978).
14. Nierman W. C., Chamberlin M. J.: J. Biol. Chem. *254*, 7921 (1979).
15. Gamper H. B., Hearst J. E.: Cell *29*, 81 (1982).
16. Šebesta K., Horská K.: Biochim. Biophys. Acta *209*, 357 (1970).
17. Šebesta K., Sternbach H.: FEBS (Red. Eur. Biochem Soc.), Lett. *8*, 233 (1970).
18. Kalvoda L., Horská K., Šebesta K.: This Journal *46*, 677 (1981).
19. Horská K., Rosenberg I., Holý A., Šebesta K.: This Journal *49*, 1352 (1983).
20. Burgess R. R.: J. Biol. Chem. *244*, 6160 (1969).
21. Sternbach H., Engelhardt R., Lezius A. G.: Eur. J. Biochem. *60*, 511 (1975).
22. Maxam A. M., Gilbert W.: Methods Enzymol. *65*, 495 (1980).
23. Malygin A. G., Shemyakin M. F.: FEBS Fed. Eur. Biochem. Soc., Lett. *102*, 51 (1979).
24. Yee D., Armstrong V. W., Eckstein F.: Biochemistry *18*, 4116 (1979).
25. Smagowicz W. J., Castell J. V., Clegg R. M., Scheit K. H.: Biochemistry *20*, 5538 (1981).
26. DeRiemer L. H., Meares C. F.: Biochemistry *20*, 1606 (1981).
27. Smagowicz W. J., Scheit K. H.: Nucleic Acids Res. *10*, 2397 (1981).
28. Hanna M. H., Meares C. F.: Biochemistry *22*, 3546 (1983).

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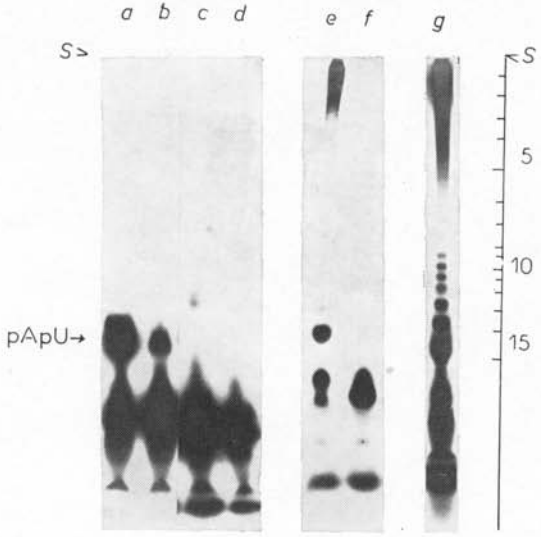

Fig. 1

Autoradiogram of denaturing polyacrylamide gel electrophoretic separation of complementary oligonucleotides Lane *a* control containing 2 mmol l^{-1} AMP and 50 μmol l^{-1} UTP; lane *b* sample containing 2 mmol l^{-1} AMP, 50 μmol l^{-1} UTP, and 2 mmol l^{-1} thuringiensin; lane *c* sample containing 2 mmol l^{-1} thuringiensin and 50 μmol l^{-1} UTP; lane *d* sample containing 2 mmol l^{-1} dephosphorylated thuringiensin and 50 μmol l^{-1} UTP; lane *e* control containing 50 μmol l^{-1} ATP and 5 μmol l^{-1} UTP; lane *f* sample containing 50 μmol l^{-1} ATP, 5 μmol . . l^{-1} UTP, and 2 mmol l^{-1} thuringiensin; lane *g* representation of treatment of gel for Table I