THE EFFECT OF RIFAMPICIN ON DNA-DEPENDENT RNA POLYMERASE FROM *Mycobacteria*

P.MIŠOŇ and L.TRNKA

Research Institute of Tuberculosis and Respiratory Diseases, Prague 8

Received December 10th, 1970

The purification and properties of DNA-dependent RNA polymerase from the *Mycobacterium bovis* strain BCG 725-Prague and *Mycobacterium smegmatis* strain 607 have been described and the inhibitory action of the antibiotic rifampicin on the both RNA polymerase systems has been examined. Its effect is similar to that reported for DNA-dependent RNA synthesis by *Escherichia coli* RNA polymerase which suggest that rifampicin is a specific inhibitor of initiation also for mycobacterial enzyme. Whereas the growth of *M. bovis* str. BCG 725 is inhibited by a 500 times smaller concentration of rifampicin than that required for inhibition of *M. smegmatis* str. 607, the two enzymes do not differ significantly in their sensitivity towards the antibiotic. The possibility of resistance due to differences of the cell-wall composition of some mycobacterial strains is discussed.

Rifamycins are known to inhibit the DNA-dependent synthesis of RNA in *Escherichia coli* and of some other bacterial species[1-5]. They form a stable complex with RNA polymerase[6,7] and thus prevent the binding of the enzyme to template DNA, *i.e.* they inhibit the polymerization reaction in the initiation phase[8-10]. In resistant strains this binding and hence inhibition do not take place.

Although rifamycins and especially their derivative rifampicin belong to important antimycobacterial drugs a similar mechanism of action has not yet been demonstrated in mycobacteria. The sensitivity of various species of mycobacteria to rifampicin varies from species to species – it is rather high in *Mycobacterium tuberculosis* and *Mycobacterium bovis* but very low with some atypical and saprophytic strains. The relationship between this sensitivity and that of mycobacterial RNA polymerase is treated in the present communication.

EXPERIMENTAL

Material. Adenosine 5′-triphosphate was obtained from Boehringer, Mannheim, guanosine 5′-triphosphate was from Schwarz, Orangeburg, cytidine 5′-triphosphate, dithiothreitol and protamine sulfate were from Calbiochem, Lucerne, and calf thymus DNA from Sigma, St. Louis. Uridine 5′-triphosphate-[U-^{14}C] (135 mCi/mmol) was obtained from the Institute for Research, Production and Uses of Radioisotopes, Prague, DEAE-cellulose (DE 11) and CM-cellulose (CM 11) for column chromatography was from Whatman, England. Streptomycin suifate was from Medexport, Moscow, and rifampicin from Lepetit, Milan.

Preparation of the DNA-*dependent* RNA *polymerase.* RNA polymerase was isolated from the S 105 fraction of strains *Mycobacterium bovis sp.* BCG 725-Prague, and *Mycobacterium smegmatis*[11] *sp.* 607 (50—70 g wet weight) grown in Sauton's medium and disintegrated with alumina. The cultivation and preparation of the S 105 fraction were described previously[12] but for the extraction of the crushed material 0·05M-Tris-HCl (pH 7·9) with 0·01M-$MgCl_2$, 0·006M 2-mercaptoethanol and 10^{-4}M-EDTA was used. All the work described was performed at 0—4°C unless otherwise stated. Protein concentration was determined by measuring the absorbance at 260 and 280 nm. Purification of RNA polymerase was done by a modified method of Chamberlin and Berg[13] and Weiss[14].

Isolation from the S 105 *fraction.* DNA was precipitated from the S 105 fraction (12—15 mg protein/ml) with freshly prepared 10% streptomycin sulfate added in 1/20 the amount of the original S 105 fraction. After centrifugation, the enzyme from the supernatant was precipitated with protamine sulfate. RNA polymerase was extracted from the precipitate with 0·05M-Tris-HCl (pH 7·9) containing 0·01M-$MgCl_2$, 0·006M 2-mercaptoethanol, 10^{-4}M-EDTA and 0·15M-$(NH_4)_2SO_4$ and, after centrifugation, precipitated from the supernatant by an addition of ammonium sulfate to 55% saturation. The precipitate was separated by centrifugation, dissolved in the same Tris-HCl buffer containing 0·06M ammonium sulfate and 5% glycerol. The solution was passed through a layer of CM-cellulose prepared according to Weiss[14].

Purification on DEAE-*cellulose.* Eluate after purification on CM-cellulose was loaded onto a column (1 × 20 cm) of DEAE-cellulose. Inactive proteins were washed out with 0·05M-Tris-HCl (pH 7·9) containing 0·01M-$MgCl_2$, 0·006M 2-mercaptoethanol, 10^{-4}M-EDTA, 0·13M-KCl and 5% glycerol, and RNA polymerase was then eluted from the column with the same buffer but containing 0·23M-KCl and 10% glycerol. Fractions containing the main portion of enzyme activity were combined, the enzyme was precipitated with ammonium sulfate (to 55% saturation), the precipitate was dissolved in 0·05M Tris-HCl (pH 7·9) containing 0·3M ammonium sulfate and 10^{-4}M-EDTA. The same volume of glycerol was added to the solution and the preparation was stored at —20°C.

Estimation of RNA *polymerase.* The reaction mixture for estimating RNA polymerase contained in 0·25 ml: 12·5 μmol Tris-HCl (pH 7·9), 1·5 μmol $MgCl_2$, 0·5 μmol $MnCl_2$, 1·0 μmol dithiothreitol, 0·0625 μmol nucleoside 5′-triphosphate with the exception of 1 nmol uridine-U-^{14}C 5′-triphosphate, 50 μg calf thymus DNA and 10—100 μg of the enzyme fraction. The mixture was incubated for 10 min at 30°C. The reaction was stopped by adding 0·1 ml saturated sodium pyrophosphate and 2 ml 5% trichloroacetic acid. The precipitate was collected on membrane filters (Sartorius, TB 2, 2 cm diameter) which were kept in 8 mM adenosine 5′-triphosphate. Filters with precipitate were washed and suspended in 6 ml scintillation medium and their radioactivity counted in a Tritio-Matic 3100 (ACEC, Charleroi) counter with an efficiency of about 36%.

RESULTS AND DISCUSSION

Typical values of specific activity of mycobacterial DNA-dependent RNA polymerase at individual stages of isolation are shown in Table I. The yield varied about 1% referred to total protein content of the S 105 fraction and the specific activity rose about 100 times. Up to the stage of purification on CM-cellulose the enzyme is very labile. The temperature optimum of the reaction is at about 30°C although the temperature dependence curve is very flat and results at 37°C do not differ substantially from the optimum. A greater drop is observed at 45°C and more.

TABLE I

Purification of Mycobacterial DNA-Dependent RNA Polymerase

Specific activity is expressed in pmol uridine-[U-^{14}C] 5'-triphosphate incorporated by 1 mg of enzyme in 10 min at 30°C into trichloroacetic acid-insoluble material.

Fraction	Specific activity		Extent of purification	
	BCG 725	M 607	BCG 725	M 607
S 105	2·9	2·4	(1)	(1)
Fractionation with				
streptomycin	28	36	10	15
protamine	125	68	43	28
CM-cellulose	260	92	90	38
DEAE-cellulose	352	229	121	95

TABLE II

General Characteristics of Mycobacterial RNA Polymerase (BCG 725)

Incorporation is expressed in pmol uridine-[U-^{14}C] 5'-triphosphate incorporated by 60 μg enzyme under conditions of estimation described in the text.

System	pmol	%
Complete	20·4	100
Without Mg^{2+}	13·9	68·2
Mn^{2+}	3·6	17·6
Mg^{2+} and Mn^{2+}	1·0	4·9
dithiothreitol	10·7	52·5
ATP, GTP and CTP	0·8	4·2
DNA	0·2	1·0
enzyme	0·2	1·0

The enzyme activity is markedly dependent on the presence of magnesium and manganese ions and of all the four nucleotides. Dithiothreitol increases the activity of the preparation, the dependence on DNA is very pronounced. The general characteristics of the preparation are shown in Table II.

Rifampicin inhibits completely this polymerization reaction at concentrations as low as 1 μg/ml (*i.e.* $1{\cdot}2 \cdot 10^{-6}$ M) and a considerable inhibition is found even at much lower concentrations (Fig. 1). Complete inhibition is observed only in the case that the antibiotic is present in the reaction mixture before the onset of polymerization. On adding it after the onset of synthesis the inhibitory effect of rifampicin is

greatly reduced so that the reaction course cannot be distinguished from the control (Fig. 2). This different effect before and after onset of synthesis suggest that rifampicin is probably a specific inhibitor of initiation for the mycobacterial RNA polymerase as well as for the *E. coli* enzyme[8,9]. The slight inhibition observed after an earlier addition to the reaction mixture (after 5 min) suggests that at a high substrate concentration initiation continues for a certain period[3].

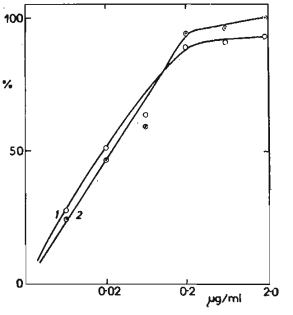

FIG. 1

Inhibition of Mycobacterial DNA-Dependent RNA Polymerase by Rifampicin

The reaction conditions are described in the text. ⊙ RNA polymerase from *M. bovis sp.* BCG 725 — Prague, ○ RNA polymerase from *M. smegmatis sp.* 607. % Inhibition of reaction; μg/ml, amount of rifampicin in the reaction mixture.

It is of interest, however, that the sensitivity of RNA polymerase toward rifampicin is about the same for the two mycobacterial strains although the minimum concentrations inhibiting growth of the two strains *in vitro* are rather different (0·2 μg/ml

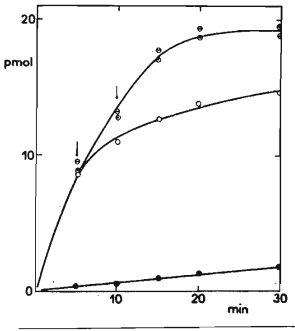

FIG. 2

Time Course of Inhibition of DNA-Dependent Synthesis of RNA in *M. bovis sp.* BCG 725 — Prague

The reaction mixture contained the components used for standard estimations (with 150 μg enzyme/ml) and 0·25 ml samples were removed from it at the shown time intervals and processed in the usual way. Rifampicin to $2{\cdot}4 \cdot 10^{-7}$ M (0·2 μg/ml) was added at times shown by the arrow. ⊙ Control reaction (without rifampicin), ○ rifampicin added 5 min after onset of incubation; ⊖ rifampicin added 10 min after onset of incubation; ● enzyme preincubated for 10 min with rifampicin. Reaction started by adding [^{14}C]-UTP and DNA at time 0. pmol, amount of incorporated uridine 5′-triphosphate; *t* time of incubation at 30°C in min.

for BCG 725 and 100 μg/ml for M 607). The high primary resistance of *M. smegmatis* is thus not caused by an altered enzyme structure but is probably due to differences in the structure of the cell envelopes of the two strains, either of the wall or of the membrane. The increased resistance occurs primarily in the group of atypical mycobacteria. It is less represented with photochromogens, scotochromogens, nonphotochromogens and avian strains, most pronounced being in rapidly growing mycobacteria[15–19]. Values found for some saprophytic strains (more than 200 μg/ml) were similarly observed in nocardias[15] which are often difficult to distinguish from mycobacteria. The increased resistance to rifampicin is not a characteristic feature of these atypical strains, so that *e.g.* even for different strains of the fourth group of Runyon the sensitivity varies from 1 μg to 200 μg/ml. It may be assumed that the resistant mutants with altered RNA polymersae were formed by induction mostly from the sensitive mycobacterial strains although a combination of both types of resistance cannot be excluded.

REFERENCES

1. Hartmann G., Hornikel K. O., Knüsel F., Nüesch J.: Biochim. Biophys. Acta *145*, 843 (1967).
2. Umezawa H., Mizuno S., Yamazaki H., Nitta K.: J. Antibiotics *21*, 234 (1968).
3. Straat P. A., Ts'o P. O.: Biochemistry *9*, 926 (1970).
4. Wehrli W., Knüsel F., Staehelin M.: Biochim. Biophys. Res. Commun. *32*, 284 (1968).
5. Goldthwait D. A., Anthony D. D., Wu Ch.-W.: *Proc. 1st Internat. Lepetit Coll. on the RNA-Polymerase and Transcription* (L. Silvestri, Ed.), p. 10. North-Holland, Amsterdam, London 1970.
6. Wehrli W., Knüsel F., Schmid K., Staehelin M.: Proc. Natl. Acad. Sci. US *61*, 667 (1968).
7. Mauro E. di, Snyder L., Marino P., Lamberti A., Coppo A., Tocchini-Valentini G. P.: Nature *222*, 533 (1969).
8. Anthony D. D., Zeszotek E., Goldthwait D. A.: Proc. Natl. Acad. Sci. US *56*, 1026 (1966).
9. Lill H., Lill U., Sippel A., Hartmann G.: *Proc. 1st Internat. Lepetit Coll. on the RNA-Polymerase and Transcription* (L. Silvestri, Ed.), p. 55. North-Holland, Amsterdam, London 1970.
10. Sippel A., Hartmann G.: Biochem. Biophys. Acta *157*, 218 (1968).
11. Gordon R. E., Smith M. M.: J. Bacteriol. *66*, 41 (1953).
12. Trnka L., Mišoň P.: Fol. Microbiol. (Prague) *16*, 91 (1971).
13. Chamberlin M., Berg P.: Proc. Natl. Acad. Sci. US *48*, 81 (1962).
14. Weiss S. B. in the book: *Methods in Enzymology*, Vol. XII B (L. Grossman, K. Moldave, Eds). Academic Press, New York-London 1968.
15. Arioli V., Pallanza R., Furesz S., Carniti G.: Arzneimitt.-Forsch. *17*, 523 (1967).
16. Pallanza R., Arioli V., Furesz S., Bolzoni G.: Arzneimitt.-Forsch. *17*, 529 (1967).
17. Lorian V., Finland M.: Appl. Microbiol. *17*, 202 (1969).
18. McClatchy J. K., Waggoner R. F., Lester W.: Am. Rev. Resp. Dis. *100*, 234 (1968).
19. Hobby G. L., Lenert T. F., Majer-Engallena J.: Am. Rev. Resp. Dis. *99*, 453 (1969).

Translated by A. Kotyk.