ADP-Ribosylation as an Intermediate Step in Inactivation of Rifampin by a Mycobacterial Gene

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Received 9 March 1998/Returned for modification 20 October 1998/Accepted 4 November 1998

Mycobacterium smegmatis DSM43756 inactivates rifampin, and the inactivated antibiotic product recovered from culture medium was ribosylated on the 23-OH group. To study this process, the gene responsible for the inactivation was expressed at high levels by the lac promoter in Escherichia coli conferring resistance to >500 μg of rifampin per ml. Cell homogenates generated a novel derivative designated RIP-TAs; in this study, we determined that RIP-TAs is 23-(O-ADP-ribosyl)rifampin. Our results indicated that RIP-TAs is an intermediate in the pathway leading to ribosylated rifampin and that the previously characterized gene encodes a mono(ADP-ribosyl)transferase which, however, shows no sequence similarity to other enzymes of this class.

Rifampin is a valuable antibiotic and one of the principal chemotherapeutic agents used to combat tuberculosis and other pathogenic mycobacteria. Its antimicrobial activity is due to inhibition of prokaryotic DNA-dependent RNA polymerases, and most rifampin-resistant Mycobacterium tuberculosis and Mycobacterium leprae strains have been reported to have an alteration in the β-subunit of this enzyme (9, 18). However, most rifampin-resistant clinical isolates of Mycobacterium avium and Mycobacterium intracellulare do not have any mutations in the rpoB gene (6), which suggests that there are other genes which are able to confer some degree of resistance. Hetherington et al. (8) showed that Mycobacterium smegmatis, which is naturally resistant to the antibiotic, had no identifiable rpoB mutations and suggested that another mechanism of resistance is at work in this species.

During our studies on the mechanisms of rifampin resistance in acid-fast bacteria, we found that the antibiotic was modified to give a glucosylated or phosphorylated derivative in pathogenic Nocardia spp. (23–25). We also found that M. smegmatis DSM43756 has the ability to inactivate rifampin by ribosylation (3), the first reported case of such a mechanism. Gene disruption experiments showed that ribosylative inactivation of rifampin is a major contributor to the low susceptibility of M. smegmatis to rifampin and that this is the principal rifampin inactivation mechanism in this bacterium (20). We have also reported that many mycobacteria including M. chelonae subsp. abscessus, M. flavescens, M. vaccae, and M. parafortuitum as well as Gordona and Tsukamurella strains inactivate rifampin by ribosylation (23). The ribosylated antibiotic was purified from culture broth of M. smegmatis or Rhodococcus rhodochrous carrying the cloned gene responsible (20), so in this work, we determined the inactivated form of the antibiotic produced by cell homogenates in vitro when the gene was expressed at a high level by using the lac promoter in Escherichia coli.

Rifampin was generously provided by CIBA-Geigy Pharmaceuticals. MICs were determined by an agar dilution method with brain heart infusion agar (Difco Laboratories, Detroit, Mich.) medium. Strains, plasmids, and other methods were as described previously (20).

The cloned mycobacterial gene was found in E. coli MM294-4 to confer a twofold increase in rifampin resistance when expressed off its own promoter, but in vitro antibiotic inactivation by homogenates was not detected with this transformant. By using the previously sequenced 1.1-kb SaII fragment (20) as starting material, various subclones were obtained in E. coli plasmid pGEM3Zf(−). One construct, in which a 600-bp BstNI fragment blunt ended with Klenow enzyme was cloned into the HindIII site of the vector, conferred resistance to >500 μg of rifampin per ml and was used for in vitro studies. E. coli carrying this clone, pGEM3Z-Bst49, was grown in a 500-ml Erlenmeyer flask containing 200 ml of Luria broth (LB) with 100 μg of ampicillin per ml on a Brunswick rotary shaker for 18 h at 37°C. Cells were collected by centrifugation and resuspended in 5 ml of extraction buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5 mM dithiothreitol). The cell suspension on ice was sonicated for 30 s three times each time (Bioruptor; Cosmo Bio, Tokyo, Japan). To 4 ml of this cell homogenate, 4 ml of reaction mixture containing 60 mg of rifampin and 100 mg of NADH was added. After incubation at 37°C for 3 h, the reaction mixture was centrifuged and the supernatant was freeze-dried. Dried samples were extracted with 5 ml of methanol, and the extract was chromatographed on a LH-20 Sephadex column. From 60 mg of rifampin, 43 mg of purified inactivated compound (designated RIP-TAs) was obtained. The purification state of RIP-TAs was checked by reverse-phase high-pressure liquid chromatography [LiChrospher 100, RP-18(e), Cica-Merck; column dimensions, 4.6 by 150 mm; 38% acetonitrile (MeCN) with 0.05% trifluoroacetic acid] with a detection system at UV 270 nm. Reverse-phase thin-layer chromatography [LiChrospher 100, RP-18(e), Cica-Merck; column dimensions, 4.6 by 150 mm; 38% acetonitrile (MeCN) with 0.05% trifluoroacetic acid] with a detection system at UV 270 nm. Reverse-phase thin-layer chromatography (KC18F; J. T. Baker, Inc.) with a development system of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan, and Genetics Department, University of the Witwatersrand, Johannesburg 2050, South Africa1

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tion of the nitrogen-fixing enzyme dinitrogenase reductase in the photosynthetic bacterium *Rhodospirillum rubrum* mediates the reversible inactivation of the enzyme and is influenced by light (12). T4 and related bacteriophages code for two ADP-ribosylating activities, the Alt and Mod gene products, whose main target is the host DNA-dependent RNA polymerase altering promoter specificity (10).

Endogenous mono(ADP-ribosylation) has been demonstrated in several bacteria including *Pseudomonas aeruginosa* (11, 19), but little is known about the physiological role of this modification process (11). In these cases, the acceptors were proteins. However, in the present study the acceptor was a low-molecular-weight antibiotic, rifampin, and to our knowledge, this is the first case of ADP-ribosylation as a mechanism of antibiotic inactivation. Moreover, the ADP-ribosyl moiety is joined to an oxygen atom, in contrast to the examples cited above, where it is a nitrogen atom.

We find that NAD is the preferred source of the ADP-ribosylation, both in vivo and in vitro, so the ADP-ribosylated rifampin (RIP-TAs) may be hydrolyzed by an ADP-ribose phosphohydrolase, as previously described (4). This work was partly supported by a Grant-in-Aid for Scientific research (C) from the Ministry of Education, Sciences, Sports, and Culture of Japan to Y.M.

**REFERENCES**


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**FIG. 2.** 1H NMR spectrum of RIP-TAs in CD3 OD. Peaks caused by impurity or methanol are indicated by asterisks.


