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Role of Glycosylation and Deglycosylation in Biosynthesis of and Resistance to Oleandomycin in the Producer Organism, *Streptomyces antibioticus*

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Cell extracts of *Streptomyces antibioticus*, an oleandomycin producer, can inactivate oleandomycin in the presence of UDP-glucose. The inactivation can be detected through the loss of biological activity or by alteration in the chromatographic mobility of the antibiotic. This enzyme activity also inactivates other macrolides (rosaramicin, methymycin, and lankamycin) which contain a free 2'-OH group in a monosaccharide linked to the lactone ring (with the exception of erythromycin), but not those which contain a disaccharide (tylosin, spiramycin, carbomycin, josamycin, niddamycin, and relomycin). Interestingly, the culture supernatant contains another enzyme activity capable of reactivating the glycosylated oleandomycin and regenerating the biological activity through the release of a glucose molecule. It is proposed that these two enzyme activities could be an integral part of the oleandomycin biosynthetic pathway.

Resistance to macrolides in producing actinomycetes has been studied in some detail, and several resistance genes have been cloned (1, 3, 4, 12, 15, 20). Analysis of the biochemical basis of the resistance indicates that ribosomal modification by means of methylation at specific sites in the 23S rRNA seems to be a quite general resistance mechanism (13, 14, 22, 23). However, enzymatic activities capable of inactivating macrolides have not been detected in macrolide producers, although they have been reported in clinical isolates (11) and in some other streptomycetes (8, 9, 21).

Oleandomycin is a macrolide antibiotic structurally closely related to erythromycin. It contains a 14-membered lactone ring (oleandolide) with one amino sugar (desosamine) and one sugar (oleandrose) attached (Fig. 1). However, while *Saccharopolyspora erythraea* (an erythromycin producer) possesses ribosomes which are constitutively resistant to erythromycin (13), the oleandomycin producer, *Streptomyces antibioticus*, does not contain ribosomes resistant to oleandomycin during the cell cycle, even during oleandomycin biosynthesis (5). Therefore, another resistance mechanism must exist. Celmer et al. (2) reported the presence of glucosylated oleandomycin in the fermentation broths of *S. antibioticus*, which we believed might form the basis for a new mechanism of resistance.

Here, we report the existence of two enzymatic activities in *S. antibioticus*: one that is capable of inactivating oleandomycin using UDP-glucose as a cofactor and another that is able to reactivate the molecule.

MATERIALS AND METHODS

Microorganisms and culture conditions. *S. antibioticus* ATCC 11891, an oleandomycin producer, was used throughout this study. To obtain spores, it was grown on GAE solid medium plates (6) for 7 days at 30°C. For growth in liquid culture, the microorganism was grown in tryptone soy broth (TSB) medium (Oxoid) at 30°C with shaking (200 rpm) in an orbital Gallenkamp incubator for 48 h. *Micrococcus luteus* ATCC 10240 was used as the indicator organism; it was grown overnight at 37°C in TSB medium and then diluted 1:100 in TSB containing 50% (wt/vol) glycerol and stored at −20°C until needed.

Preparation of cell extracts. TSB medium (50 ml) was inoculated with a spore suspension of *S. antibioticus* and incubated as described above. The mycelia were collected by centrifugation, washed twice in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 50 mM NH_4Cl, and 2 mM 2-mercaptoethanol, and finally resuspended in 5 ml of the same buffer. The cells were broken by ultrasonic treatment (five pulses of 30 s each) while on ice-cold water. After centrifugation at 18,000 rpm (30,000 × *g*) for 30 min, the supernatant was dialyzed against two changes of the above-mentioned buffer and used as a source of enzymatic activity.

Preparation of extracellular enzymatic activities. A spore suspension of *S. antibioticus* was used to inoculate 300 ml of TSB medium. After incubation at 30°C for different time intervals, the culture was centrifuged and the supernatant was filtered. Proteins in the filtrate were precipitated with ammonium sulfate at 80% saturation. After centrifugation at 15,000 rpm for 30 min at 4°C, the precipitates were resuspended in 10 ml of the buffer described above and dialyzed against three 500-ml changes of the same buffer. The resultant material was used as a source of extracellular enzymatic activities.

In vitro inactivation of oleandomycin. Oleandomycin (6.6 μg/ml; 8.3 μM) was incubated with 50 μl of dialyzed cell extract in the presence of 1 mM UDP-glucose at 30°C in a final volume of 150 μl. Both at zero time and after 6 h of incubation, samples were removed and boiled for 2 min. After cooling, the residual antibiotic activity was determined by bioassay against *M. luteus* as described previously (18). In some experiments, UDP-D-[6-^{3}H]glucose (20 μCi/ml; 2 mM) was used instead of the nonlabelled compound, and the products of the reaction mixture were analyzed by paper chromatography as described below.

In vitro reactivation of oleandomycin. For assays of reactivation of oleandomycin, tritiated inactive oleandomycin was used as a substrate after preparation as follows. An inactivation assay was carried out using UDP-[^{3}H]glucose as

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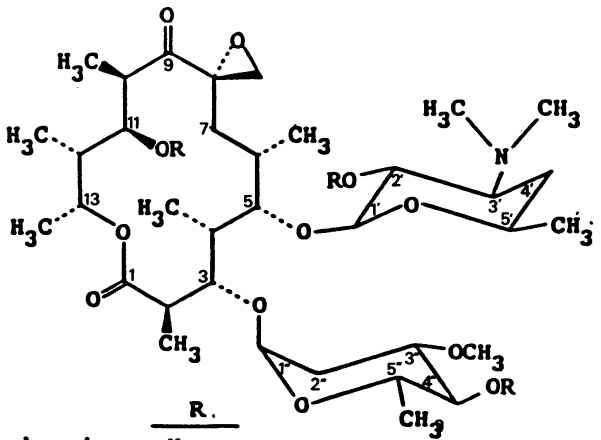

FIG. 1. Chemical structures of oleandomycin and its triacetylated derivative, troleandomycin.

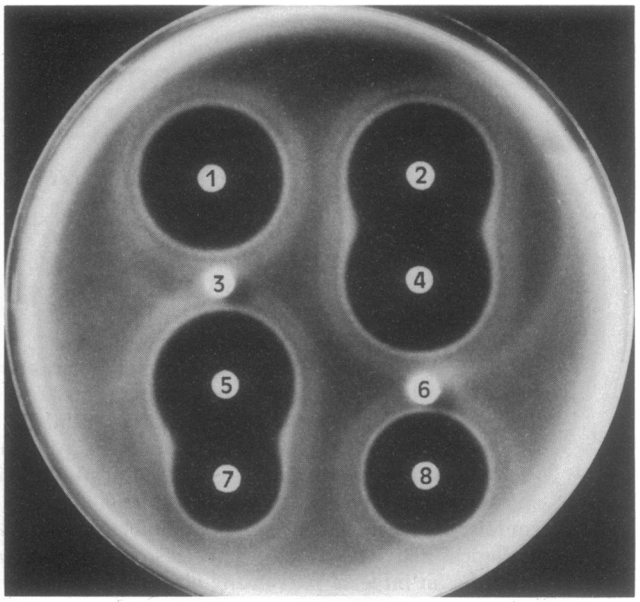

FIG. 2. Assay of oleandomycin inactivation by cell extracts of *S. antibioticus* as determined by bioassay against *M. luteus*. Oleandomycin (6.6 μg/ml; 8.3 μM) was incubated with a cell extract of *S. antibioticus* in the absence or in the presence of different cofactors (all at 1 mM) as indicated below. At zero time and after 6 h of incubation at 30°C, samples were removed and antibiotic activity was determined by bioassay against *M. luteus*. 1, oleandomycin (control); 2, soluble fraction plus oleandomycin; 3, soluble fraction plus UDP-glucose; 4, complete reaction (soluble fraction plus oleandomycin plus UDP-glucose, i.e., the soluble fraction heated at 100°C for 2 min before the start of the reaction); 5, complete reaction (at zero time); 6, complete reaction (after 6 h of incubation); 7, complete reaction with TDP-glucose (after 6 h of incubation); 8, complete reaction with ADP-glucose (after 6 h of incubation).

a cofactor and the labelled inactive and modified oleandomycin was eluted from the paper strips after paper chromatography by immersion in absolute methanol and incubation at room temperature overnight with gentle shaking. The eluate was lyophilized and dissolved in a small volume of methanol; then aliquots (approximately 8,000 cpm) were incubated with 40 to 50 μl of the ammonium sulfate precipitates from the culture supernatant (without addition of any cofactor) in a final volume of 100 μl at 30°C for 2 h. The reaction products were analyzed by counting the radioactivity in 1-cm strips after paper chromatography.

Thin-layer chromatography. Samples of the inactivation assays (50 μl; 15 μg of oleandomycin) were applied to silica gel F_{254} plates (Merck) and subjected to ascending chromatography with methanol as the solvent. The plates were developed and then stained with a mixture of anisaldehyde-concentrated sulfuric acid-ethanol (1:1:9) and heating at 100°C for 2 min (16). Parallel samples (without staining) were assayed by bioautography against *M. luteus*.

Paper chromatography. For determination of the radioactive labelling of the reaction products, samples (10 to 30 μl) were spotted onto Whatman 3MM paper and developed by descending chromatography using *n*-butanol–acetic acid–water (3:1:1, by volume). The dried chromatograph was cut into 1-cm strips, and the radioactivity in each was determined.

Chemicals. Sources of chemicals were as follows: oleandomycin, troleandomycin, erythromycin, and spiramycin, Sigma Chemical Co.; tylosin, Lilly; carbomycin, Pfizer Inc.; josamycin, Yamanouchi International Ltd.; relomycin, American Cyanamid; rosaramicin, Schering Corp.; methymycin, Squibb; niddamycin, Hoechst; and lankamycin, Taisho. All macrolides were dissolved in methanol-distilled water (1:1). UDP-D-[6-^{3}H]glucose (specific activity, 156 GBq/mmol; 4.2 Ci/mmol) and D-[6-^{3}H]glucose (specific activity, 19.6 GBq/mmol; 527 mCi/mmol) were from Amersham International.

RESULTS

Inactivation of oleandomycin by *S. antibioticus*. When dialyzed cell extracts of *S. antibioticus* were incubated at 30°C with oleandomycin and the residual antibiotic activity was determined with *M. luteus* as a test organism, no inactivation of the antibiotic was detected. However, if UDP-glucose was present as a cofactor in the reaction assay, the antibiotic activity dramatically decreased (Fig. 2). Other glucose nucleotides tested as possible cofactors for inactivation, such as TDP-glucose and ADP-glucose, were not as effective for oleandomycin inactivation as was UDP-glucose (Fig. 2). No inactivation was observed after heat treatment of the extract at 100°C for 2 min.

Oleandomycin inactivation was accompanied by an alteration in the chromatographic mobility of the antibiotic in thin-layer chromatography. Oleandomycin showed R_f values of about 0.21 in the chromatographic solvent system used; after inactivation, it showed higher R_f values (about 0.50).

Experiments were carried out to further characterize the process. The inactivation assays were done in the presence of UDP-glucose labelled with tritium in the hydroxyl group of the 6′ carbon. After the assay, the reaction products were separated by paper chromatography and the radioactivity in the different paper strips was counted. When the incubation was carried out in the absence of oleandomycin (Fig. 3A), only one labelled peak (R_f of 0.28) was observed. However, when oleandomycin was added to the reaction assay (Fig. 3B), this peak clearly diminished and a new radioactive peak (R_f of 0.69), corresponding to the mobility of the inactive oleandomycin, was detected. These experiments indicated that the radioactivity from the sugar moiety of UDP-glucose had been transferred into oleandomycin and suggested that

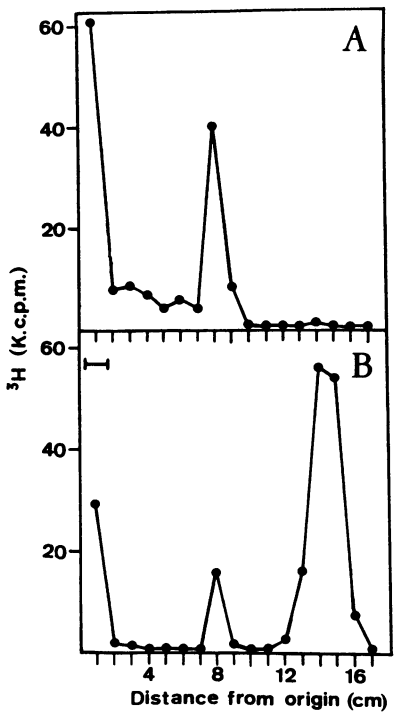

FIG. 3. Analysis of the reaction products of an inactivation assay. Cell extracts of *S. antibioticus* were incubated with UDP-D-[6-^{3}H]glucose (20 μCi/ml; 2 mM) in the absence (A) or in the presence (B) of oleandomycin (6.6 μg/ml; 8.3 μM). After incubation at 30°C for 6 h, the reaction products were analyzed by paper chromatography and the radioactivity was counted as described in Materials and Methods. The bar indicates the chromatographic mobility of UDP-[6-^{3}H]glucose.

glucose had been transferred during the inactivation. In addition, the presence of 1 mM UMP completely abolished transfer of radioactivity from UDP-glucose.

Oleandomycin was not the only macrolide that could be inactivated by *S. antibioticus* cell extracts (Table 1). Some macrolides (methymycin, rosaramicin, and lankamycin) were good substrates for the inactivating activity, while others (erythromycin, carbomycin, josamycin, spiramycin, niddamycin, relomycin, and tylosin) were not. This inactivation correlates very well with the presence of a 2'-OH group on a monosaccharide, except in the case of erythromycin, which has a 2'-OH group but is not inactivated. To test the hypothesis that this position could be the target for the inactivating activity, we used as a substrate troleandomycin. This antibiotic is an active triacetylated oleandomycin derivative lacking the 2'-OH group in the desosamine molecule (Fig. 1). Therefore, we assayed the ability of the extracts to inactivate this molecule. Initial experiments indicated that commercial preparations of troleandomycin in methanol-water were partially inactivated by cell extracts. Furthermore, we found that these solutions were very unstable (troleandomycin being converted to oleandomycin), as observed by thin-layer chromatography. Consequently, we took the precaution of using freshly prepared solutions of troleandomycin (0.5 mg/ml) made in water to be used for the assays. In these conditions, it was found that no inactivation was observed and only 1.2% of the tritiated glucose from UDP-glucose was transferred (Table 1).

The possible existence of a similar activity in other macrolide producers was also assayed. Cell extracts of *Streptomyces ambofaciens* (spiramycin), *S. halstedii* (carbomycin), *S. venezuelae* (methymycin), *S. fradiae* (tylosin), and *Saccharopolyspora erythraea* (erythromycin) were prepared, and their ability to inactivate either oleandomycin or the antibiotic that each organism produces was tested. In none of these cases was inactivation of oleandomycin or of any other macrolide observed (data not shown).

Reactivation of oleandomycin by *S. antibioticus*. The possible existence of a second enzymatic activity able to reactivate the modified oleandomycin was also assayed. Tritiated inactive oleandomycin, prepared as described in Materials and Methods, was incubated with the ammonium sulfate precipitates from the culture supernatant (without the addition of cofactor). The reaction products were then analyzed by paper chromatography. After incubation, the peak corresponding to the inactive molecule greatly diminished and another slowly migrating peak, with the same R_f value as glucose, appeared (Fig. 4). These experiments indicate the presence of a second activity in *S. antibioticus* capable of removing glucose from the inactive oleandomycin. Furthermore, the product of this reaction recovered its antibiotic activity against *M. luteus* (data not shown).

TABLE 1. Substrate profile of the macrolides-inactivating activity of *S. antibioticus*

Macrolide	No. of elements in the lactone ring	2'-OH group[a]	Bioassay[b]	Amt (nmol) of [^{3}H]glucose transferred	Stoichiometry of inactivation[a] (nmol of [^{3}H]glucose transferred/nmol of antibiotic)
Methymycin	12	+, M	+	16.82	0.52
Oleandomycin	14	+, M	+	11.70	0.53
Troleandomycin	14	−, M	−	0.20	0.02
Erythromycin	14	+, M	−	0.16	0.01
Lankamycin	14	+, M	+	5.21	0.28
Carbomycin	16	+, D	−	0.06	0
Josamycin	16	+, D	−	0.30	0.02
Niddamycin	16	+, D	−	0	0
Relomycin	16	+, D	−	0.04	0
Rosaramicin	16	+, M	+	19.50	0.75
Spiramycin	16	+, D	−	0	0
Tylosin	16	+, D	−	0.10	0

[a] Presence of a 2'-OH group in either a monosaccharide (M) or a disaccharide (D) substituent.
[b] Inactivation of the antibiotic was assayed by bioassay against *M. luteus*. +, inactivation; −, no inactivation.

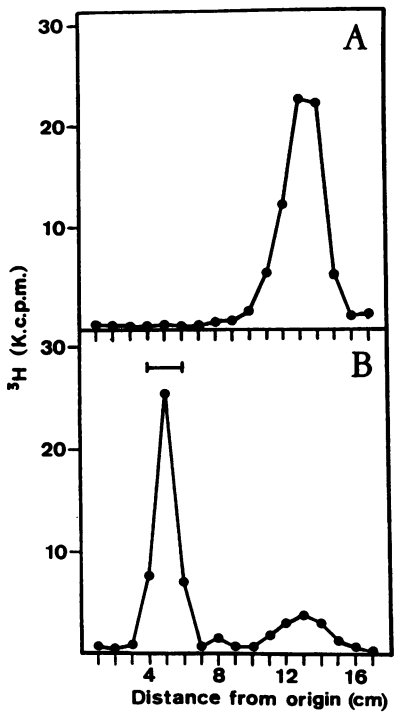

FIG. 4. Analysis of the reaction products of a reactivation assay. Inactive glucosylated oleandomycin (approximately 8,000 cpm) prepared as described in Materials and Methods was incubated with an ammonium sulfate precipitate of the culture supernatant of *S. antibioticus*. At zero time (A) and after 6 h of incubation (B), the reaction products were analyzed by paper chromatography and the radioactivity was counted as described in Materials and Methods. The bar indicates the chromatographic mobility of D-[6-^{3}H]glucose.

DISCUSSION

Antibiotic-inactivating enzymes are widespread among clinical isolates of gram-positive and gram-negative bacteria, in which they are responsible for resistance to different groups of antibiotics. Similar activities have been found in many antibiotic-producing organisms capable of inactivating the produced antibiotic (for a review, see reference 3). All of these enzyme activities either are dependent on the presence of different cofactors such as ATP, acetyl coenzyme A, or glutathione or, in some cases, require no cofactor. Our finding of a macrolide-inactivating activity dependent on glucose nucleotides as a cofactor represents a new mechanism of antibiotic resistance not previously encountered in a producing organism. However, glycosylation of erythromycin A by *Streptomyces vendargensis* has been described (8), although there appears to be no connection between this enzyme activity and antibiotic biosynthesis, since this strain is not a macrolide producer. In this case, the site of glycosylation in the erythromycin molecule has been identified at the C-2′ position of the desosamine moiety. It is interesting that in the case of the enzyme of *S. antibioticus*, macrolide antibiotics belonging to the 12-, 14-, or 16-membered groups can be inactivated, but all of the macrolides (except erythromycin) that were good substrates in the inactivation assay contain a free 2′-OH group either in the desosamine molecule (oleandomycin and methymycin) or in another sugar (chalcose in lankamycin) or amino sugar (mycaminose in rosaramicin). The fact that all macrolide antibiotics that contain a monosaccharide moiety react with the inactivating enzyme and those which contain a disaccharide do not suggests that in the latter case, this hydroxyl group is not available for reaction with the inactivating enzyme. Probably the disaccharide moiety creates a steric hindrance to modification. Additional evidence pointing to the 2′-OH group as the target for the modification came from the inability of the enzymatic activity to inactivate troleandomycin, in which this hydroxyl group is blocked. Interestingly, erythromycin (which has a free 2′-OH group) was not inactivated by the enzymatic activity. A possible explanation of this fact could be the presence of an extra hydroxyl group in the C-6 of the erythromycin lactone ring which is not present in the other macrolides that were substrates (methymycin, oleandomycin, and rosaramicin). This hydroxyl group could, in some way, alter the structural conformation of the molecule, thus avoiding the inactivation process. All of these results strongly suggest that inactivation of oleandomycin occurs via a glycosylation event which could take place at the 2′-OH group of the desosamine molecule of oleandomycin.

Producing organisms must protect themselves against intracellular toxicity during antibiotic biosynthesis. Obviously, those organisms synthesizing xenotoxic antibiotics face no such problem since the target site for the antibiotic is not present in the organism (19). On the other hand, specific resistance mechanisms must be developed for survival during the biosynthesis of potentially autotoxic antibiotics. The existence of antibiotic-inactivating activities in producer strains could fulfill this requirement, but the question arises of whether these enzyme activities really represent antibiotic-inactivating activities or whether they should be considered enzymes participating in the antibiotic biosynthetic pathway. In some cases, evidence exists that these enzymes could play a role in the producer strains both in self-defense and in antibiotic biosynthesis (7, 10, 17). The macrolide-inactivating activity that we report here could constitute an enzyme of the oleandomycin biosynthetic pathway that is able to glycosylate a precursor in the route. Although oleandomycin is a substrate for the enzyme in cell extracts, one could assume that the natural substrate for this activity is an intermediate molecule of the oleandomycin biosynthetic pathway and that the glycosylation of this precursor could avoid the presence of active antibiotic during biosynthesis in the cytoplasm, where oleandomycin-sensitive ribosomes exist (5). UDP-glucose could be the donor of the glucose moiety; however, although this glucose nucleotide was a better cofactor for the inactivation, we cannot exclude the possibility that TDP-glucose, or even ADP-glucose, is the natural cofactor. In this respect, it must be kept in mind that sugars and amino sugars of macrolides are synthesized as TDP intermediates. Whatever the nature of the cofactor, the last intracellular molecule synthesized in the cytoplasm (presumably glycosylated oleandomycin) would lack antibiotic activity and would be excreted. The existence of such a molecule has been reported in the culture supernatants of *S. antibioticus* during oleandomycin fermentations (2). A second enzyme activity would be necessary to activate the molecule either during or after export of the inactive molecule. In the case of *S. antibioticus*, the enzyme activity that we found in ammonium sulfate precipitates of the culture supernatant of *S. antibioticus* would fill this role. This enzyme could represent a β-glucosidase, since the ammonium sulfate precipitates of the culture supernatants in which reactivating activity was detected also contained β-glucosidase but not α-glucosidase activity (data not shown). Experiments now in progress will attempt to clarify the role of these two enzyme activities in oleandomycin biosynthesis.

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