Characterization in \textit{Micromonospora inyoensis} of Aminoglycoside Acetyltransferase Activity Not Previously Encountered among Actinomycetes

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(Received 25 July 1984; revised 31 October 1984)

Extracts of \textit{Micromonospora inyoensis} contain aminoglycoside acetyltransferase activity not previously encountered among actinomycetes. Neomycin and its derivatives (excluding butirosin) are good substrates as is the novel aminoglycoside apramycin, whereas the gentamicins and kanamycins are utilized poorly if at all. Sisomicin, which is produced by \textit{M. inyoensis}, is not acetylated. When neomycin was modified by extracts of \textit{M. inyoensis} the product, most probably 3-N-acetylneomycin, was inactive against cell-free protein synthesis.

\section*{INTRODUCTION}

Aminoglycoside antibiotics are produced, almost exclusively, by actinomycetes (in particular, by members of the genera \textit{Streptomyces} and \textit{Micromonospora}) and considerable attention has been given to the manner in which such organisms tolerate their products. Until relatively recently, this was thought to be due primarily to their possession of aminoglycoside-modifying enzymes, which phosphorylate or acetylate the drugs at specific sites within the molecules and thereby (usually) inactivate them (for reviews see Davies & Smith, 1978; Phillips & Shannon, 1984). Obviously, this could not be the whole story (otherwise the organisms in question would not be producers of antibiotics!) but the occurrence of similar modifying enzymes in clinical isolates of aminoglycoside-resistant bacteria was, at least, consistent with the hypothesis (Benveniste & Davies, 1973; Davies \textit{et al.}, 1979). More recently, however, it has become apparent that resistance is exerted at the level of the ribosome in the \textit{Streptomyces} species which produce istamycin (Yamamoto \textit{et al.}, 1981), the nebramycin complex (Yamamoto \textit{et al.}, 1982) and kanamycin (Murakami \textit{et al.}, 1983; Nakano \textit{et al.}, 1984). Accordingly, since some of these strains also possess enzymes capable of modifying the endogenous antibiotics, alternative roles for those enzymes must be considered in processes not necessarily associated with drug resistance (for a review see Cundliffe, 1984). On the other hand, the producers of streptomycin and neomycin do not appear to be capable of desensitizing their ribosomes towards their products (Hotta \textit{et al.}, 1981; Sugiyama \textit{et al.}, 1980, 1981). Those organisms may well depend upon their antibiotic-modifying enzymes to eliminate biologically active metabolites from the cytoplasm during idiophase. Thus, there appear to be several possible functions for aminoglycoside-modifying enzymes and these may vary from one organism to another. When the present work began, the resistance mechanisms in the \textit{Micromonospora} species which produce gentamicin and related compounds, including sisomicin, were unknown. None of these strains had been shown to possess antibiotic-modifying enzymes and none were known to contain drug-resistant ribosomes although, shortly thereafter, the latter were reported to be present in \textit{Micromonospora purpurea}, the gentamicin producer (Piendl & Böck, 1982). The

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Abbreviation: AAC, aminoglycoside acetyltransferase.
present work describes aminoglycoside acetyltransferase (AAC) activity present in *Micromonospora inyoensis* (ostensibly the producer of sisomicin) and not previously observed in actinomycetes.

**METHODS**

**Organisms.** *Micromonospora inyoensis* NRRL 3292 was obtained from the NRRL Collection, US Department of Agriculture, Peoria, Ill., USA. According to the ATCC catalogue, strain NRRL 3292 should be the same as strain ATCC 27600 — the latter being described as a producer of sisomicin. *Streptomyces coelicolor* A3(2) was obtained from Professor D. A. Hopwood, John Innes Institute, Norwich, UK. Both actinomycetes were grown in medium containing (per litre): 10 g glucose, 2 g yeast extract (Difco), 2 g Casamino acids (Difco) and 1 g beef extract (Oxoid, Lab-Lemco). The pH of the medium was adjusted to 7-0 with NaOH.

**Growth of cells and preparation of extracts.** Erlenmeyer flasks (2 litre) containing 1 litre of medium were inoculated with spore suspensions and shaken at 200 r.p.m. and 37°C for 24 h (*S. coelicolor*) or for 48 h (*M. inyoensis*). Cells were then harvested by centrifugation at 12000*g* for 10 min and washed twice with RS buffer (10 mM-Tris/HCl pH 7-6 at 20°C, 10 mM-MgCl₂, 50 mM-NH₄Cl and 3 mM-2-mercaptoethanol). Cell breakage was carried out by grinding with alumina (2 g per g of mycelial wet weight) in the presence of small quantities of RS buffer. After breakage, DNAase (5 µg ml⁻¹ final concentration) was added and unbroken cells plus debris were removed by centrifugation at 30000*g* for 1 h. The supernatant (S30) was stored at −70°C as small samples.

**Aminoglycoside acetyltransferase assay.** The assay used routinely depended upon the ability of aminoglycosides and their derivatives to bind to cation-exchange paper (Ozanne et al., 1969). A typical reaction mixture (in 100 µl RS buffer) contained: 30 µM-antibiotic, 0-025 mM-[¹⁴C]acetyl-coenzyme A (51-6 mCi mmol⁻¹, 2-29 GBq mmol⁻¹) and 30-70 µl *M. inyoensis* S30. After incubation at 30°C for various times, 20 µl samples were removed and pipetted onto squares (2 x 2 cm) of Whatman P81 phosphocellulose paper. These were then washed twice with deionized water to remove unreacted acetyl-CoA and then twice with acetone. Radioactivity bound to the paper was then estimated by liquid scintillation spectrometry.

On occasion, acetyltransferase activity was also determined by a colorimetric assay using 5,5'-dithiobis(2-nitrobenzoic acid) as previously described (Benveniste & Davies, 1971).

**Partial purification of an acetyltransferase.** All procedures were carried out at 2-4°C.

Crude S30 obtained from 20 g wet weight of *M. inyoensis* mycelium was centrifuged at 100000*g* for 4 h. To the resultant supernatant, solid (NH₄)₂SO₄ was added batchwise to give 30% saturation. The suspension was stirred for 30 min and then centrifuged at 30000*g* for 30 min. Additional (NH₄)₂SO₄ was added to the supernatant to give 60% saturation followed by stirring and centrifugation as above. Each of the protein fractions precipitated by (NH₄)₂SO₄ was dissolved in 2 ml RS buffer and dialysed against 3 x 2 litres of the same. More than 90% of the acetyltransferase activity (measured with neomycin as substrate using the colorimetric assay) was recovered among the proteins precipitated by (NH₄)₂SO₄ between 30 and 60% saturation. This material was then applied to a DEAE-cellulose column (10 x 2 cm), previously equilibrated with RS buffer containing 5% (v/v) glycerol, at a flow rate of 30 ml h⁻¹ and washed with 100 ml of the same buffer. Then, material was eluted from the column with 50 ml RS buffer containing NaCl as a linear concentration gradient (0 to 0-4 M) applied at 50 ml h⁻¹. The eluate was passed through a UV monitor (Pharmacia) and the A₄₅₀ was monitored continuously. Fractions (2 ml each) were collected and assayed for acetyltransferase activity by the colorimetric method using neomycin as substrate. Active fractions were pooled, dialysed against 3 x 2 litres RS buffer containing 20% (v/v) glycerol and then stored as small samples at −70°C.

**Preparation of acetylneomycin.** Neomycin was acetylated in bulk using S30 of *M. inyoensis* as described above except that the reaction mixture was scaled up to 2 ml and incubation was for 4 h. Then, DEAE-cellulose (Whatman DE 52) was added to the mixture which, after standing for 10 min at room temperature, was centrifuged at 12000*g* for 5 min. The pellet contained unreacted [¹⁴C]acetyl-CoA bound to the DEAE-cellulose whereas the supernatant contained [¹⁴C]acetylneomycin. The supernatant was lyophilized and the residue taken up in a few drops of water. This was then applied to Whatman 3MM paper and developed by descending chromatography for about 16 h using n-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.). After drying, the chromatogram was cut into 1 cm strips and the radioactivity in each was determined. Authentic neomycin was run in a parallel track and located using a spray reagent containing 1 g ninhydrin, 85 ml acetone and 15 ml of a solution made by dissolving 1 g CaCl₂ in 50 ml acetic acid plus 100 ml distilled water. Acetylneomycin was eluted from paper strips containing radioactive material using 2% (v/v) formic acid at 37°C for 2 h. The eluate was lyophilized, dissolved in a few drops of water and stored at −20°C.

**Alkaline degradation of [¹⁴C]-modified neomycin.** (a) To distinguish [¹⁴C]acetyl- from [¹⁴C]carboxymethylneomycin we used vigorous digestion with alkali. Radioactivity from the former should be released as [¹⁴C]acetic acid which would not be expected to adhere to phosphocellulose paper. In contrast, radioactivity from [¹⁴C]carboxymethylneomycin should still be bound to the negatively charged matrix. Thus, [¹⁴C]-modified neomycin (about 25 pmol in 20 µl H₂O) was incubated with an equal volume of 1 M-NaOH at 100°C for 30 min.
Aminoglycoside modification in Micromonospora

The solution was neutralized by the addition of 18 μl 1 M-HCl followed by 40 μl 1 M-Tris/HCl (pH 7.6 at 20 °C) and applied to phosphocellulose paper. This was washed as described above and the residual radioactivity was estimated by liquid scintillation spectrometry.

(b) Mild digestion with base was used to distinguish O-acetylation of neomycin from N-acetylation, since [14C]acetyl groups should be much more readily released from the former. Therefore, 10 μl 0.1 M-hydroxylamine in 0.1 M-sodium bicarbonate buffer pH 9.0 was added to [14C]acetyl-neomycin (approximately 25 pmol in 10 μl H2O) and the mixture was incubated at 30 °C for 30 min. Then 20 μl 1 M-Tris/HCl (pH 7.6 at 20 °C) was added and the resultant material was applied to phosphocellulose paper for estimation of bound radioactivity as above.

Assay of cell-free protein synthesis. Mycelium of S. coelicolor was ground with alumina and S30 was prepared as described above for M. inyoensis. This was then layered over a one-third volume of RS buffer containing 1 M-NH4Cl plus 40% (w/v) sucrose and centrifuged at 100000 g for 4 h at 2 °C. The ribosomal pellet was resuspended in RS buffer. The supernatant (S100) was dialysed at 4 °C against 2 x 1 litre RS buffer. Both ribosomes and S100 were sub-divided and stored at −70 °C until use. Synthesis of polyphenylalanine in response to poly(U) was carried out as previously described (Cundliffe et al., 1979).

Chemicals. Radiolabelled materials were obtained from Amersham, fine chemicals were from Sigma, (NH4)2SO4 (enzyme grade) was from Merck. The antibiotics used in this work were kindly supplied by Dr Julian Davies, Biogen SA, Geneva, Switzerland.

RESULTS

Modification of aminoglycosides by cell extracts

In preliminary experiments not presented here, extracts (S30) of M. inyoensis failed to cause modification of aminoglycoside antibiotics either by phosphorylation or by adenylylation. However, various aminoglycosides were radioactively modified when such extracts were supplemented with [14C]acetyl-CoA (Table 1). Subsequently (see below), it was ascertained that the drugs were being acetylated (rather than carboxymethylated) in these experiments. Hence, M. inyoensis possesses aminoglycoside acetyltransferase (AAC) activity which is particularly active against the neomycin family of antibiotics (including paromomycin, lividomycin, ribostamycin and neamine) together with the novel compound apramycin. Conversely, the kanamycins and gentamicins were very poor substrates while G418 was the only antibiotic tested against which no activity was detected. In particular, since some strains of M. inyoensis produce sisomicin, it was notable that this drug was not a good substrate for AAC activity in extracts of our strain.

Effect of pH and temperature on AAC activity

Before the AAC activity in M. inyoensis could be further characterized, it was desirable to establish whether the substrate–activity profile represented in Table 1 resulted from the action of a single enzyme or of several. Accordingly, the influence of pH and temperature on the AAC

<table>
<thead>
<tr>
<th>Antibiotic (30 μM)</th>
<th>Percentage substrate activity (normalized)*</th>
<th>Antibiotic (30 μM)</th>
<th>Percentage substrate activity (normalized)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>100</td>
<td>Kanamycin A</td>
<td>2</td>
</tr>
<tr>
<td>Neamine</td>
<td>106</td>
<td>Amikacin</td>
<td>1</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>88</td>
<td>Kanamycin B</td>
<td>2</td>
</tr>
<tr>
<td>Lividomycin</td>
<td>34</td>
<td>Tobramycin</td>
<td>8</td>
</tr>
<tr>
<td>Ribostamycin</td>
<td>68</td>
<td>Apramycin</td>
<td>18</td>
</tr>
<tr>
<td>Butirosin</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin C1</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin C1a</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin C2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sisomicin</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin B</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G418</td>
<td>0</td>
<td></td>
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</tr>
</tbody>
</table>

* Relative to neomycin: 100% represents 1242 pmol [14C]acetyl groups transferred in 30 min.
activity of unfractionated S30 extracts was examined using neomycin, kanamycin and gentamicin as substrates. In one set of experiments (data not given), AAC activity against all three antibiotics responded similarly to variations in pH over the range 5.5-8.5. In each case, activity was optimal at slightly alkaline pH and fell away dramatically at acid pH. Moreover, this pattern was preserved throughout the temperature range 30-45°C. In other experiments, the influence of heat treatment upon AAC activity was also studied. Again, similar results were obtained with neomycin, kanamycin and gentamicin as substrates (data not given). In each case, AAC activity was reduced by about 50% after about 30 min at 55°C, about 1-5 min at 60°C or about 0.5 min at 65°C. While not definitive, these data suggested that only a single AAC enzyme might be present in *M. inyoensis*.

**Partial purification of the AAC enzyme**

With S100 from *M. inyoensis* as starting material and neomycin as substrate, AAC activity was purified about 70-fold by a combination of ammonium sulphate precipitation and ion-exchange chromatography. A major peak of AAC activity was eluted from DEAE-cellulose columns by NaCl in the concentration range 0.1-0.15 M (Fig. 1). At each stage of the purification process, active material was also assayed with kanamycin and gentamicin as substrates; low-level AAC activity (about 2-5% of that seen with neomycin) was still present (data not given). This final preparation also retained substantial activity against apramycin. These results were again compatible with the idea that a single AAC enzyme might be responsible for the modification of aminoglycosides in extracts of *M. inyoensis*.

**Preparation and characterization of modified neomycin**

Radioactively-modified neomycin was prepared in bulk using the partially purified AAC preparation together with [14C]acetyl-CoA and was separated from unmodified drug and the cofactor by descending paper chromatography (see Methods). This material was then confirmed to be N-[14C]acetylneomycin by alkaline digestion. First, it was necessary to determine the type of modification since acetyl-CoA can function as a donor of acetyl or carboxymethyl groups. To resolve this question, 14C-modified neomycin was digested with 0.5 M-NaOH at 100°C for 30 min before the mixture was neutralized and applied to phosphocellulose paper to retain neomycin and/or positively charged degradation products. Radioactivity bound to the paper was then estimated (Table 2). Since carboxymethyl groups should not be cleaved from neomycin or its products by such treatment, the loss of 60% of the radioactivity from 14C-modified neomycin indicated that the bulk of the latter was not carboxymethylated and, presumably therefore, carried [14C]acetyl substituents.
Fig. 2. Effect of neomycin and acetylneomycin on polyphenylalanine synthesis. Polyphenylalanine synthesis was assayed as described in Methods using ribosomes and S100 from S. coelicolor. ●, Control; Δ, plus 1 ng neomycin ml⁻¹; □, plus 2 ng neomycin ml⁻¹; △, plus 5 ng acetylneomycin ml⁻¹; ■, plus 10 ng acetylneomycin ml⁻¹.

Table 2. Analysis of the type of modification produced by the M. inyoensis enzyme

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity from [¹⁴C]acetylneomycin retained on phosphocellulose paper*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Assay of acetylation versus carboxymethylation</td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>25240</td>
</tr>
<tr>
<td>1 M-NaOH at 100 °C for 30 min</td>
<td>10155</td>
</tr>
<tr>
<td>(b) Assay of N-versus O-acetylation</td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>20270</td>
</tr>
<tr>
<td>0.1 M-NH₄OH at 30 °C for 30 min</td>
<td>18335</td>
</tr>
</tbody>
</table>

* The specific radioactivity of [¹⁴C]acetylneomycin was about 800 c.p.m. pmol⁻¹.

A separate experiment was carried out to differentiate between N- and O-acetylation. In this case, much milder digestion with base (0.1 M-hydroxylamine at 30 °C for 30 min) was used to distinguish between possible N-acetylation (which should have been resistant) and O-acetylation (which should have been labile with the release of neomycin and [¹⁴C]acetic acid). Since, after digestion and neutralization, virtually all the radiolabelled material still bound to phosphocellulose paper (Table 2), we concluded that the AAC enzyme in M. inyoensis most probably acts by N-acetylation in common with all other known AAC enzymes. This point remains to be established unequivocally as does our tentative conclusion that a single AAC enzyme in M. inyoensis modifies both neomycin and apramycin.

Finally, we assayed the biological activity of the acetylneomycin by introducing it into a cell-free protein synthesizing system and comparing its effects with those of unmodified neomycin. The system we chose was derived from S. coelicolor and produced polyphenylalanine in response to poly(U). Neomycin is an extremely potent inhibitor of such systems (Fig. 2) but acetylneomycin, produced in extracts of M. inyoensis, had evidently lost at least 90% of that activity. Since it is conceivable that our preparation of acetylneomycin might have been contaminated with traces of unmodified drug, we concluded that the acetylneomycin retained very little, if any, biological activity.

**DISCUSSION**

Several types of AAC activity have previously been reported (Davies & Smith, 1978; Davies et al., 1979; Coombe & George, 1982), differing in various parameters including their substrate range and site of action. Usually, they are classified according to the latter, although sub-groups
exist so that, even among enzymes which share a common site of action within a given drug molecule, substrate ranges may vary. So far, however, each AAC enzyme characterized has been shown to acetylate a single primary amino group located (for substrates in the neomycin, kanamycin and gentamicin families of drugs) either on ring I or on ring II (deoxystreptamine). This is probably also true for the enzyme(s) described here, although there are several features which reveal that this type of AAC activity has not hitherto been encountered among actinomycetes. Indeed, there has been only one other report (and that very recently) of an AAC enzyme which is highly active against neomycin and its derivatives but virtually inactive against kanamycin and gentamicin. That enzyme was found in *Escherichia coli* (Hedges & Shannon, 1984) and its substrate–activity profile was essentially identical with that of the enzyme from *M. inyoensis*. It would, therefore, be interesting to know the extent to which the two enzymes (or, more appropriately, their genes) might be related.

The position within the neomycin molecule which is modified in extracts of *M. inyoensis* must be on ring I or II since neamine, which lacks rings III and IV of neomycin, is a perfectly good substrate. So also is paromomycin, which differs from neomycin only in having an hydroxyl group instead of an amino group at position 6' on ring I. Therefore, *M. inyoensis* does not appear to possess AAC(6') activity. The only other amino groups in neamine are located at position 2' on ring I and at positions 1 and 3 on ring II (deoxystreptamine) and several lines of reasoning suggest that it was the latter site which was modified here. First, when acetylneomycins were produced in extracts of *M. inyoensis* and *Streptomyces spectabilis* and subjected to paper chromatography, they migrated differently (data not given). Since the latter organism possesses an AAC(2') enzyme (Benveniste & Davies, 1973), the *M. inyoensis* enzyme would appear to act differently. Secondly, butirosin was only a poor substrate. This antibiotic is derived from ribostamycin (which comprises rings I, II and III of neomycin and is a good substrate) by substitution of the 1-amino group of ring I with hydroxyaminobutyric acid. Typically, such substitution inhibits AAC(3) enzymes but not those of the AAC(2') type (Chevereau et al., 1974; Biddlecombe et al., 1976). Thirdly, and perhaps crucially, the novel antibiotic apramycin, was acetylated in extracts of *M. inyoensis*. This drug is not related in structure to other aminoglycosides, except in possessing a deoxystreptamine residue. Nor is it a substrate for any of the known aminoglycoside-modifying enzymes other than certain AAC(3) enzymes (Davies & O'Connor, 1978; Hedges & Shannon, 1984). Thus, if *M. inyoensis* possesses only a single AAC activity (as we have assumed), it seems most probable that it is of the AAC(3) type. However, we have not formally excluded the possibility that the enzyme might act at position 1 of ring II in conventional aminoglycosides (a type of action not previously observed).

Given that neomycin was such a good substrate for AAC activity and that the acetylated product was virtually inactive in *vivo*, we were somewhat surprised to discover that our strain of *M. inyoensis* was quite sensitive to neomycin (MIC 5 µg ml⁻¹). This is quite unlike the situation in strains of *E. coli* possessing the ‘neomycin–aprampyacin acetyltransferase’; they are resistant to fairly high concentrations of either drug (Hedges & Shannon, 1984). Such observations underline the importance of factors other than the mere possession of aminoglycoside-modifying enzymes in the expression of resistance (see Cundliffe, 1984 for a review). They do not, however, render the presence of the AAC activity in *M. inyoensis* less enigmatic.

Finally, we have recently been informed that *Micromonospora inyoensis* strain ATCC 27600 is highly resistant to sisomicin and contains drug-resistant ribosomes, although it is not known whether AAC activity similar to that described here is also present (A. Böck, personal communication). From those and the present data, it is obvious that strains NRRL 3292 and ATCC 27600 are not identical. The latter organism, apparently constitutively resistant to sisomicin, may therefore be capable of producing higher levels of the drug than our strain.

This work, which was carried out while J. A. S. was on leave of absence from the University of Oviedo, was supported by an EMBO Short Term Fellowship (to J. A. S.) and by a Project Grant from the Medical Research Council (to E. C.). We thank Jill Thompson and Dino Bedlington for many helpful suggestions.
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REFERENCES


