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New Plasmid-Mediated Aminoglycoside Adenylyltransferase of Broad Substrate Range That Adenylylates Amikacin

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The same aminoglycoside 2″-adenylyltransferase was isolated from four gram-negative species which were among a random group of gentamicin-resistant isolates from the same hospital. The enzyme was partially purified from a crude extract which also contained a second modifying enzyme identified as APH(3′)-I. The substrate range of the new aminoglycoside 2″-adenylyltransferase included the newer aminoglycosides sisomicin and amikacin, but showed much-reduced activity against gentamicins C2 and C1a. The pH optimum was 7.8 to 8.0 for every substrate, and the molecular weight of the enzyme molecule was estimated at approximately 29,000. Genetic experiments clearly established that both enzymes were expressed by a conjugative plasmid.

Since the introduction of the deoxystreptamine aminoglycosides to chemotherapy, many modifying enzymes and isozymes have been isolated and characterized (3). These enzymes are named and classified according to (i) the position of modification on the drug molecule and (ii) the full substrate profile and other biochemical properties. The adenylyltransferases catalyze *O*-adenylylation (or *O*-nucleotidylation) of the 2″-hydroxyl (2) or the 4′- and 4″-hydroxyl groups (11) of deoxystreptamine-containing aminoglycosides.

Until now, only one type of aminoglycoside 2″-adenylyltransferase [AAD(2″)] has been described (2, 4, 11), and its substrate range includes kanamycin, gentamicin, and tobramycin. This paper reports the isolation, purification, and characterization of a new plasmid-mediated AAD(2″) from *Klebsiella pneumoniae*, *Escherichia coli*, *Serratia marcescens*, and *Proteus vulgaris*. In each instance the enzyme was accompanied by APH(3′)-I which was also plasmid determined. The two enzymes were successfully separated from the same bacterial extract by affinity chromatography.

This investigation was part of a routine screen of many aminoglycoside-resistant isolates from a semiclosed hospital environment. The study of the enzyme which is the subject of this paper was initiated by the recognition of its unique substrate profile.

MATERIALS AND METHODS

Antibiotics. Gentamicin and sisomicin were gifts from Schering Corp. (Essex Laboratories, Australia). Tobramycin was a gift from Eli Lilly (Australia), and amikacin was a gift from Bristol Laboratories (Australia). Kanamycin and neomycin were purchased from Boehringer Mannheim Corp., New York. Gentamicin C complex, kanamycins, and neomycin complex were separated into respective components by methods described elsewhere (A. M. George, Ph.D. thesis, University of Sydney, Australia, 1980).

Bacteria. Aminoglycoside-resistant clinical isolates were obtained from D. Groot Obbink and V. Ackerman of the Royal North Shore Hospital, Sydney (October 1977 and May 1978). *E. coli* GM119 *rif*^{r} for transfer studies was a gift from K. D. Brown (School of Biological Sciences, University of Sydney).

Preparation of crude enzyme. S105 extracts were prepared by sonication and ultracentrifugation as described elsewhere (7). The S105 designation refers to the 105,000 × *g* supernatant from a sonicated bacterial suspension. Cells were grown in tryptone soy broth (Oxoid Ltd., London) and were washed, suspended, and disrupted in TMED buffer [10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8) containing 10 mM $MgCl_2$, 0.15 mM ethylenediaminetetraacetate, and 1 mM dithiothreitol]. This buffer was also used routinely in column chromatography and enzyme assays. pH optima were determined in sodium acetate (pH 4.0 to 6.5) and tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.1 to 9.0) buffers.

Enzyme assays. The radiochemical phosphocellulose paper binding assay (7) was employed for the measurement of adenylyltransferase activity. Each assay mixture contained 10 μl of [*U*-^{14}C]adenosine triphosphate (10 μCi/μmol per ml; Amersham), 10 μl of antibiotic (1 mg/ml), 30 μl of TMED buffer (10 times strength; pH 7.8), and 50 μl of enzyme sample. The reaction was initiated by the addition of enzyme, and incubation was continued for 20 min at 34°C. After heating at 90°C for 3 min, 50-μl samples were applied to phosphocellulose paper (7) and counted. Controls with no added substrate or no added enzyme were

routinely included. One unit of enzyme activity adenylylated 0.1 nmol of kanamycin A per min under optimal conditions (0.1 nmol was equivalent to 2,000 cpm). Phosphotransferase activity was estimated by bioassay with a sensitive test organism (*Bacillus subtilis* subsp. *niger*). Samples (50 μl) from reaction mixtures were heated (90°C, 3 min) and applied to sterile paper disks (Whatman, 13 mm) which were placed onto the surface of seeded tryptone soy agar plates. Zones of inhibition were compared with appropriate standards.

Protein determination. Protein was estimated by the method of Lowry et al. (9) and by ultraviolet absorption at 260 and 280 nm (13).

Preparation of immobilized gentamicin. Gentamicin-Sepharose for affinity chromatography was prepared from CNBr-activated Sepharose 4B (Pharmacia) as described elsewhere (George, Ph.D. thesis).

Polyacrylamide disk gel electrophoresis. Column fractions containing enzyme activity were pooled and dialyzed overnight at 4°C against distilled water to remove salts. The volume of the sample was then reduced by packing the dialysis bag in Sephadex G200 (4°C, four changes, 12 h). When the volume had reduced to about 50 μl it was combined with 25 μl of 25% sucrose containing 1% bromophenol blue. The sample was divided and run in two identical 7% acrylamide (Eastman) gels for 2 h at 120 V with tris(hydroxymethyl)aminomethane-glycine buffer (pH 8.3). One gel was stained with 0.1% naphthol blue black (in glacial acetic acid) and destained in 7% glacial acetic acid. The second gel was sliced and homogenized in TMED buffer and assayed for adenylyltransferase activity.

Preparation of plasmid DNA. Cleared lysis supernatants (6) were used to prepare crude ethanol-precipitated DNA (10). Dye-buoyant density gradient centrifugation was essentially as described previously (1). Crude and purified DNA samples and *Eco*RI digests (George, Ph.D. thesis) were run on vertical 0.8% agarose gels in tris(hydroxymethyl)aminomethane-borate buffer (10).

Conjugation and transformation. Conjugation and transformation were carried out by methods described elsewhere (George, Ph.D. thesis), using *E. coli* GM119 *rif*^{r} as the recipient. Selective plates contained rifampin (50 μg/ml) as the counterselecting drug and sisomicin (10 μg/ml) as the selecting agent for resistant transconjugants or transformants.

RESULTS

Purification of AAD(2″). *K. pneumoniae* was selected as representative of the four species which harbored the two enzymes. A crude supernatant (S105) from an extract was chromatographed on a Sephacryl S-200 column (1.6 by 80 cm; flow rate, 15 ml/h in 7-ml fractions) (Fig. 1). The elution profiles of the enzymes partially overlapped, although APH(3′)-I was eluted just before the AAD(2″) peak. Fractions containing either enzyme were combined and applied to a gentamicin-Sepharose column (0.9 by 11 cm; 10 ml/h in 5-ml fractions) which was eluted with a gradient of sodium chloride (0 to 1.0 M) in TMED buffer (120 ml). AAD(2″) was eluted in 0.35 to 0.5 M NaCl, but APH(3′)-I was not adsorbed to the affinity column and was recovered from the pregradient washings. AAD(2″) fractions were combined, dialyzed against TMED buffer (overnight, 4°C), and then applied to a diethylaminoethyl-Sephacel column (0.9 by 10 cm; 10 ml/h in 5-ml fractions) which was eluted with a gradient of ammonium sulfate (0 to 0.65 M) in TMED buffer (100 ml). The enzyme was recovered in fractions (6 to 9) in about 0.1 M $(NH_4)_2SO_4$ (Fig. 2).

The purification steps are summarized in Table 1. Calculated yields of enzyme activity exceeded 100% in all three stages of purification. Similar results have been obtained by others who have suggested that the progressively higher percentages at each stage reflect the increasing purity of the AAD(2″) preparation by

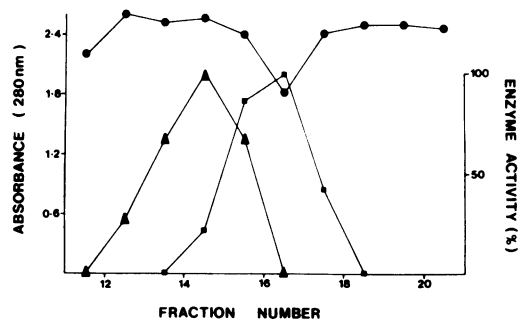

FIG. 1. *Gel filtration of APH(3′)-I and AAD(2″)-II on Sephacryl S-200. The plot represents: protein (●), APH(3′)-I activity (▲), and AAD(2″)-II activity (■). The column was 1.6 by 80 cm and eluted at 15 ml/h in 7-ml fractions. The sample was 5 ml of crude S105 supernatant concentrate from 4 liters of late-log-phase K. pneumoniae.*

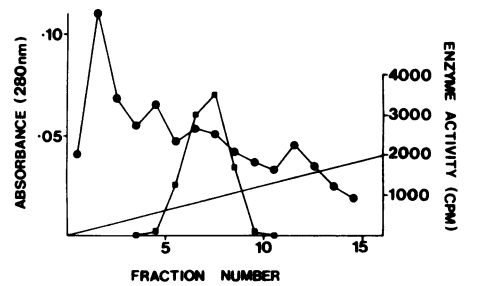

FIG. 2. *Ion-exchange chromatography of AAD(2″)-II (from affinity column) on diethylaminoethyl-Sephacel. The plot represents: protein (●) and AAD(2″)-II activity (■). The unbroken line is an ammonium sulfate gradient (0 to 0.65 M). The column was 0.9 by 10 cm and eluted at 10 ml/h in 5-ml fractions.*

TABLE 1. *Purification of AAD(2")-II from K. pneumoniae*

Procedure	Vol (ml)	Enzyme (total U)	Protein (mg/ml)	Sp act (U/mg of protein)	Yield (%)	Purification
S105 supernatant	5	160	12	2.7	100	1
Gel filtration	28	288	1.4	7.4	180	3
Affinity chromatography	30	323	0.24	45	202	17
Ion-exchange chromatography	20	238	0.028	425	149	157

removing contaminating proteins (5), phosphotransferases (4), and adenosine triphosphatase (12). If the 202% apparent yield from the affinity chromatography step (Table 1) is assumed to represent the total activity in the original crude sample (step 1), then the approximate recovery of AAD(2") from the ion-exchange column is 74%, with a purification of 157.

Fractions 7 and 8 from the ion-exchange column (Fig. 2) were combined, reduced in volume, and applied to polyacrylamide gels. Gel electrophoresis of purified AAD(2") depicted five protein bands with the enzyme apparently comprising more than 80% of the total protein (Fig. 3) since the major protein band was coincident with adenylyltransferase activity as determined by assay of homogenized slices of a second identical (unstained) gel.

Molecular weight determination. The apparent molecular weight of AAD(2") was estimated by gel filtration through Sephacryl S-200, and was about 29,000 for both crude and purified passages of the enzyme (elution volume, 94 ml). Variation of flow rate (5 to 30 ml/h) and sample volume (1 to 15 ml) did not significantly affect the reproducibility. The column was the same as the one used in the purification by gel filtration (Fig. 1). The column was calibrated with the following standard globular proteins: cytochrome *c* (molecular weight, 12,700; elution volume, 119 ml); α-chymotrypsinogen (molecular weight, 24,000; elution volume, 99 ml); ovalbumin (molecular weight, 45,000; elution volume, 80 ml); and bovine serum albumin (molecular weight, 67,000; elution volume, 68 ml).

pH optimum. The activity of the adenylyltransferase enzyme was measured at various pH's in acetate and tris(hydroxymethyl)-aminomethane buffers. The pH optimum was the same for every substrate tested (kanamycin A, gentamicin C1, tobramycin, sisomicin, and amikacin) and was located in a narrow peak at 7.8 to 8.0. The activity fell away sharply on either side of the optimum band.

Substrate studies. Aminoglycoside substrates of purified AAD(2") are represented in Table 2. The substrate profile of this enzyme differs quite markedly from that of AAD(2")-I (2, 12). Common substrates (kanamycin A, gentamicin C1, tobramycin, and kanamycin B) of the two enzymes are modified at different rates. AAD(2")-I also modifies gentamicins C2 and C1a, but not amikacin; the best substrates are tobramycin and sisomicin (4). Our AAD(2") enzyme recognized gentamicins C2 and C1a as very poor substrates or nonsubstrates, where amikacin was a good substrate; the best substrates were the kanamycins and gentamicin C1. Dibekacin (3',4'-dideoxykanamycin B) and netilmicin (1-*N*-ethylsisomicin) should also be substrates of this enzyme, but they were not tested.

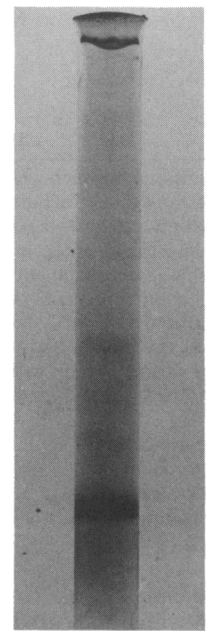

FIG. 3. *Polyacrylamide disk gel electrophoresis of purified AAD(2")-II from K. pneumoniae. The intense protein band at the lower end of the gel was coincident with adenylyltransferase activity.*

On substrate evidence alone it seemed that this adenylyltransferase was a new member of the AAD(2") group since the 2"-hydroxy position was the only universally available *O*-adenylylating site in all substrates of the enzyme. The site of modification presumed from substrate evidence was supported by mass spectral analysis of per *N*-acetylated adenylylsisomicin

TABLE 2. *Adenylylation of aminoglycosides by AAD(2″)-II*

Substrate[a]	Relative efficiency (%)[b]		
	AAD(2″) -II	AAD(2″) -I[c]	AAD(2″) -I[d]
Kanamycin A	100	65	31
Kanamycin B	97	ND[e]	17
Gentamicin C1	73	78	61
Tobramycin	50	ND	62
Sisomicin	24	ND	ND
Amikacin	19	0[f]	2
Gentamicin C2	4	28	100
Gentamicin Cla	1	25	81
Neomycins A,B,C	0	0	0
Gentamicin A	ND	100	7

[a] Additional compounds which were tested but were found to be nonsubstrates included: kasugamycin, streptomycin, streptamine, 2-deoxystreptamine, D-glucosamine, 3-*N*-acetylsisomicin, per *N*-acetylkanamycin A, and per *N*-acetylneomycin B.

[b] Substrate efficiency represents rate of inactivation of substrate per minute at 34°C expressed as a percentage relative to the best substrate and at the pH optimum. The substrate concentrations were 40 μM, and reactions were terminated in the linear range of the assay.

[c] Data from reference 2.

[d] Data from reference 12.

[e] ND, Not determined.

[f] This result was determined in a later experiment.

(R. G. Coombe and A. M. George, Aust. J. Chem., in press) which indicated that sisomicin was adenylylated in the garosamine ring. The enzyme was given the designation AAD(2″)-II.

Isolation of plasmid deoxyribonucleic acid (DNA). Figure 4 shows agarose gel electrophoresis of crude lysates from three resistant bacterial isolates. The two major bands in the centre of each gel slot represent plasmid DNA. Agarose gel electrophoresis of purified plasmid DNA from cesium chloride gradients showed a single plasmid band. Restriction endonuclease digestion of this purified band indicated at least seven DNA fragments on agarose gels (unpublished data).

Transfer studies. After conjugal matings of recipient (*E. coli* GM119 *rif*[r]) and donors [AAD(2″)- and APH(3′)-containing hosts], presumptive sisomicin-resistant *E. coli* transconjugants were picked from selective plates and tested for adenylyltransferase and phosphotransferase activities (crude S105 supernatants from late log phase cultures of *E. coli* survivors were assayed for enzyme activities as described above). The results indicated that *E. coli* transconjugants had acquired and expressed both aminoglycoside-modifying enzymes. Ampicillin and streptomycin resistances were also exhibited in the new phenotype of *E. coli*.

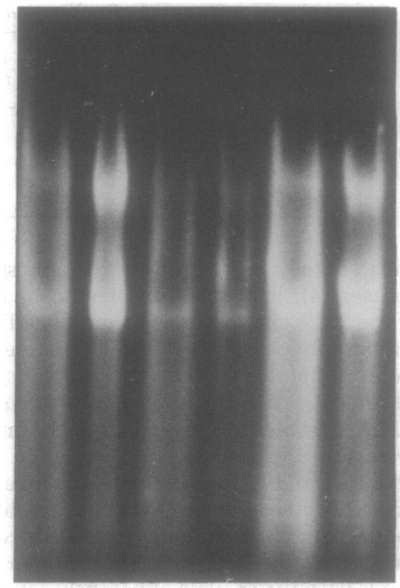

FIG. 4. *Agarose gel electrophoresis of crude lysates from K. pneumoniae (slots 1 and 2), S. marcescens (slots 3 and 4), and E. coli (slots 5 and 6). One liter of each stationary-phase culture produced 1 ml of crude lysate; 5 μl of each preparation was applied to the gel.*

Transformation with crude plasmid DNA from *K. pneumoniae* and *S. marcescens*, and *E. coli* GM119 *rif*[r] as recipient, produced transformants with phenotypes identical to those of the *E. coli* transconjugants. Again, both enzymes were present in crude S105 extracts from transformed *E. coli*, and ampicillin and streptomycin resistances were expressed in the phenotype. This result indicated that the crude plasmid DNA (Fig. 4) transformed the ability of previously sensitive *E. coli* to express the modifying enzymes.

DISCUSSION

In a random screen of 26 aminoglycoside-resistant clinical isolates we found four different species expressing the same two modifying enzymes. In addition, APH(3′)-I was present as the only modifying enzyme in several of the screened cultures (Coombe and George, unpublished data). However, AAD(2″)-II was never isolated alone and, instead, was always accompanied by APH(3′)-I in crude extracts. These observations were reflected in DNA studies. Restriction endonuclease digests of plasmid DNA from APH(3′)-I-bearing isolates indicated four discrete DNA bands in agarose gels (Coombe and

George, unpublished data), whereas DNA digests from the two-enzyme isolates indicated at least seven DNA fragment bands. This is suggestive evidence of the presence of a larger antibiotic resistance plasmid or two R-plasmids in the two-enzyme cultures.

We have achieved the separation of APH(3')-I and AAD(2")-II from the same crude supernatant extract of *K. pneumoniae*. The absorption of AAD(2")-II, but not APH(3')-I, to gentamicin-Sepharose makes this a useful step in the purification process. After additional chromatography on a diethylaminoethyl-ion-exchange resin, the resulting AAD(2")-II preparation showed five bands on polyacrylamide gels, with the major band apparently comprising at least 80% of the total protein and coinciding with adenylyltransferase activity. This compares very favorably with seven protein bands and 50% of the total protein for AAD(2")-I purified on diethylaminoethyl-agarose (5). The activity of the purified enzyme, unlike the crude extract, was considerably more stable against denaturation in solution at 4°C or frozen at −20°C with or without the added protection of substrate (sisomicin).

AAD(2")-II is the smallest adenylyltransferase protein reported so far. AAD(2")-I has a molecular weight of 35,000 (12), and AAD(4',4") is still larger at 47,000 (11). The use of Sephacryl S-200 for molecular weight estimation of aminoglycoside-modifying enzymes has not been reported previously. We consider this to be a very satisfactory chromatographic process since the same molecular weight approximation was achieved for repeated passages of crude and purified enzyme through the same column, even after 1 year.

A gentamicin adenylyltransferase isolated from *P. aeruginosa* by Kabins et al. (8) was closer in substrate profile to AAD(2")-II than is AAD(2")-I. However, the authors ascribed some of the substrate activities to an additional enzyme, probably a kanamycin phosphotransferase in the crude extract. This situation could not have occurred in the AAD(2")-II profile since the adenylyltransferase and phosphotransferase enzymes were separated by affinity chromatography before the final substrate study. This argument raises the question of the validity of preliminary substrate profiles of crude supernatant extracts in situations where two or more modifying enzymes are present; then, the sharing of common substrates between enzymes—and the binding of poor substrates and nonsubstrates—must produce incomplete or biased profiles.

Initially, AAD(2") was known to inactivate kanamycin, gentamicin, and tobramycin (2). Later investigators (4) expanded its substrate range by noting that the same enzyme also modified sisomicin and netilmicin. In this study we report a new AAD(2") with activity against amikacin in addition to previously known substrates of AAD(2"). Remarkably, the closely related compounds gentamicin C1a and sisomicin differ quite significantly in their affinity for the enzyme.

The expansion of the substrate range of established enzymes and the evaluation of new modifying enzymes are not new in antibiotic chemotherapy. Accordingly, of much concern to investigators is the emergence and incidence of aminoglycoside-modifying enzymes in a pattern which uncomfortably parallels the clinical usage of old and new aminoglycoside antibiotics. Thus, AAD(2")-II is a new enzyme with activity against most of the clinically important aminoglycosides, and its demonstrated partnership with APH(3')-I enables the host cell to enjoy an unusually broad degree of resistance in a strong drug environment. Conjugal transfer studies indicated that the two modifying enzymes are determined by a transmissible plasmid(s), which explains the ubiquitousness of the enzymes among a diverse bacterial population.

ACKNOWLEDGMENTS

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