Properties of a Bifunctional Bacterial Antibiotic Resistance Enzyme That Catalyzes ATP-Dependent 2″-Phosphorylation and Acetyl-CoA-Dependent 6′-Acetylation of Aminoglycosides

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Received December 13, 1996

Widespread use of antibacterial agents became possible just over 50 years ago. Since then, a number of significant contributions to chemotherapy of bacterial infections have been made.[1] These advances have been countered effectively by bacteria in a number of ways, such that bacteria resistant to a wide range of antibiotics have become common and indeed seriously compromise clinical options in their treatment.[2] Aminoglycosides were among the first antibacterials to find clinical use, and, as such, bacteria have acquired a myriad of enzymes that modify the structure of these antibiotics and, by so-doing, render them inactive.[3] Whereas the identities of many of these enzymes are known at the present,[3] very few have been characterized beyond the knowledge of their respective gene sequences.

A family of such aminoglycoside resistance enzymes which has been studied in much detail recently is that of the aminoglycoside 3′-phosphotransferases [APH(3′)s].[4,5] These enzymes phosphorylate a wide range of aminoglycosides to manifest their biological function. This family of enzymes has been important historically for resistance to aminoglycosides in Gram-negative bacteria, although a variant in Gram-positive organisms is gaining significance.[5] In this respect, an interesting enzyme has been described which confers resistance to a broad spectrum of aminoglycoside antibiotics primarily in Gram-positive bacteria.[6] Initially, this enzyme was thought to be found only in Gram-positive organisms. However, Kettner *et al.* have recently reported the presence of this enzyme in Gram-negative bacteria as well.[7] This report is quite significant and demonstrates the ability of a plasmid-encoded Gram-positive enzyme to be transferred and expressed in Gram-negative bacteria, whereby the resistance phenotype is disseminated to other nonrelated and previously aminoglycoside-sensitive bacterial strains. It is remarkable that DNA-hybridization studies in Europe and Asia have revealed that Gram-positive pathogens predominantly exhibit the presence of the gene for this enzyme.[8]

This novel enzyme confers resistance to the older aminoglycosides as well as many newer ones that are currently in clinical use. These include gentamicin, tobramycin, dibekacin, amikacin, isepamicin, 5-episisomicin, and fortimicin, most of which are relatively poor substrates for the commonly found APH(3′)s. The enzyme was suggested to have two enzymic activities (phosphotransferase and acetyltransferase), based on the substrate profile and investigation of its gene with DNA probes.[3,6] This is in fact a unique aminoglycoside-modifying enzyme with an unprecedented breadth of activity, presumably because of the complementary spectrum of the two enzymic reactions, which in effect render the organisms harboring it into "super" resistant organisms.

We have purified this enzyme to apparent homogeneity in two chromatographic steps (cibacron blue and gentamicin-Sepharose columns; please see the purification table in the Supporting Information section).[9] The enzyme turned over aminoglycosides with transfer of either the γ-^{32}P-phosphate moiety from ATP or the [^{3}H]-acetyl moiety from acetyl coenzyme A. We employed conditions that recycled either ATP or acetyl coenzyme A for the preparation of two distinct products for turnover of kanamycin A (**1**) during either the phosphotransferase or the acetyltransferase activities. We also prepared the product of kanamycin A (**1**) turnover which was modified twice, as a result of both enzymic activities (please see the Supporting Information).

The ^{1}H-NMR spectrum of the product of the acetyltransferase activity revealed a single peak at 1.84 ppm (s, 3H) which corresponded to an acetyl resonance. Multiple homodecoupling and NOE experiments allowed assignment of the entire spectrum. Comparison of the 2D H-H COSY spectra of kanamycin A and the acetylated product, at pH 12.7, showed a downfield shift in the signals for the 6′-methylene protons $H_{6'R}$ and $H_{6'S}$, from 2.62 and 2.86 ppm to 3.18 and 3.50 ppm, respectively. This shift indicated acetylation of kanamycin A at the 6′-amino group. This observation was supported also by the 2D H-C COSY spectrum for the acetylated product, which showed coupling of the $C_{6'}$ signal (40.3 ppm) with the proton signals at 3.18 and 3.50 ppm. Therefore, structure **2** corresponds to the product of the acetyltransferase activity. Assignments of the proton signals in the ^{1}H-NMR spectra for the two products of the phosphorylation reaction (single and double modifications) were also performed by extensive homodecoupling and NOE experiments. The ^{31}P-NMR spectrum for the singly modified phosphorylation product showed a resonance at 3.23 ppm, which was indicative of a phosphate monoester.[10] The 2D H−H COSY spectrum, at pH 13, revealed, with respect to kanamycin

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(1) Lancini, G.; Parenti, F.; Gallo, G. G. *Antibiotics: A Multidisciplinary Approach*; Plenum Press: New York, 1995. Goodman Gilman, A.; Goodman, L. S.; Rall, T. W.; Murad, F. In *Pharmacological Basis of Therapeutics*, 7th ed.; Macmillan: New York, 1985; pp 1066−1218.

(2) Levy, S. B. *The Antibiotic Paradox. How Miracle Drugs Are Destroying the Miracle;* Plenum Press: New York, 1992. Garrett, L. *The Coming Plague*; Farrar, Straus and Giroux Press: New York: 1994, pp 411−456. H. C. Neu *Science* **1992**, *257*, 1064. Davies, J. *Science* **1994**, *264*, 375. Nikaido, H. *Science* **1994**, *264*, 382. Spratt, B. G. *Science* **1994**, *264*, 388.

(3) Shaw, K. J.; Rather, P. N.; Hare, R. S.; Miller, G. H. *Microbiol. Rev.* **1993**, *57*, 138.

(4) (a) Siregar, J. J.; Lerner, S. A.; Mobashery, S. *Antimicrob. Agents Chemother*. **1994**, *38*, 641. (b) Siregar, J. J.; Miroshnikov, K.; Mobashery, S. *Biochemistry* **1995**, *34*, 12681. (c) Roestamadji, J.; Grapsas, I.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 80. Roestamadji, J.; Grapsas, I.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 11060.

(5) McKay, G. A.; Thompson, P. R.; Wright, G. D. *Biochemistry* **1994**, *33*, 6936. McKay, G. A.; Wright, G. D. *Biochemistry* **1996**, *35*, 8680. Thompson, P. R.; Hughes, D. W.; Wright, G. D. *Chemistry & Biology* **1996**, *3*, 747. McKay, G. A.; Roestamadji, J.; Mobashery, S.; Wright, G. D. *Antimicrob. Agents Chemother*. **1996**, *40*, 2648.

(6) Martel, A.; Masson, M.; Moreau, N.; Le Goffic, F. *Eur. J. Biochem*. **1983**, *133*, 515. Ferretti, J. J.; Gilmore, K. S.; Courvalin, P. *J. Bacteriol*. **1986**, *167*, 631. Rouch, D. A.; Byrne, M. E.; Kong, Y. C.; Skurray, R. A. *J. Gen. Microb*. **1987**, *133*, 3039.

(7) Kettner, M.; Macickova, T.; Kremery, V. J. In *Antimicrobial Chemotherapy in Immunocompromised Host*; International Congress of Chemotherapy, Berlin, 1991; pp 273−275.

(8) French, G. L.; Ling, J.; Ling, T.; Hui, Y. W. *J. Antimicrob. Chemother*. **1988**, *21*, 581. Lovering, A. M.; Bywater, M. J.; Holt, H. A.; Champion, H. M.; Reeves, D. S. *J. Antimicrob. Chemother*. **1988**, *22*, 823. Weems, J. J.; Lowrance, J. H.; Baddour, L. M.; Simpson, W. A. *J. Antimicrob. Chemother*. **1989**, *24*, 121. Ounissi, H.; Deriot, E.; Carlier, C.; Courvalin, P. *Antimicrob. Agents Chemother*. **1990**, *34*, 2164.

(9) The gene encoding the bifunctional enzyme was obtained by cloning a *Bsp*RI-*Hpa*II fragment (7.8 kb) from streptococcal pGB3013. An *Alu*I fragment (1.5 kb) was subsequently subcloned into the *Sma*I site of the plasmid pUC8 to form plasmid pSF815A (Feretti, J. J.; Gilmore, K. S.; Courvalin, P. *J. Bacteriol*. **1986**, *167*, 631). *E. coli* JM 109(pSF815A) was the generous gift of Dr. Joseph Chow. The nucleotide sequence of the gene revealed an open reading frame that encodes a protein of 479 amino acids, giving a molecular weight of 56 850 Da for the translation product, which corresponded well with a 56 kDa size estimate for the purified protein by SDS-PAGE. The enzyme behaved as a monomer according to PAGE under nondenaturing condition.

(10) Gorenstein, D. G. In *Phosphorus-31 NMR*; Gorenstein, D. G., Ed.; Academic Press: Orlando, FL, 1984.

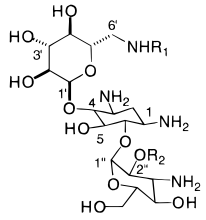
S0002-7863(96)04278-3 CCC: $14.00

Table 1. Kinetic Parameters for Phosphorylation and Acetylation of Aminoglycosides[a]

	acetyltransferase activity			phosphotransferase activity			MIC (μg/mL)[b]	
substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	*E. coli* JM109	*E. coli* JM109(pSF815A)
kanamycin A	4.5 ± 0.1	0.4 ± 0.1	$(8.2 \pm 1.9) \times 10^4$	23.9 ± 1.6	176.7 ± 18.7	$(7.4 \pm 0.9) \times 10^6$	4	2048
isepamicin	1860.0 ± 74.0	34.9 ± 1.0	$(1.9 \pm 0.1) \times 10^4$	24.3 ± 1.0	158.1 ± 16.7	$(6.5 \pm 0.8) \times 10^6$	1	8
tobramycin	16.2 ± 0.6	0.5 ± 0.1	$(3.0 \pm 0.1) \times 10^4$	130.5 ± 10.4	861.4 ± 77.5	$(6.6 \pm 0.8) \times 10^6$	2	1024
dibekacin	702.0 ± 28.0	9.0 ± 0.3	$(1.3 \pm 0.1) \times 10^4$	178.6 ± 14.3	195.2 ± 21.2	$(1.1 \pm 0.2) \times 10^6$	8	512
amikacin	477.0 ± 14.0	4.1 ± 0.2	$(8.6 \pm 0.6) \times 10^3$	298.6 ± 1.3	104.6 ± 9.4	$(3.7 \pm 0.4) \times 10^6$	2	32

[a] The K_m values for acetyl coenzyme A and ATP were 23.2 ± 0.9 and 58.0 ± 3.0 μM, respectively. [b] The MIC is defined as the lowest concentration of antibiotic that prevented growth.

A, a downfield shift for the 2″-H signal from 3.39 (dd) to 3.65 ppm (td). This triplet of doublets is due to splitting of the signal for the 2″-H by 3″-H, 1″-H, and the phosphorus nucleus, which is consistent with the $J_{P-O-C-H}$ coupling constant reported by Naganawa *et al.* for other phosphorylated aminoglycosides.[11] Hence, compound **3** corresponds to the product of phosphorylation of kanamycin A. The product of double modification showed a single signal at 3.31 ppm in the ^{31}P-NMR spectrum as well as a resonance at 1.85 ppm (s, 3H) in the ^{1}H-NMR spectrum. These observations indicated that kanamycin A was modified both by phosphorylation and acetylation to give compound **4**. Both the H−H COSY and the H−C COSY spectra for this doubly modified kanamycin A were consistent with this structure assignment. Full analyses of the structures of these products of enzymic reactions are documented in the Supporting Information.



1 $R_1 = R_2 = H$
2 $R_1 = COCH_3$, $R_2 = H$
3 $R_1 = H$, $R_2 = PO_3^{2-}$
4 $R_1 = COCH_3$, $R_2 = PO_3^{2-}$

With the exception of kanamycin A [which has been rendered clinically obsolete by APH(3′)s], the remainder of aminoglycosides studied by us, namely isepamicin, tobramycin, dibekacin, and amikacin, are of interest clinically. The minimum inhibitory concentrations for these antibiotics for the background plasmidless *E. coli* JM109 are in the order of 1−8 μg/mL, which are elevated by 8−512-folds for *E. coli* JM109(pSF815A), which expresses the bifunctional enzyme (Table 1). Interestingly, the phosphotransferase activity appears to be more significant for turnover of the substrates that we have studied, as evidenced by substantially higher k_{cat} values for this activity than those for the acetyltransferase function (Table 1). Nonetheless, it would appear from the values for k_{cat}/K_m that both activities are performed quite competently by the enzyme.

Having prepared and analyzed the structures of the products of turnover of kanamycin A by this enzyme, this presented an opportunity for us to investigate which of these products is observed *in vivo* in light of the fact that compartmentalization of the enzyme or availability of the second substrate (ATP or acetyl coenzyme A) at the enzyme locale may play a role. Kanamycin A was given to a growth medium of *E. coli* JM109-(pSF815A) at a concentration below the MIC value (1024 μg/mL). The cells were allowed to grow for 8 h, after which they were harvested and lysed by boiling. The samples were prepared as described in the Supporting Information. The three products of modification of kanamycin A give distinct tlc profiles (R_f = 0.58, 0.12, 0.32, for **2**, **3**, and **4**, respectively; EtOH:MeOH:HOAc:H_2O, 5:5:4.5:4.5). We were able to detect only the product of phosphorylation (i.e., **3**; also verified by mass spectral analysis) with kanamycin A in this *in vivo* experiment.

The issue of the evolution of this enzyme is a matter of considerable interest. We note that whereas 3′-phosphotransferases are common,[3,12] and indeed a few other types of aminoglycoside phosphotransferases are known,[3] 2″-phosphotransferases had not been identified until very recently.[13] The DNA sequence of the carboxy-terminal domain of the bifunctional enzyme is homologous with that of this newly discovered phosphotransferase, which in turn is distinct from those for the family of APH(3′)s. Hence, a free standing 2″-phosphotransferase as a potential ancestor for the 2″-phosphotransferase activity of the bifunctional enzyme does exist, and it simply had evaded detection until recently. It would appear that the gene for the ancestral bifunctional enzyme may have arisen by the fusion of the corresponding genes for a 6′-acetyltransferase—of which several[3] are already known—and a 2″-phosphotransferase. Recombination, deletion, and loss of transposons can all contribute to loss of genes from plasmids. Whereas occurrence of bifunctional enzymes is not common in bacteria, the existence of such a bifunctional resistance enzyme represents an extra level of sophistication in evolution of a bacterium to ensure that the two activities are retained, even if only one activity may prove sufficient for the survival of the organism at any given time. The selection for the organisms that harbor this novel enzyme would provide a distinct advantage as the bifunctional enzyme possesses an unprecedented breadth of substrate profile.

Acknowledgment. This work was supported by the National Institutes of Health. We thank Dr. Stephen Lerner for drawing our attention to the enzyme studied in this work. In addition, we are indebted to Dr. Joseph Chow for making available to us *E. coli* JM109-(pSF815A).

Supporting Information Available: Detailed procedures for enzyme purification, assays for the two activities of the enzyme, kinetic determinations, preparation and isolation of compounds **2**, **3**, and **4**, and their spectroscopic characteristics, MIC determination and detection of the product of turnover of kanamycin A in the *in vivo* experiment (15 pages). See any current masthead page for ordering and Internet access instructions.

JA964278W

(11) Naganawa, H.; Kondo, S.; Maeda, K.; Umezawa, H. *J. Antibiot.* **1971**, *24*, 823.

(12) A recent survey of 3412 bacterial clinical isolates collected from several countries worldwide, including the U.S., investigated by oligonucleotide probe hybridization revealed that one-half of the isolates showed high-level kanamycin A resistance associated with 3′-phosphorylation modification (Shaw, K. J.; Hare, R. S.; Sabatelli, F. J.; Rizzo, M.; Cramer, C. A.; Naples, L.; Kocsi, S.; Munayyer, H.; Mann, P.; Miller, G. H.; Verbist, L.; van Landuyt, H.; Glupczynski, Y.; Catalano, M.; Woloj, M. *Antimicrob. Agents Chemother.* **1991**, *35*, 2253).

(13) Chow, J. W.; Zervos, M. J.; Jaworaki, D. D.; Thal, L. A.; Donabedim, S.; Shaw, K. J.; Miller, G. H.; Clewell, D. B. *35th Interscience Conference on Antimicrobial Agents and Chemotherapy*; San Francisco, CA, September 1995; Abstr C82.