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Basicity of the Amino Groups of the Aminoglycoside Amikacin Using Capillary Electrophoresis and Coupled CE−MS−MS Techniques

Ravi S. Kane,[†] Peter T. Glink,[†] Robert G. Chapman,[†] J. Cooper McDonald,[†] Pamela K. Jensen,[‡] Hongying Gao,[‡] Ljiljana Paša-Tolić,[‡] Richard D. Smith,[‡] and George M. Whitesides*,[†]

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, P.O. Box 999, MS: K8-98, Richland, Washington 99352.

This paper describes the use of capillary electrophoresis (CE), and coupled CE and mass spectrometric techniques, to measure the values of the pK_a of the amino groups of the aminoglycoside antibiotic amikacin and of its acetylated derivatives. These values of pK_a (8.4, 6.7, 9.7, 8.4) were determined by measuring the electrophoretic mobilities of the molecules as a function of pH; they are within 0.7 unit of certain values reported in the literature (by ^{13}C and ^{15}N NMR spectroscopies) but resolved ambiguities left by these earlier studies. The range of values of pK_a of amino groups also indicates the complex dependence of the acidity of a functional group (and thus the extent of ionization at a specified value of pH) on the molecular environment of that group.

Aminoglycosides are an important family of antibiotics that bind RNA.[1] Members of this family include broad-spectrum antimicrobial drugs such as neomycin and streptomycin, which inhibit bacterial protein synthesis by binding to bacterial ribosomes.[2] Aminoglycosides inhibit substrate cleavage by hammerhead ribozymes[3] and the group I intron self-splicing reaction[4] and block binding of HIV rev protein to its viral RNA recognition element.[5] The electrostatic properties of aminoglycosides are particularly interesting, since the charge of an aminoglycoside influences its permeability across the membrane of the cell, and ionic interactions (such as those between the positively charged ammonium groups of the aminoglycoside and the negatively charged phosphate groups of RNA) are important for aminoglycoside−RNA complexation. The values of pK_a of the amino groups of amikacin have been determined by both ^{13}C NMR spectroscopy[4] and ^{15}N NMR spectroscopy,[6] but there are discrepancies in the values reported by these two methods. For instance, there are considerable differences in the values of the pK_a that have been reported for amino groups 3 (10.1,[4] 8.13[6]) and 4 (8.5,[4] 9.7[6]).

The combination of CE and charge ladders is a useful tool for measuring the electrostatic properties (such as the charge [7−9] and the values of pK_a of ionizable groups[10,11]) of proteins and for determining the contribution of global electrostatic interactions to the free energy of binding of a protein to a charged ligand.[12,13] A charge ladder is a set of derivatives of a protein produced by the partial acetylation of its Lys ϵ-NH_3^+ groups.[12,14] These derivatives are often resolved by capillary electrophoresis (CE) into a set of distinct peaks. Each peak (or "rung") of the charge ladder usually consists of derivatives of the protein having the same number of acetylated lysine residues and approximately the same value of charge. We have previously used the combination of charge ladders and CE to study the electrostatic properties of insulin.[10] In this work, we used the combination of acetylation and CE to investigate the electrostatic properties of small molecules—amikacin and its derivatives (Table 1). We used CE−MS−MS to assign structures to the distinct peaks resolved by CE. The acetylated derivatives can be separated by CE to generate a library of potentially pharmacologically relevant small molecules.

EXPERIMENTAL SECTION

Reagents. All chemicals used were reagent grade unless stated otherwise. Amikacin was purchased from Sigma (St. Louis, MO). Anhydrous dioxane, acetic anhydride, 10% Pd/C, benzyl

* To whom correspondence should be addressed: (telephone) (617) 495-9430; (telefax) (617) 495-9857; (e-mail) gwhitesides@gmwgroup.harvard.edu.

[†] Harvard University.

[‡] Pacific Northwest National Laboratory.

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Table 1. Determination of Structure of Acylated Derivatives of Amikacin, and of the Values of pK_a for These Compounds

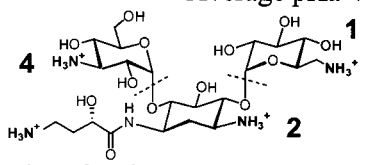
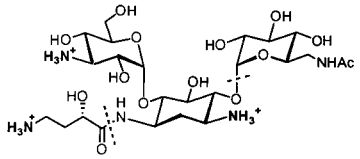
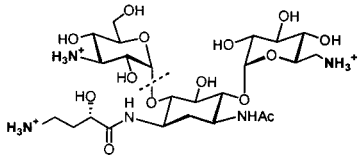
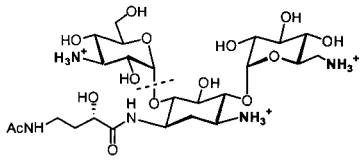
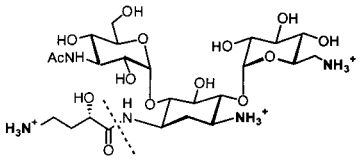
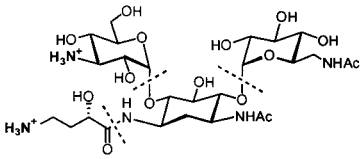
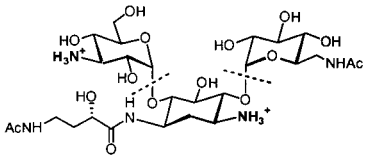
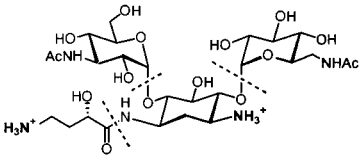
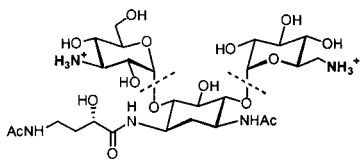
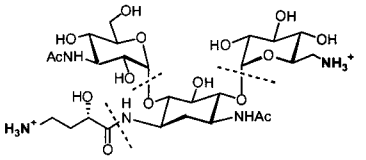
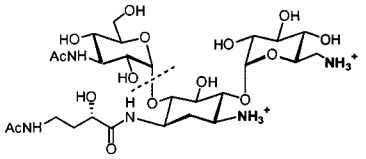
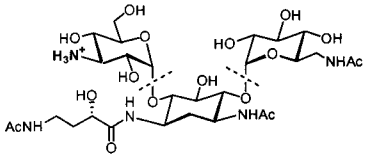
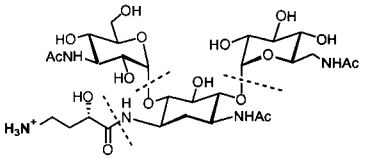
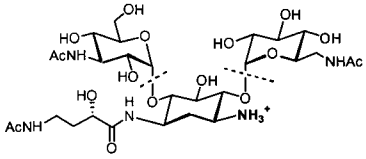
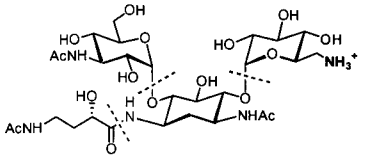
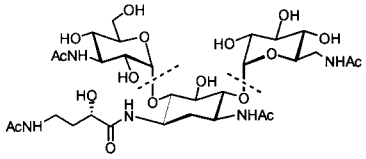
Structure[a] and Position #[b]	Letter	Acetylated at #	Charge Ladders[c] Figure 3a	Charge Ladders[c] Figure 3b	pKa Value[d] at Position Number 1	2	3	4	CE-MS-MS[a] Parent Mass *Major Fragment Masses* [Acetylated at #]
Average pKa Values					8.7	6.9	10.1	8.1	
	**a**		•	•	8.4	6.7	9.7	8.4	*720 ♦*447*, ♦*581* [NA]
3 Amikacin									
	**b**	1	•			6.7	9.5	8.2	627 *527, 467* [1, 2, or 4]
	**c**	2	•		8.9		9.1	7.7	627 *467* [1, 2, 3, or 4]
				•					*762 *661, 601, 467* [2]
	**d**	3		•					*762 *601* [3]
	**e**	4		•					*762 *661* [4]
	**f**	1, 2	•				9.9	7.9	669 *569, 509, 467, 306* [1, 2 or 2, 4]
	**g**	1, 3	•			6.9		8	669 *509, 306* [1, 3 or 3, 4]
	**h**	1, 4	•			7	10.6		669 *569, 467* [1, 4]
	**i**	2, 3		•	8.8			8	*804 *643, 509, 348* [2, 3]

Table 1 (Continued)

Structure[a] and Position #[b]	Letter	Acetylated at #	Charge Ladders[c] Figure 3a	3b	pKa Value[d] at Position Number 1	2	3	4	CE-MS-MS[a] Parent Mass *Major Fragment Masses* [Acetylated at #]
	**j**	2, 4		•	8.7		10.3		*804 *703, 601, 509* [2, 4]
	**k**	3, 4		•					*804 *601* [3, 4]
	**l**	1, 2, 3	•					7.9	711 *551, 509, 348* [1, 2, 3 or 2, 3, 4]
	**m**	1, 2, 4	•				10		711 *611, 509* [1, 2, 4]
	**n**	1, 3, 4	•			7.1			711 *509* [1, 3, 4]
	**o**	2, 3, 4		•	8.7				*846 ♦*725*, ♦*665*, ♦*573* [2, 3, 4]
	**p**	1, 2, 3, 4	•	•					753 *551* [1, 2, 3, 4]

[a] Structure determinations were made by CE−MS−MS; the fragmentation of the parent structure as measured by CE−MS−MS is indicated with a dashed line. The mass redundancy of the exterior aza sugars of amikacin did not allow the CE−MS−MS to conclusively determine the structure for all signals; for the charge ladder of amikacin, we made the assumption that position 1 (the most reactive amino group) was acetylated in all cases except for the second monoacetylated derivative labeled "**c**". The CE−MS−MS results for the charge ladder of mono-Z-amikacin were extrapolated for the structure determination of this charge ladder after deprotection of the Z group. The asterisk (*) indicates that the parent mass (and therefore some of the masses of the major fragments) include the Z-protecting group. The diamond (♦) indicates that the mass of the fragment includes Na^+. [b] The structure of amikacin shows the numbering sequence used for the amino groups. [c] The members of the charge ladder for amikacin (Figures 3a and 4a) and the charge ladder for deprotected mono-Z-amikacin (Figures 3b and 4b) are indicated with bullets. [d] The values of pK_a were determined by the titration curves of Figure 4a and b. Values of pK_a were not assigned to the amino groups of derivatives **d**, **e**, and **k** because the intensities of these peaks were very low, and it was difficult to identify these peaks in every electropherogram (i.e., at every value of pH).

chloroformate (Z-Cl), and acetic acid were purchased from Aldrich (Milwaukee, WI). Sodium borate buffer (80 mM, pH 8.9) and tris−glycine (25 mM/192 mM) buffer were freshly prepared in distilled, deionized water and filtered through 0.22-μm filters prior to use.

The ^{1}H NMR spectrum was recorded at 400 MHz on a Bruker spectrometer. Chemical shifts are reported in parts per million referenced with respect to residual solvent ($CHCl_3$ = 7.26 ppm). The fast atom bombardment (FAB) mass spectrum for mono-Z-

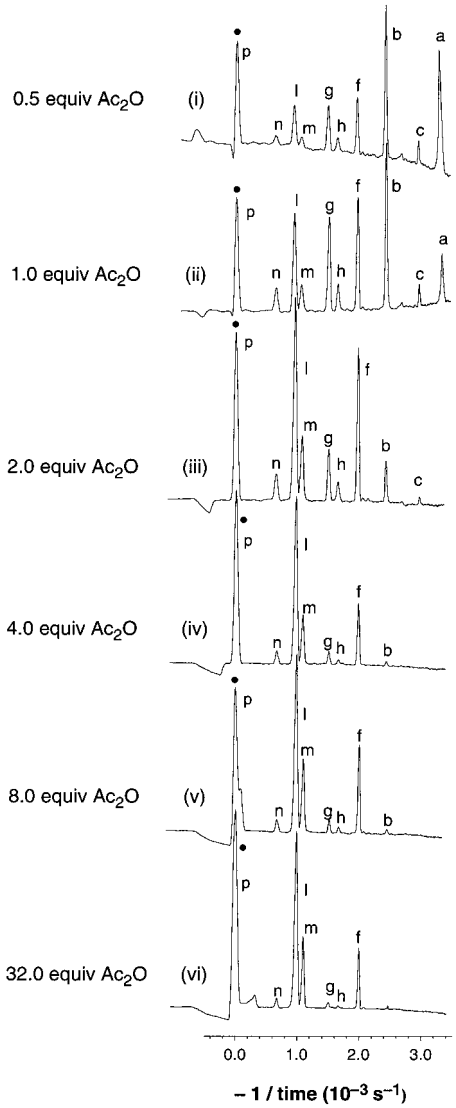


Figure 1. Electropherograms (in the 1/time domain) of the mixtures obtained after reaction of amikacin with different equivalents of acetic anhydride in 80.0 mM aqueous borate buffer (pH 8.9). The distance between two peaks on the 1/time axis is linearly related to the difference in their electrophoretic mobilities. CE analyses were performed on a Polybrene-coated capillary using 25 mM tris−192 mM gly buffer at pH 6.9. Key: (i) 0.5, (ii) 1.0, (iii) 2.0, (iv) 4.0, (v) 8.0, and (vi) 32 equiv of Ac_2O. Up to nine peaks are observed in these electropherograms, corresponding to triacetylated (**l**−**n**), diacetylated (**f**−**h**), monoacetylated (**b**, **c**), and unreacted amikacin (**a**). The peak corresponding to N,N′,N″,N‴-tetraacetylated amikacin (**p**) coelutes with the neutral marker (*p*-methyoxybenzyl alcohol, ●).

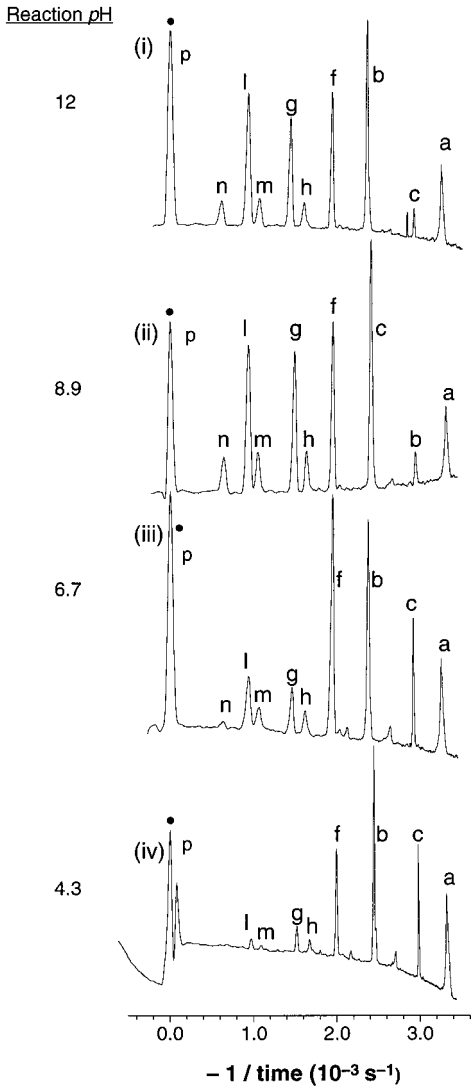


Figure 2. Electropherograms (in the 1/time domain) obtained for the reaction of amikacin with 1 equiv of acetic anhydride at different values of pH. Analyses were performed on a Polybrene-coated capillary using 25 mM tris−192 mM Gly (pH 6.9) buffer. Acetic anhydride (530 mM) in dioxane was added to 24 mM amikacin in (i) 60 mM sodium phosphate buffer (pH 12), (ii) 80 mM borate buffer (pH 8.9), (iii) 100 mM sodium phosphate buffer (pH 6.7), and (iv) 200 mM sodium acetate buffer (pH 4.3).

amikacin was recorded on a JEOL AX-505 mass spectrometer by the Harvard University Mass Spectrometry Laboratory.

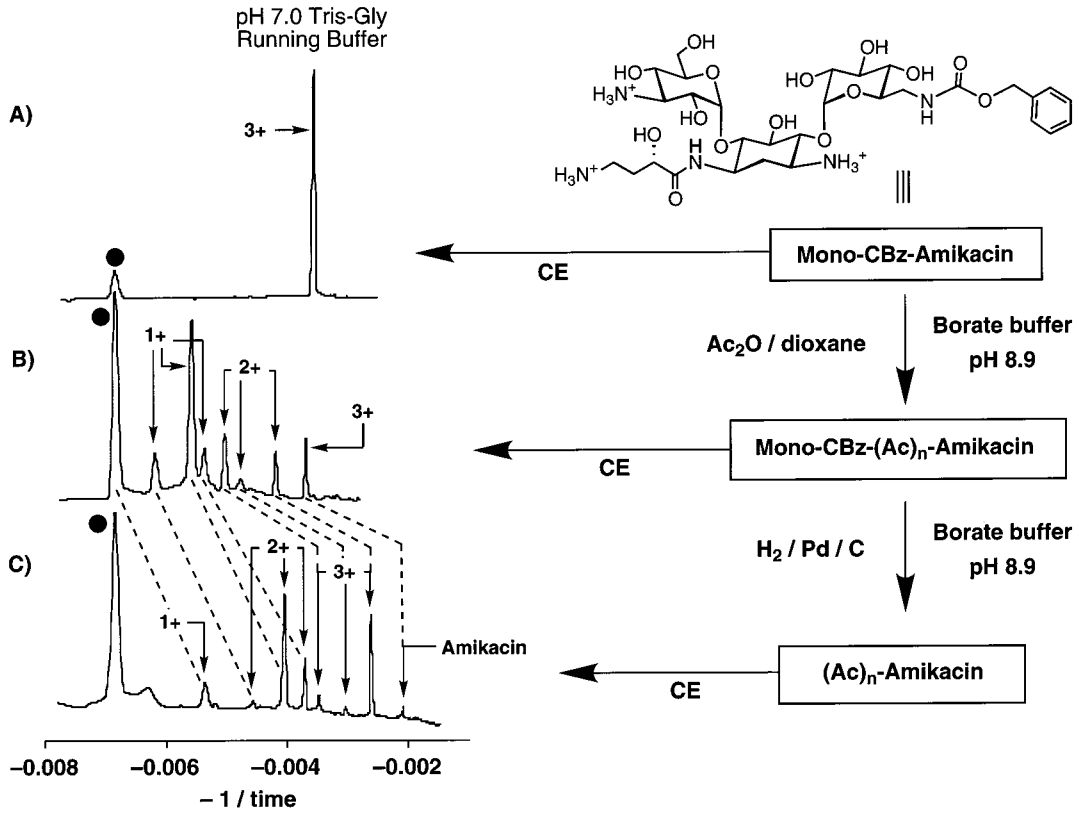
Acetylation of Amino Groups of Amikacin. Amikacin was dissolved in 80 mM sodium borate buffer (pH 8.9) at a concentration of 18 mg/mL, 24 mM; 0.5−32 equiv of acetic anhydride dissolved in dioxane (530 mM) was added to the amikacin solution; and the reactants were mixed quickly by vortexing. Reaction mixtures were allowed to stand at room temperature for 1−15 min before being diluted in electrophoresis buffer (25 mM tris−192 mM glycine at appropriate pH) prior to analysis by CE.

Capillary Electrophoresis. CE experiments were conducted on a Beckman P/ACE 5000 instrument. Reaction products derived from modification of amikacin were analyzed at 25 °C in reverse polarity on a Polybrene-coated capillary[11] (L_{tot}= 47 cm, L_{det} = 40 cm, 50-μm internal diameter), using 25 mM tris−192 mM glycine buffer at pH 8.4 (unless stated otherwise) and an applied voltage of 20 kV.

Measurement of Mobility as a Function of pH. Running buffers of different values of pH (between 5.43 and 9.78) were obtained by adding either acetic acid or 0.1 N NaOH to a stock solution of 25 mM tris−192 mM glycine buffer (pH 8.4). The mixture formed from the reaction of amikacin (24 mM in 80 mM borate buffer, pH 8.9) with 2 equiv of acetic anhydride (530 mM in dioxane) was used as the analyte. Values of the electrophoretic mobility (μ) ($cm^2\ kV^{-1}\ s^{-1}$) were determined from the CE data using eq 1. By referencing all mobilities to a neutral marker, we removed artifacts due to rates of electroosmotic flow.

CE−FTICR MS. On-line coupling of CE with ESI-FTICR employed a Crystal 310 CE system (ThermoQuest, San Jose, CA)

Scheme 1. Generation of the Remaining Acetylated Derivatives of Amikacin by a Z-Protection−Deprotection Strategy[a]



[a] Amikacin was reacted with benzyl chloroformate and the monoprotected product was isolated (electropherogram A). The charge ladder obtained after acetylating the mono-Z-protected derivative is shown in electropherogram B. The charge ladder obtained after deprotecting the Z group is shown in electropherogram C.

using 100-cm-long (50-μm-i.d., 192-μm-o.d.) fused-silica capillaries (Polymicro Technologies, Inc., Phoenix, AZ) which were mounted within the electrospray probe of a standard Finnigan MAT (San Jose, CA) ESI source utilizing a coaxial liquid sheath flow configuration.[15] The inner surface of the fused-silica capillary was statically modified using Polybrene cross-linked with dicumyl peroxide (10% Polybrene was incubated into the capillary and baked at 110 °C under nitrogen for 20 min, and then the capillary was injected with 2% dicumyl peroxide in dichloromethane and baked at 110 °C under nitrogen for 20 min). The run buffer of 20 mM ammonium acetate (pH 7.0) was used to prerinse the capillary for 15 min before the CE run and to wash the capillary for 6 min between runs. Samples were injected by low pressure (100 mbar) for 6 s. A negative 30-kV potential was applied at the inlet buffer reservoir for separation. A low pressure of 60 mbar was used to assist the reversed electroosmotic flow.

The ESI mass spectra were acquired with a 7-T ESI-FTICR mass spectrometer equipped with an Odyssey data system (Finnigan FTMS, Madison, WI).[16] Ions were transferred to the trap from the ESI interface through an rf quadrupole for collisional focusing (at ~200 mTorr) and two subsequent sets of rf-only quadrupoles (~750 kHz, ~500 V_{pp}) located in higher vacuum regions. Mass spectra were obtained by utilizing standard experimental sequences (i.e., ion injection and accumulation, pump down, and excitation/detection) with a total sequence time of ~2.75 s. Ion accumulation was accomplished by ~10^{-5} Torr N_2 injected into the trap via a piezoelectric pulse valve (Lasertechniques Inc., Albuquerque, NM). Background pressure in the ICR trap was maintained at ~10^{-9} Torr by a custom cryopumping assembly that provides effective pumping speeds of ~10^5 L/s and thus allows rapid transition between in-trap ion accumulation (i.e., 10^{-5} Torr) and high-performance ion excitation/detection (i.e., 10^{-9} Torr) events. The Odyssey data station provided ICR trap control, ion excitation (i.e., broadband chirp excitation over a 360-kHz bandwidth with a 35 Hz/μs sweep rate), data acquisition (128K data points at 900 kHz), and storage.

CE−FTICR MS−MS. On-line CE−FTICR MS−MS experiments utilize an ancillary PC that controls the instrument through an Ethernet link, with the Odyssey data station providing most of the common experimental parameters, similar to that described previously for automated dynamic range enhancement.[17] Automated CE−FTICR MS−MS employs stored-waveform inverse Fourier transform (SWIFT) ion isolation followed by sustained off-resonance irradiation-collisionally induced dissociation (SORI-CID, typically ~15 V_{pp} at a frequency 1 kHz lower than the reduced ICR frequency of the selected ions). Dipolar excitation waveforms (i.e., SWIFT and SORI) were generated by use of a PC board

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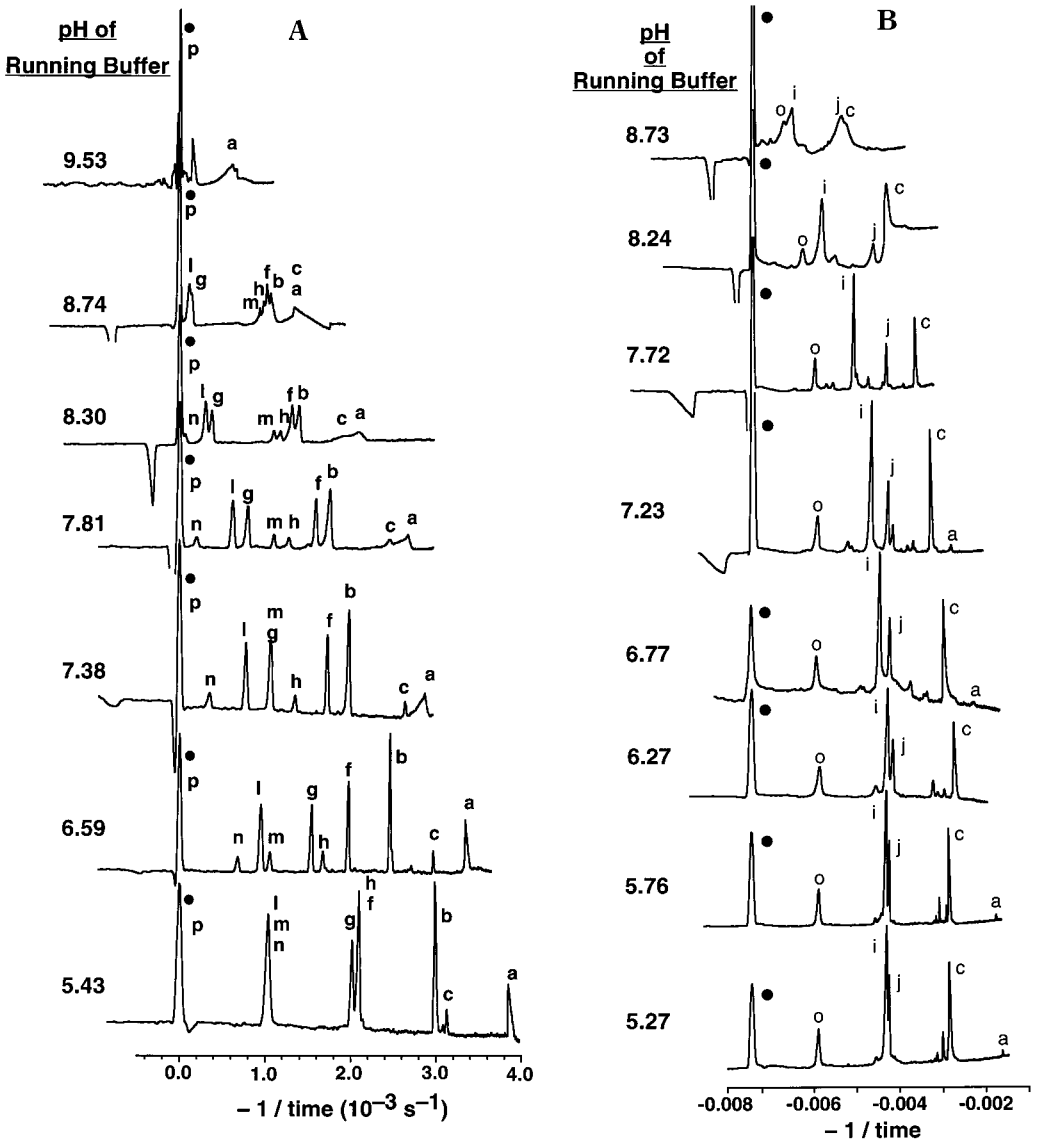

Figure 3. (A) Electropherograms of a mixture of amikacin and eight of its acetylated derivatives obtained with running buffers at different values of pH. Nine peaks are observed in the electropherogram, corresponding to tetraacetylated (**p**), triacetylated (**l**−**n**), diacetylated (**f**−**h**), monoacetylated (**b**, **c**), and unreacted amikacin (**a**). The peak corresponding to tetra-N-acetylated amikacin (**p**) has the same electrophoretic mobility as the neutral marker (*p*-methoxybenzyl alcohol, ●). (B) Electropherograms of the mixture of amikacin and its acetylated derivatives (after the Z-deprotection) obtained with running buffers at different values of pH. The peaks labeled in the electropherograms correspond to triacetylated (**o**), diacetylated (**i**, *and* **j**), monoacetylated (**c**), and unreacted amikacin (**a**).

(PCIP-AWFG, 5 MHz, 12 bit, Keithley Metrabyte Co., Taunton, MA) based on the most abundant signal detected in the prior MS sequence. Total time taken for the MS and MS/MS sequences was ∼5.5 s.

Mono-Z-Amikacin. Sodium hydroxide (3 M aqueous solution, 0.58 mL, 1.7 mmol) and benzyl chloroformate (0.24 mL, 1.7 mmol) were added in portions over 10 min to a rapidly stirred solution of amikacin dihydrate (1.0 g, 1.6 mmol) in DMF−water (1:1, 50 mL) at 0 °C, and the reaction was stirred for 18 h.[18] The reaction mixture was concentrated in vacuo, and water (5 mL) was added. This mixture was filtered to remove a small amount of insoluble material. The filtrate was purified using preparatory HPLC (Rainin, reverse phase) using a gradient of water and acetonitrile to afford mono-Z-amikacin (protected at position 1, 0.12 g, 10%) and di-Z-amikacin (protected at positions 1 and 2, 0.05 mg, 4%) as colorless oils. Mono-Z-amikacin: ^{1}H NMR (DMSO, 400 MHz) Hz 1.59 (m, 1H), 1.74 (m, 1H), 1.99 (m, 2H), 2.88 (m, 2H), 3.04, (m, 4H), 3.33−3.60 (m, 10 H), 3.71 (m, 1H), 3.76 (m, 1H), 3.85 (m, 2H), 3.98 (m, 1H), 4.99 (m, 1H), 5.03 (s, 2H), 5.05 (s, 1H), 8.22 (d, J = 8 Hz, 1H); MS-FAB m/z 720 (M + H, 100)$^+$, 559 (M − aza sugar + H, 40)$^+$, 425 (M − Z-aza sugar + H, 20)$^+$, 264 (M − Z-aza sugar − aza sugar + H, 30)$^+$.

Deprotection of the Mono-Z-Amikacin Charge Ladder. A catalytic amount of Pd/C was added to an aqueous solution of the mono-Z-amikacin charge ladder, and H_2 gas was allowed to bubble through the solution for 2 h. To remove the Pd/C, the sample was centrifuged, and the supernatant, containing the charge ladder, was removed by pipet. Small aliquots of this charge

(18) Kawaguchi, H.; Naito, T.; Nakagawa, S.; Fujisawa, K. *J. Antibiot.* **1972,** *25*, 695−708.

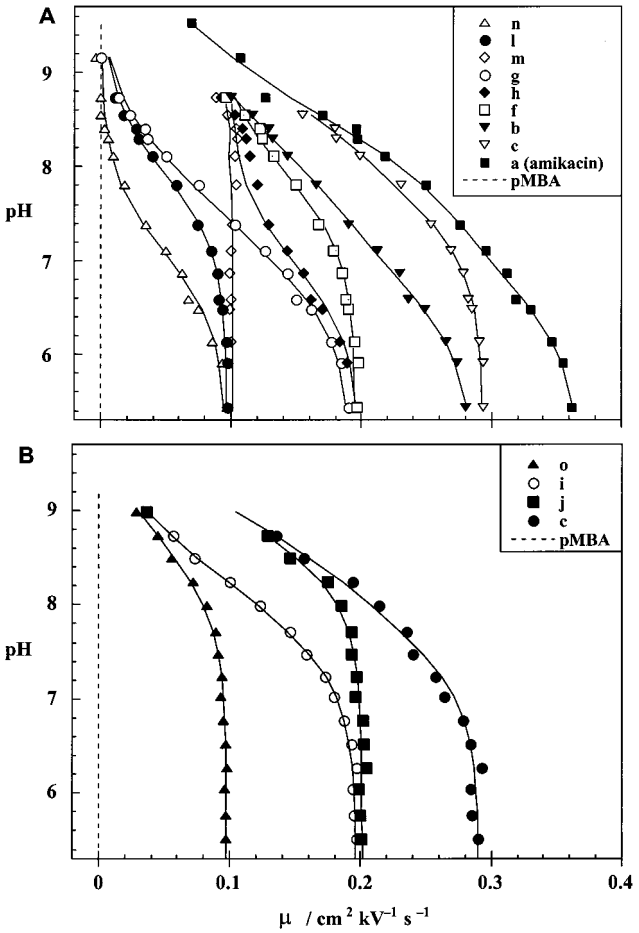

Figure 4. (A) Titration plot of electophoretic mobilities (μ) of amikacin and eight of its acetylated derivatives as a function of the pH of the running buffer. The data represent triacetylated (**n**−**m**), diacetylated (**g**, **h**, **f**), monoacetylated (**b**, **c**), and unreacted amikacin (**a**). The curves superimposed upon these data were obtained from a nonlinear least-squares fit using eqs 1 and 2. The values of pK_a of each of the NH_3^+ ions obtained by this method are listed in Table 1. (B) Titration plot of electophoretic mobilities (μ) of amikacin and its acetylated derivatives (after the Z-deprotection) as a function of the pH of the running buffer. The data represent triacetylated (**o**), diacetylated (**i**, **j**), and monoacetylated (**c**) amikacin. The curves superimposed upon these data were obtained from a nonlinear least-squares fit using eqs 1 and 2. The values of pK_a of each of the NH_3^+ ions obtained by this method are listed in Table 1.

ladder solution were diluted in the appropriate buffer prior to analysis by CE.

RESULTS AND DISCUSSION

Acetylation with Acetic Anhydride. Figure 1 shows electropherograms of the mixtures obtained after reaction of amikacin with different numbers of equivalents of acetic anhydride in 80 mM aqueous borate buffer (pH 8.9). In principle, the reaction of amikacin with acetic anhydride can result in 15 *N*-acetyl derivatives (**b**−**p**) that vary in the position and number of acetylated amino groups (Table 1). As can be seen from Figure 1, we observed only nine such derivatives (**b**, **c**, **f**−**h**, **l**−**n**, **p**), in addition to unreacted amikacin (**a**). As expected for a molecule with no net charge at values of pH between 5 and 10, the tetra-N-acetylated amikacin (**p**) had an electrophoretic mobility indistinguishable from that of the neutral marker, *p*-methoxybenzyl alcohol. We hypothesized that one amino group of amikacin has a reactivity toward acetylation that is significantly greater than the others and is always acetylated in the diacetylated, triacetylated, and tetra-acetylated derivatives of amikacin obtained by the reaction with acetic anhydride. If this hypothesis were true, eight of the nine derivatives observed would represent all the derivatives of amikacin in which the most reactive amine is acetylated. The remaining peak would represent another monoacetylated derivative of amikacin.

Varying the pH (at which the reaction of acetic anhydride (1 equiv) and amikacin was performed) resulted in only modest differences in the distribution of acetylated derivatives (Figure 2). No significant amounts of new derivatives of amikacin were observed beyond the eight derivatives presented in Figure 1.

Protection of Amikacin with Benzyl Chloroformate (Z-Cl). To test our hypothesis that one of the amino groups of amikacin had a reactivity toward acetylation significantly greater than the others and to obtain the remaining acetylated derivatives of amikacin, we allowed amikacin to react with Z-Cl and isolated the major monoprotected product. The ^{1}H NMR spectrum and FAB-MS spectrum of the major mono-Z-protected amikacin derivative isolated was consistent with Z-protection of the amino group 1 (Table 1). We reasoned that we could generate the remaining derivatives of amikacin—the ones not observed in the charge ladder resulting from its direct acetylation—by acetylating the mono-Z-protected amikacin derivative, followed by Pd/H_2 deprotection of the Z group (Scheme 1). Electropherograms of the acetylated amikacin derivatives before and after the Z-deprotection are also shown in Scheme 1. The Z protection−deprotection strategy allowed the isolation of the remaining acetylated derivatives of amikacin.

Assignment of Structures by CE−MS−MS. Table 1 summarizes the CE−MS−MS results. We include the structure, show a dashed line to indicate the major fragments observed, and give the parent mass and the major fragment masses observed. An asterisk is used when the parent mass (and therefore the masses of some of the major fragments) include the Z-protecting group.

Assignment of Structures to the Peaks in the Mono-Z-Protected Charge Ladder. The mass fragments observed in CE−MS−MS for the mono-Z-amikacin charge ladder (Scheme 1) allowed the assignment of structures to the peaks in the electropherograms without any additional assumptions. The use of the Z-protected ladder eliminates mass redundancies of the two exterior aza sugars of amikacin and makes structure determination straightforward. We used the CE−MS−MS results (and the resulting structure assignments) for the Z-protected ladder to assign structures for the peaks in the Z-deprotected ladder (as shown in Scheme 1). While we could not use CE−MS−MS results for the ladder after Z-deprotection, because the peak intensities were very low, we believe that the extrapolation depicted in Scheme 1 is appropriate for two reasons: (1) Amino group 1 is Z-protected in all of the components of the protected charge ladder. Upon deprotection, we assume that amino group 1 has roughly the same value of pK_a in each component of the deprotected charge ladder, so that at any particular value of pH each peak in this charge ladder has roughly the same partial charge associated with protonation of amino group 1, and the order of the peaks is roughly the same in both the protected and

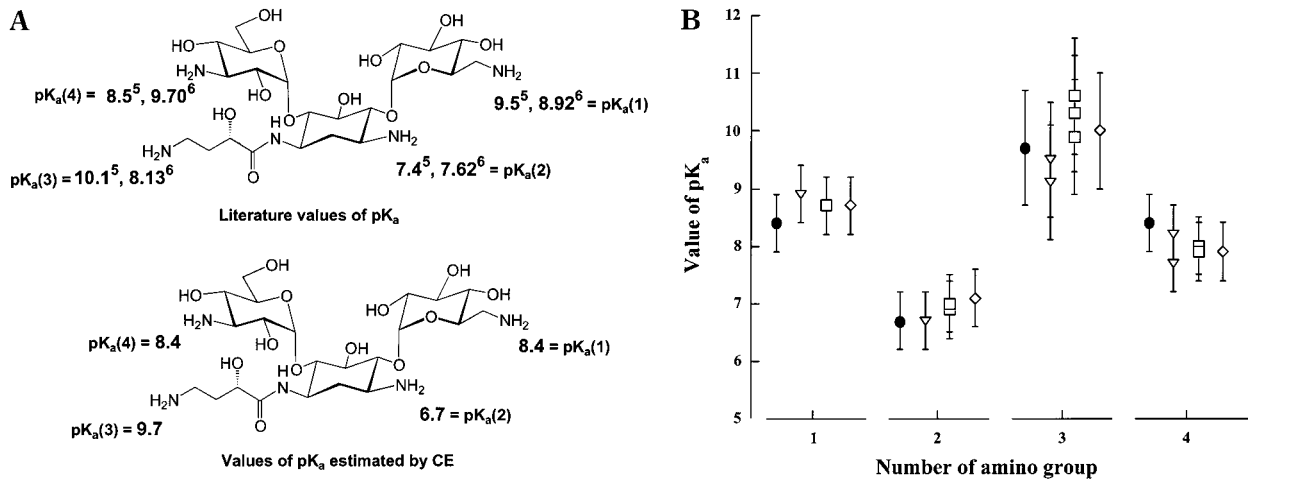

Figure 5. (A) Comparison of the values of pK_a of the amino groups in amikacin (estimated by CE) with the values reported in the literature. The values of pK_a reported are those for unacylated amikacin and are indicated in (B) with the filled symbol (●). (B) Values of pK_a (estimated by CE) for the amino groups in triacetylated (◇), diacetylated (□), monoacetylated (▽), and unacylated amikacin (●). These values are taken from Table 1.

deprotected charge ladders. (2) The intensities of the series of signals in the deprotected charge ladder correlate well with the intensities of the series of signals in the Z-protected charge ladder.

Assignment of Structures to the Peaks in the Amikacin Charge Ladder. The mass fragments observed in CE−MS−MS for the amikacin charge ladder allowed the assignment of structures to the peaks labeled **h**, **m**, and **n**, without any additional assumptions. Table 1 indicates that the mass fragments do not uniquely define the structure of the other peaks because of the mass redundancy of the two exterior aza sugars. To assign structures to these peaks, we have made the assumption that the amino group in position 1 is the most reactive and that this amino group is acetylated in all diacetylated and triacetylated derivatives of the ladder. Our assumption is based on our isolation of the mono-Z-amikacin derivative (found to be modified at amino group 1) as the major product. The literature also suggests that amino group 1 is highly reactive, as it must be protected in the synthesis of amikacin from kanamycin.[18] These assumptions allow us to assign structures to the remaining peaks of the ladder. The values of pK_a determined for these derivatives match those determined for the derivatives (**h**, **m**, **n**) whose structures are uniquely defined.

Analysis: Determination of Values of pK_a of the Amino Groups. To estimate the values of pK_a of the amino groups of amikacin and of its acetylated derivatives, we obtained electropherograms of the acetylated amikacin derivatives for values of the pH of the running buffer between 5.4 and 9.8. The electropherograms for the pH titration are shown in Figure 3A (for the initial eight acetylated derivatives) and Figure 3B (for the derivatives obtained by Z-protection−deprotection). The electrophoretic mobility of each derivative was plotted as a function of pH (Figure 4). Since the amino groups represent the only charged functional groups in amikacin and its acetylated derivatives for values of pH between 5.4 and 9.8, the total charge on an amikacin derivative will range from 0 to $+n$ (where n is the number of free amino groups in the derivative). The total charge (Z_{tot}) on an amikacin derivative (the sum of the charges on each of the n amino groups of the derivative) can be related to the electrophoretic mobility (μ) of the derivative using eq 1.[8] In eq 1, L_{det} represents the length of the capillary from the inlet to the detector, L_{tot} represents the total length of the capillary, V is the applied voltage, t_{nm} represents the time of migration of the neutral marker from injection to detection, t represents the time of migration of the analyte, M represents the molecular weight of the analyte, and C_p and α are constants.[8] Values of pK_a for the individual amino groups can be estimated using eq 2, where $Z_{R(n)}$ is the partial positive charge associated with amino group n (Table 1), and p$K_a(n)$ is the value of pK_a for amino group n.

$$\mu = \frac{L_{det}L_{tot}}{V}\left(\frac{1}{t_{nm}} - \frac{1}{t}\right) = \frac{C_p}{M^{\alpha}}Z_{tot} \quad (1)$$

$$Z_{tot} = Z_{R(1)} + Z_{R(2)} + Z_{R(3)} + Z_{R(4)} = \frac{[R(1)NH_3^+]}{[R(1)NH_3^+] + [R(1)NH_2]} + \frac{[R(2)NH_3^+]}{[R(2)NH_3^+] + [R(2)NH_2]} + \frac{[R(3)NH_3^+]}{[R(3)NH_3^+] + [R(3)NH_2]} = \frac{1}{1 + 10^{pH-pK_a(1)}} + \frac{1}{1 + 10^{pH-pK_a(2)}} + \frac{1}{1 + 10^{pH-pK_a(3)}} + \frac{1}{1 + 10^{pH-pK_a(4)}} \quad (2)$$

Electrophoretic mobilities of each of the peaks in Figure 3 were fitted to eqs 1 and 2. From the fits to the equations, the values of pK_a listed in Table 1 were determined. The term (C_p/M^{α}) was taken to be another variable in the fit and was found to have approximately the same value (0.097 ± 0.005) in each fit.

Comparison of pK_a Values Obtained by CE with Values Reported in the Literature. Figure 5A compares the values of pK_a of the amino groups of (unacetylated) amikacin obtained by CE with the values obtained using NMR spectroscopic techniques.[5,6] The values lie within 0.7 unit of values reported in the literature. The CE experiments help to resolve ambiguities left by the earlier studies. In this case, the values determined by CE (9.7 for amino group 3 and 8.4 for amino group 4) are in good agreement with the values previously determined by ^{13}C NMR spectroscopy.[5] Figure 5B suggests that the value of pK_a of a given

amino group does not depend on the degree of acetylation of the amikacin derivative (within experimental error). The error bars in Figure 5B represent conservative estimates of the error in the calculated values of pK_a and correspond to the range of values of pK_a over which only small changes were observed in the difference between the calculated and experimental titration curves. The range of values of pK_a estimated by CE and the uncertainty in the value of pK_a is greatest for amino group 3 (which has the highest value of pK_a), perhaps because the maximum value of pH used in our titrations (9.5) is comparable in magnitude to the value of pK_a of amino group 3, and the titration curves are less completely defined than for the other amino groups.

CONCLUSIONS

This work demonstrates that CE and CE−MS−MS are powerful tools for studying the electrostatic properties of small molecules. The values of pK_a of the amino groups of amikacin determined by CE were in reasonable agreement with certain values reported in the literature and also resolved ambiguities left by the earlier studies. The combination of CE and CE−MS−MS also enabled the determination of the values of pK_a of the amino groups in the acetylated derivatives of amikacin. The combined use of CE and CE−MS−MS may enable a greater understanding of the dependence of the degree of ionization of an ionizable group on its environment.

The acetylation of amikacin—a pharmacologically relevant small molecule—yields a library of potentially active compounds. Knowledge of the values of pK_a of the ionizable groups in these compounds would be useful in studies that compare the pharmacological efficacy of these derivatives with their electrostatic properties.

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