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Protonation of kanamycin A: Detailing of thermodynamics and protonation sites assignment

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ABSTRACT

Protonation of an aminoglycoside antibiotic kanamycin A sulfate was studied by potentiometric titrations at variable ionic strength, sulfate concentration and temperature. From these results the association constants of differently protonated forms of kanamycin A with sulfate and enthalpy changes for protonation of each amino group were determined. The protonation of all amino groups of kanamycin A is exothermic, but the protonation enthalpy does not correlate with basicity as in a case of simple polyamines. The sites of stepwise protonation of kanamycin A have been assigned by analysis of $^{1}H-^{13}C$–HSQC spectra at variable pH in D_2O. Plots of chemical shifts for each H and C atom of kanamycin A vs. pH were fitted to the theoretical equation relating them to pK_a values of ionogenic groups and it was observed that changes in chemical shifts of all atoms in ring C were controlled by ionization of a single amino group with pK_a 7.98, in ring B by ionization of two amino groups with pK_a 6.61 and 8.54, but in ring A all atoms felt ionization of one group with pK_a 9.19 and some atoms felt ionization of a second group with pK_a 6.51, which therefore should belong to amino group at C3 in ring B positioned closer to the ring A while higher pK_a 8.54 can be assigned to the group at C1. This resolves the previously existed uncertainty in assignment of protonation sites in rings B and C.

1. Introduction

The solution conformations of aminoglycoside antibiotics and their interactions with target biomolecules depend on their state of protonation. For this reason the knowledge of thermodynamics of protonation of aminoglycosides together with exact assignment of the protonation sites is necessary for interpretation of their functions. Recently the importance of establishing of a thermodynamic database involving a description of temperature and salt effects on protonation equilibria of aminoglycosides was emphasized [1].

Our interest to the protonation of kanamycin A stems from its possible application as a selective anion complexing agent [2]. Previously this and some other amynoglycoside antibiotics were employed as chiral selectors in capillary electrophoresis [3]. The advantages of kanamycin A [4] for applications in this area are that it is commercially available as sulfate in sufficiently pure form and its crystal structure [5], complete assignment of NMR spectra [6,7], solution properties, including conformational equilibria [7,8], have already been reported. There is, however, some uncertainty in the assignment of protonation sites, which were previously assigned tentatively by analogy with amikacin and some other related aminoglycosides as follows [7a]: first protonation (with the highest pK_a) involves the amino group of ring A, second and third protonations involve amino groups in rings B or C, and the last protonation involves one of the amino groups in ring B. To best of our knowledge no temperature and salt effects on protonation constants of kanamycin A were reported so far.

The purposes of this paper are to complete the thermodynamic characterization of kanamycin A protonation by studying salt and temperature effects as well as association of protonated forms of the antibiotic with sulfate anion, important since it is usually used as sulfate, and to obtain a definite assignment of protonation sites by performing detailed NMR pH-titration of kanamycin A in D_2O.

2. Experimental

2.1. Materials

All reagents were purchased from Aldrich and used as received. The purity of kanamycin A sulfate was confirmed by NMR spectroscopy and by potentiometric titrations.

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2.2. Potentiometry

Potentiometric titrations were performed in a 50 mL thermostated cell kept under nitrogen at desired temperature with 1–3 mM kanamycin A sulfate solution to which slightly more than two equivalents of HCl were added to produce a completely protonated form of the antibiotic at the start of titration. Measurements of pH were carried out using a Thermo Orion model 920Aplus pH-meter equipped with an Orion 8102U combination electrode while the titrant (CO_2–free NaOH) solution was added to the system in small increments with a piston type burette. The glass electrode was calibrated as a hydrogen-ion concentration probe by titration of previously standardized amounts of HCl with CO_2–free NaOH solutions. The details of electrode calibration were described previously [9]. The program HYPERQUAD 2003, version 3.0.51 [10], was used to calculate all equilibrium constants. The mean values of logarithms of protonation and association constants with standard errors were calculated by averaging the values obtained in at least three independent titration experiments. Errors in values of logarithms of constants as fitting parameters in each titration experiment were less than ±0.01. Species distribution diagrams were calculated by using HYSS 2000 software [10].

2.3. NMR

The NMR spectra were recorded on Varian UNITY INOVA-400 spectrometer. The following parameters were used to acquire the ^1H–^{13}C–HSQC spectra: relaxation delay of 1.0 s, spectral widths in F2 and F1 of 2410.8 and 10205.4 Hz, respectively, acquisition times in $t2$ of 0.212 s. For the $t1$ dimension, 2048 complex points were acquired using 16 scans per increment. The coupling constant between protons and carbons was 135 Hz. The spectra were obtained at 298 K in D_2O containing 0.08 M kanamycin A sulfate and pH was adjusted to the desired value with DCl or NaOD. The experimental data were fitted to theoretical Eqs. (4) and (5) using non-linear least-squares regression with Microcal Origin 5 program.

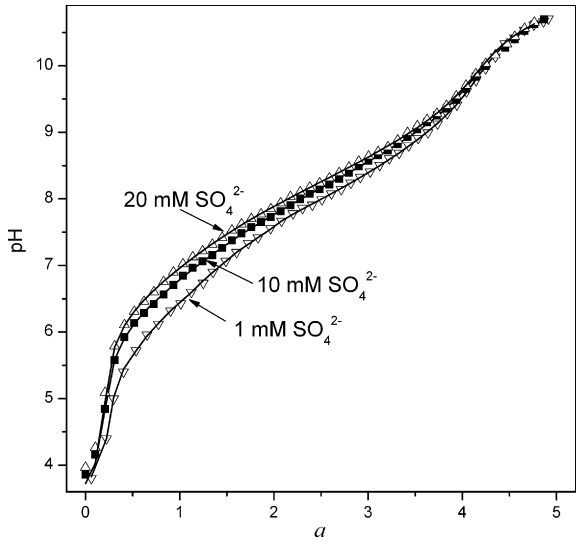

Fig. 1. Potentiometric titration curves of 1 mM kanamycin A sulfate with 0.05 M NaCl as the background electrolyte at 25 °C with increased concentrations of sulfate; a is the number of added equivalents of NaOH. The first break in pH corresponds to titration of a small excess of HCl. Open down triangles – 1 mM kanamycin A sulfate alone, solid squares and open up triangles – 1 mM kanamycin A sulfate with added 9 and 19 mM Na_2SO_4, respectively. Solid lines are the fitting curves generated by HYPERQUAD.

3. Results and discussion

Fig. 1 illustrates typical titration experiments. Analysis of titration curves performed with the HYPERQUAD program allows one to calculate the overall protonation constants (β_{1i}) from which the stepwise dissociation constants (K_{ai}) can be obtained. Titrations were performed at different concentrations of background electrolyte (NaCl) and sulfate anions. The results are shown in Table 1.

The increase in NaCl concentration brings about an increase in protonation constants. This effect is well documented for mono and polyammonium compounds and is attributed to weak ion pairing of chloride anions with ammonium groups in addition to a non-specific Debye–Hückel salt effect [11]. Published values of protonation constants in 0.1 M $NaNO_3$ (Table 1, line 3) [7a] are lower than those in 0.1 M NaCl and closer to those in 0.05 M NaCl indicating a weaker ion pairing with nitrate anions.

Titration of 1 and 3 mM kanamycin A sulfate gave the same protonation constants in limits of experimental errors. This means that in this concentration range no significant association between protonated forms of kanamycin A and sulfate anions occurs. However, additions of sulfate in higher concentrations bring about a significant increase in protonation constants, Table 1. This is manifested in the upward shift of the titration curves of kanamycin A, see Fig. 1. The highest concentration of added Na_2SO_4 0.019 M increases the ionic strength from 0.05 to 0.11 M. The respective activity corrections calculated with the semiempirical Debye–Hückel type equation proposed for analysis of ionic association with polyammonium cations [11] are +0.12 for pK_{a1}, +0.08 for pK_{a2}, +0.04 for pK_{a3} and zero for pK_{a4}. As is evident from results in Table 2, the actual effect is much larger. Also pK_{ai} values measured in these conditions are significantly higher than those measured with 0.1 NaCl, which creates approximately the same ionic strength. Obviously protonated forms of kanamycin A bind sulfate anion stronger then chloride.

The fitting of titration curves in terms of a model which considers the association between sulfate and protonated forms of the antibiotic allows one to determine the overall stability constants defined by Eq. (1), which correspond to reactions (2) (charges omitted).

$$\beta_{11i} = [ALH_n]/[A][L][H]^i \quad (1)$$

$$A + L + iH \rightleftarrows AH_iL \quad (2)$$

Table 1
Logarithms of overall and stepwise protonation constants of kanamycin A at 25 °C (errors less than ±0.04).

Conditions	Log β_{11}	Log β_{12}	Log β_{13}	Log β_{14}	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
0.05 M NaCl	9.03	17.15	24.61	30.65	6.04	7.46	8.12	9.03
0.1 M NaCl	9.16	17.42	24.95	31.23	6.28	7.53	8.26	9.16
0.1 M KNO_3[a]	9.030	17.188	24.607	30.800	6.19	7.42	8.16	9.03
0.05 M NaCl and 3 mM Na_2SO_4	9.02	17.22	24.64	30.88	6.24	7.42	8.20	9.02
0.05 M NaCl and 9 mM Na_2SO_4	9.11	17.39	24.94	31.39	6.45	7.55	8.28	9.11
0.05 M NaCl and 19 mM Na_2SO_4	9.15	17.52	25.23	31.84	6.61	7.71	8.37	9.15

[a] Data from Ref. [7a].

Table 2
Logarithms of overall and stepwise stability constants of sulfate complexes of protonated kanamycin A in 0.05 M NaCl.

Reaction	Log β_{11i}	Log K_{1i}
$AH^{+} + SO_4^{2-}$	10.31 ± 0.04	1.28
$AH_2^{2+} + SO_4^{2-}$	19.02 ± 0.02	1.87
$AH_3^{3+} + SO_4^{2-}$	26.70 ± 0.07	2.09
$AH_4^{4+} + SO_4^{2-}$	33.36 ± 0.08	2.71

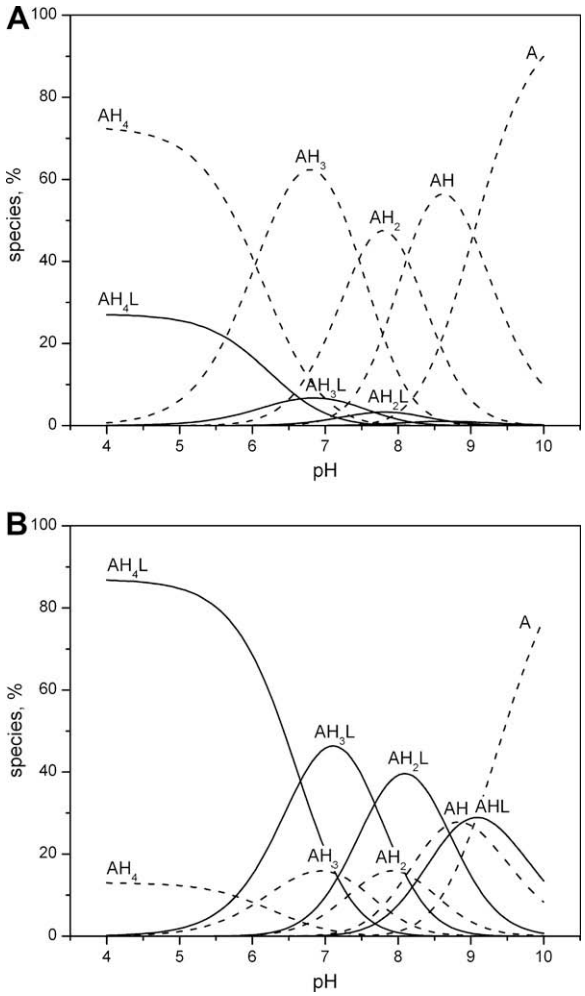

Fig. 2. Species distribution diagrams calculated with equilibrium constants given in Tables 1 and 2 for 1 mM (A) and 0.1 M (B) kanamycin A sulfate. Solid lines correspond to sulfate complexes $\left(LSO_4^{2-}\right)$ and dashed lines correspond to protonated forms of free antibiotic.

In these equations A and L stand for completely unprotonated forms of kanamycin A and the anion, respectively. Then the stepwise association constants K_i, which correspond to complex formation between individual ionic forms of kanamycin A (AH_i) and the anion, Eq. (3), can be calculated from β_{11i} with known protonation constants of kanamycin A.

$$AH_i + L \rightleftarrows AH_iL \quad (3)$$

Table 2 shows the results of fitting to this model.

As expected the binding constants increase with increasing in the degree of protonation of kanamycin A. Recently determined by isothermal calorimetry log K = 2.42 for association of sulfate with paromomycin at pH 7, when the predominant form of the antibiotic is the tetraprotonated one [1], agrees well with log K_{14} = 2.71 for tetraprotonated kanamycin A. Also similar association constant log K = 2.6 (corrected for the ionic strength 0.05 M) was reported for sulfate binding to tetraprotonated spermine [11]. Much bigger association constants with log K_{14} up to 5.5 were reported for binding of sulfate to some macrocyclic polyamines also in their tetraprotonated forms [12]. Therefore, it seems that aminoglycosides are not selective binders of sulfate and behave in this respect as other acyclic polyamines.

Even with such relatively modest affinity the degree of association of protonated forms of kanamycin A with sulfate is significant at concentrations about 0.1 M often employed for NMR studies with this and other aminoglycosides. Fig. 2 illustrates the change in the species distribution as a function of pH on going from 1 mM to 0.1 M kanamycin A sulfate. If in more dilute solution only the sulfate complex with tetraprotonated form of the antibiotic contributes significantly, but still less than 30% even at pH below 5, in more concentrated solution the sulfate complexes are the dominating species already starting form pH 8. An obvious consequence of this is that in more concentrated solutions kanamycin A sulfate as well as any other aminoglycoside sulfate will behave as a stronger base with larger pK_a values than in a dilute solution. Also it is obvious that chloride instead of sulfate salts of aminoglycosides will have lower pK_a values due to weaker complexation with chloride, as was observed in paromomycin [1] and neomycin B [13] ^{15}N NMR pH-titrations performed with either sulfates or free bases of aminoglycosides.

Protonation constants determined at different temperatures in 0.1 M NaCl are given in Table 3. As is typical for polyamines of different structures [14], they decrease on increase in temperature indicating that protonation of all amino groups of kanamycin A is exothermic. The slopes of plots of pK_a vs. temperature (dpK_a/dT) given in the Table 3 are smaller by absolute values than those for paromomycin, which lay between −0.029 and −0.023 [1]. The enthalpy and entropy changes calculated by van't Hoff analysis of protonation constants also are given in Table 3. Although determined in this way thermodynamic parameters are generally

Table 3
Logarithms of equilibrium constants (errors less than ±0.04) at different temperatures and thermodynamic parameters for stepwise protonation of kanamycin A in 0.1 M NaCl.

Log β_{10n} [a]			pK_a [b]			dpK_a/dT	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol K)	Position [c]
15 °C	25 °C	35 °C	15 °C	25 °C	35 °C				
9.4	9.16	8.98	9.4	9.16	8.98	−0.021 ±0.002	−35.7 ± 2.2	55 ± 4	Ring A, C6′
17.85	17.42	17.17	8.45	8.27	8.19	−0.013 ±0.003	−22.2 ± 4.5	84 ± 17	Ring B, C1
25.59	24.95	24.53	7.74	7.52	7.36	−0.019 ±0.002	−32.3 ± 2.3	35 ± 3	Ring C, C3″
32.04	31.23	30.61	6.45	6.28	6.08	−0.0185 ±0.0009	−31.4 ± 2.1	15 ± 1	Ring B, C3

[a] Overall protonation constants.
[b] Stepwise protonation constants.
[c] Protonation position assigned in this work, see below.

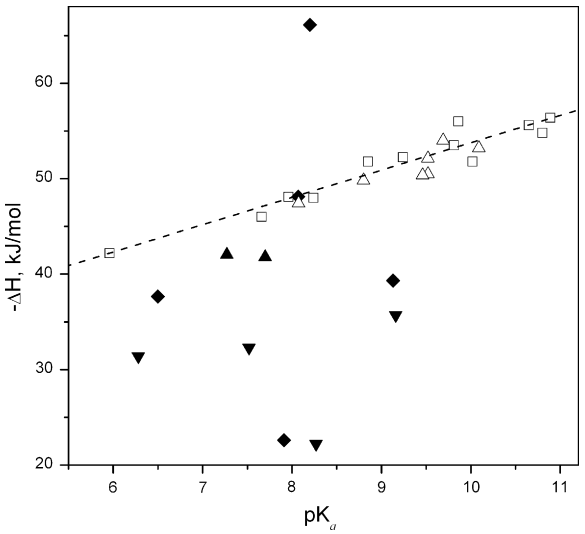

Fig. 3. Protonation enthalpies vs. pK_a for stepwise protonations of amines (open squares; spermine, spermidine, histamine, GlyOMe, $MeNH_2$ and NH_3, data from [14a] and [15]); amino alcohols (open triangles; ethanolamine, 2-hydroxypropylamine, 2-aminobutanol, 2-amino-2-methylpropanol, 3-aminopropanol, BIS, TRIS, data from [15]) and amino sugars: α- and β-glucosamines (solid up triangles, [17]), kanamycin A (solid down triangles, this work) and paromomycin (solid diamonds, [1]).

considered less accurate than those measured by calorimetry, in this particular case of multiple equilibria with strongly overlapping species distribution (see Fig. 2A) the high precision of potentiometric determination of protonation constants may even give some advantage to this method. The stepwise protonation enthalpies for linear tetraamines like spermine or triene range between −40 and −55 kJ/mol and show a regular trend of decreased absolute values for less basic amino groups [14]. The protonation of kanamycin A is less exothermic and the protonation enthalpy does not correlate with basicity, as was observed also for paromomycin [1].

A correlation between the protonation enthalpy and pK_a is expected because the protonation of amines is principally an enthalpy driven process. Fig. 3 (open squares) shows as an example a plot of ΔH^0 values vs. pK_a for stepwise protonations of a series of amines, which cover the whole range of pK_a values observed with aminoglycosides (data from Refs. [14a,15]). Amino alcohols are less basic due to the effect of hydroxyl groups and their protonation is known to be less exothermic [16]. Some typical values are included in Fig. 3 (open triangles; data from [15]) and they obviously fall on the same correlation line.

However, amino sugars do not follow the same trend. Fig. 3 (solid symbols) includes data for α- and β-glucosamines [17], kanamycin A (this work) and paromomycin [1], which mostly are below the correlation line for amines and simple amino alcohols and do not form any pattern. A theoretical analysis of the enthalpy of protonation of polyamines assigns a large contribution to the solvation enthalpy [14b]. From this point of view the most significant difference of amino sugars as compared to simple amino alcohols is a large number of vicinal hydroxyl groups surrounding amino groups, which may strongly affect their solvation by intramolecular direct and/or indirect (through a water molecule) hydrogen bonding. Indeed the occurrence of two intramolecular hydrogen bonds between OH and NH_2 groups of amikacin in aqueous solution was concluded from NMR studies [18]. Also several intramolecular hydrogen bonds between the different rings of gentamicin were identified in the antibiotic – RNA complex in solution [19]. Since hydrogen bonding is usually exothermic, disruption of intramolecular hydrogen bonds by protonation may result in a decreased observed protonation enthalpy. On the other hand, we observe that in the respect of sulfate binding protonated aminoglycosides behave as simple polyamines (see above). The reason for this is that the sulfate complexation occurs as a classical ion pairing controlled by the total charge of the polyamine [12], which is unaffected by the intramolecular hydrogen bonding.

The most direct method of assignment of protonation sites in aminoglycosides is the ^{15}N NMR pH-titration [1,20]. Results obtained by following ^1H and ^{13}C signals as a function of pH are less straightforward because chemical shifts can be affected by protonation of distant amino groups. However, as will be shown below following the signals of a large number of atoms one comes to reliable conclusions. The complete assignment of ^1H and ^{13}C signals of kanamycin A sulfate at several pH values was reported [6,7b], but in order to avoid any uncertainty in signal assignment at intermediate pH values we recorded the spectra in HSQC mode. Uncorrected for solvent isotope effect pH values were used for reasons explained below.

One-dimensional ^1H and ^{13}C spectra of kanamycin A recorded in the interval of pH from 5 to 10 are shown in Figs. S1 and S2 (Supplementary data). A typical ^1H–^{13}C–HSQC spectrum at one selected pH value is shown in Fig. 4.

Titration plots observed for C and H atoms of each ring, which undergo largest protonation induced shifts of the signals, are shown in Fig. 5. Protonation of kanamycin A induces larger mostly upfield shifts of the signals in ^{13}C spectra, which probably reflect conformational changes in the molecule [21], and smaller always downfield shifts of the signals in ^1H spectra, which reflect expected deshielding effect of protonation. As one can see some of the plots show only one inflection point corresponding to a single deprotonation process (C6′ in the ring A, C3″ and C4″ in the ring C), while signals of some atoms feel deprotonation of two ammonium groups and the respective plots show two inflection points (C1′ and C2′ in the ring A, C2, C4 and C6 in the ring B).

The experimental results were fitted to the theoretical Eqs. (4) and (5) derived for the single and double protonation processes, respectively. In these equations δ_0, δ_1 and δ_2 are the chemical shifts of the given atom in "unprotonated", "monoprotonated" and "diprotonated" forms of the molecule and K_{a1} and K_{a2} are the acid dissociation constants of the "mono" and "diprotonated" forms, respectively.

$$\delta_{obs} = (\delta_0 + \delta_1 \times 10^{(pK_{a1}-pH)})/(1 + 10^{(pK_{a1}-pH)}) \quad (4)$$

$$\delta_{obs} = (\delta_0 + \delta_1 \times 10^{(pK_{a1}-pH)} + \delta_2 \times 10^{(pK_{a1}+pK_{a2}-2pH)})/(1 + 10^{(pK_{a1}-pH)} + 10^{(pK_{a1}+pK_{a2}-2pH)}) \quad (5)$$

Obviously, the "first" and "second" protonations described by Eqs. (4) and (5) are not really the first and second processes determined by potentiometric titrations, but merely reflect one or two protonation processes, which are responsible for the shift of the signal of a given atom. In no case the fitting quality could be improved by inclusion of an additional term corresponding to a third protonation process. The pK_a values calculated for each signal are collected in Table 4 and the protonation induced changes in chemical shifts are shown in Table 5.

The pK_a values found from NMR titrations in D_2O do not coincide with those determined by potentiometric titrations in water due to the solvent isotope effect, shift of pD scale relative to uncorrected pH readings obtained with the glass electrode calibrated in aqueous buffers and much higher concentration of kanamycin A sulfate in the NMR experiment. The solvent isotope effect on association constants with sulfate should be negligible and shifts in pK_a values induced by sulfate complexation in principle can be estimated with Eq. (6) where K_{1i} and $K_{1(i-1)}$ are the binding constants of sulfate to forms of kanamycin A containing i and $i-1$ protons, respectively given in Table 2.

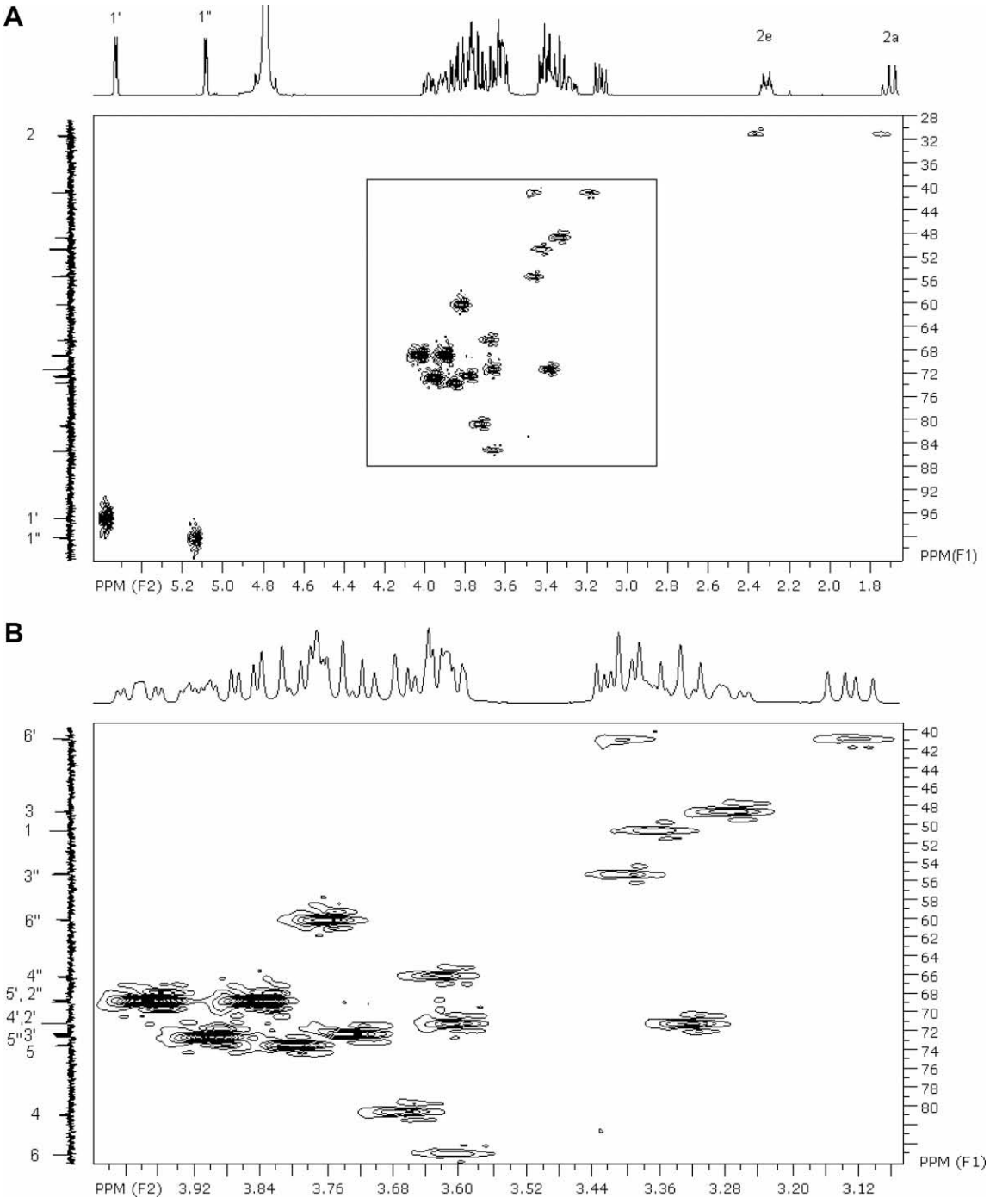

Fig. 4. (A) 298 K ^{1}H–^{13}C–HSQC NMR spectrum of 0.08 M kanamycin A sulfate in D_2O at pH 7.0; (B) zoom of the region shown by a rectangular in panel (A).

$$\Delta pK_{ai} = \log\left\{\left(1 + K_{1i}\left[SO_4^{2-}\right]\right)/\left(1 + K_{1(i-1)}\left[SO_4^{2-}\right]\right)\right\} \quad (6)$$

However, this equation involves the concentration of free rather total sulfate and cannot be applied directly since the concentration of free sulfate is variable during titration. Instead we simulated the titration profile of 0.08 M kanamycin sulfate employed in NMR experiments by using HYSS 2000 software and calculated the observed pK_a values given in the bottom line in Table 4 from this simulated curve. They are reasonably close to those found from NMR titrations in D_2O in accordance with typically observed approximate mutual cancellation of deuterium solvent isotope effect in pK_a values and the correction factor in pH scale [22].

Inspection of Table 4 leads to following conclusions. All atoms of the ring B feel two pK_a 6.61 and 8.54, which therefore can be attributed to amino groups at C3 and C1. The lower pK_a is observed also in titration curves for atoms C1′ and C2′ in the ring A (pK_a 6.51 coincides in limits of experimental errors with 6.61), which are the closest atoms to the ring B and may feel protonation of the amino group at C3. Therefore pK_a 6.61 can be attributed to NH_3^+ at C3, which is closer to ring A and may affect shifts in this ring too, and the second pK_a 8.54 belongs to NH_3^+ at C1. All atoms of the ring A feel pK_a 9.19, which therefore can be attributed to amino group at C6′. Finally all atoms of ring C feel pK_a 7.98, which can be attributed to the amino group at C3″.

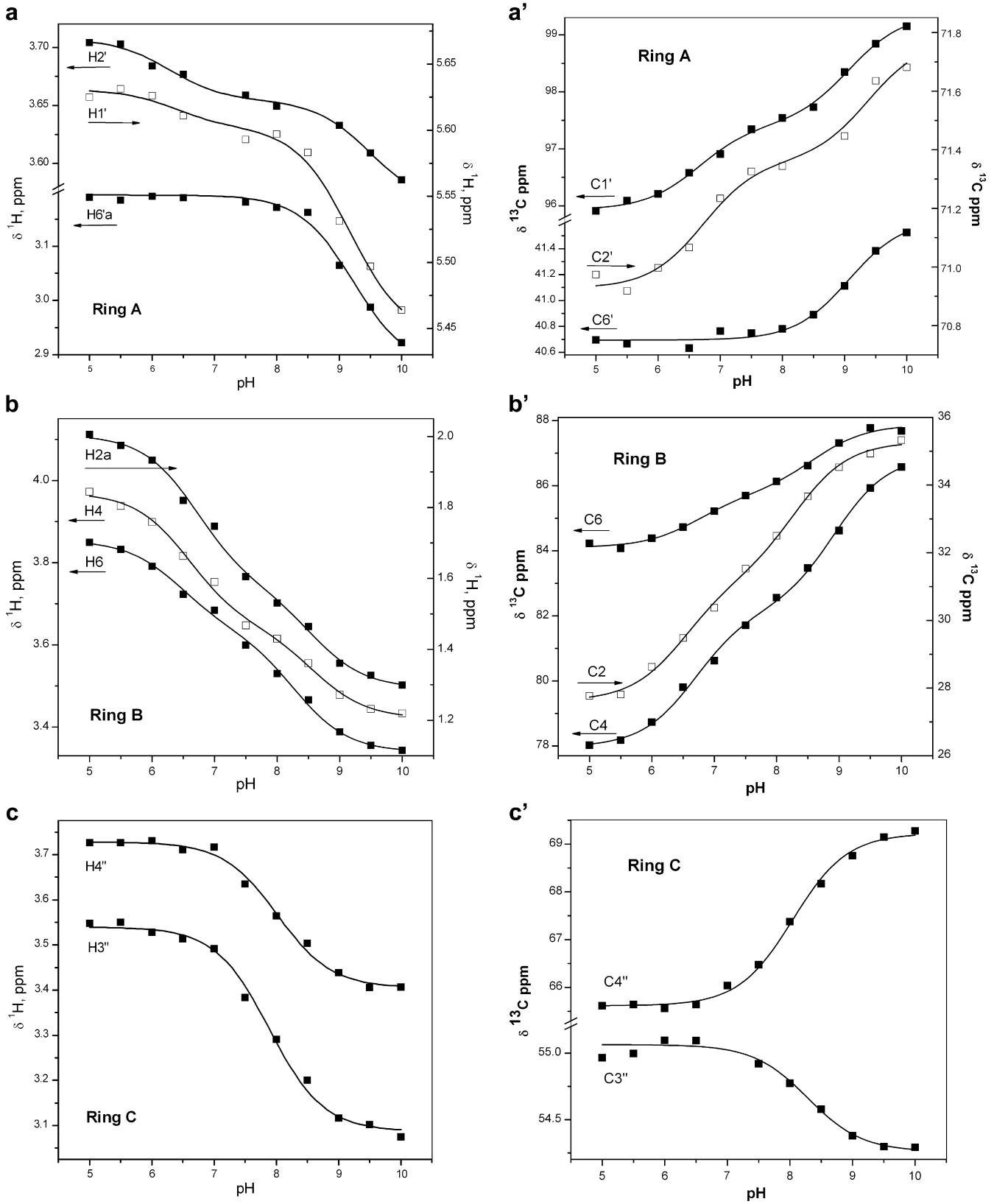

Fig. 5. Typical pH-titration plots monitored by chemical shifts of different carbon and hydrogen atoms of kanamycin A (uncorrected pH values measured with a glass electrode calibrated with aqueous buffers).

An additional analysis can be done by comparing protonation induced shifts for signals of different atoms, which are expected to be largest for atoms immediately bound to amino groups. From results collected in Table 5 one can see that the largest shift associated with pK_a 9.19 in the proton spectra indeed is observed for protons of C6′ methylene group, but in the carbon spectra the

Table 4
pK_a values obtained from pH-dependences of ^{1}H and ^{13}C NMR signals of 0.08 M kanamycin A sulfate in D_2O at 25 °C.

Ring A					Ring B					Ring C		
Atom	pK_{a1}		pK_{a2}		Atom	pK_{a1}		pK_{a2}		Atom	pK_{a1}	
	^{1}H	^{13}C	^{1}H	^{13}C		^{1}H	^{13}C	^{1}H	^{13}C		^{1}H	^{13}C
C1′	6.37	6.65	9.17	9.07	C1	6.76	6.57	8.57	9.8[a]	C1″	7.6[a]	6.9[a]
C2′	6.28	6.75	9.49	9.45	C2eq	6.71	6.59	8.56	8.37	C2″	7.75	7.87
C3′			–[b]	8.9[a]	C2ax	6.39	6.51	8.21	8.31	C3″	7.89	8.29
C4′			–[b]	–[c]	C3	6.56	6.63	8.41	8.82	C4″	8.02	8.05
C5′			8.6[a]	8.9[a]	C4	6.59	6.69	8.59	8.93	C5″	–[b]	8.04
C6′[a]			9.23	9.00	C5	6.72	ND	8.81	ND	C6″	–[b]	7.92
C6′[b]			9.12	9.07	C6	6.44	6.74	8.29	8.59			
Mean	6.51 ± 0.11		9.19 ± 0.06			6.61 ± 0.03		8.54 ± 0.07			7.98 ± 0.06	
	6.50[d]		9.16[d]			6.50[d]		8.35[d]			7.69[d]	

[a] Approximate value due to high dispersion of measurements.
[b] Too small change of the chemical shift for a reliable fitting.
[c] Signal was not resolved.
[d] Observed pK_a values from simulated titration profile of 0.08 M kanamycin A sulfate in water, see the text.

Table 5
Changes in ^{1}H and ^{13}C chemical shifts in NMR spectra of kanamycin A induced by deprotonation of ammonium groups calculated by fitting to Eqs. (4) and (5).

Atom	$\Delta\delta_1$ (^{1}H)	$\Delta\delta_1$ (^{13}C)	$\Delta\delta_2$ (^{1}H)	$\Delta\delta_2$ (^{13}C)
Ring A				
C1′	−0.024	1.489	−0.158	1.945
C2′	−0.053	0.431	−0.089	0.413
C3′			0	0.678
C4′			0	0
C5′			−0.287	3.748
C6′a			−0.318	0.977
C6′b			−0.361	0.932
Ring B				
C1	−0.299	0.619	−0.357	0.393
C2a	−0.421	3.723	−0.292	3.791
C2b	−0.269	1.801	−0.269	4.042
C3	−0.411	0.677	−0.249	0.588
C4	−0.323	4.181	−0.226	4.773
C5	−0.144	–	−0.084	–
C6	−0.22	1.665	−0.296	2.106
Ring C				
C1″	−0.061	−0.418		
C2″	−0.302	2.827		
C3″	−0.452	−0.804		
C4″	−0.323	3.608		
C5″	0	−0.551		
C6″	0	0.405		

largest shift is observed for C5′ and the signal of C6′ undergoes an intermediate shift. Similar situation is observed for signals associated with pK_a 6.6 and 8.54: largest shifts of proton signals are observed at C3 and C1, respectively, but signals of these carbons undergo actually the smallest shifts among other carbon atoms of ring B. Similarly the largest shift associated with pK_a 7.98 (ring C) is observed for the proton at C3″, but not for the same carbon atom. Thus the maximum magnitude of the protonation induced shift indicates the position of amino groups only for signals of protons, but just this criterion is not sufficient for a reliable assignment of protonation sites. For example, shifts induced in positions of signals of protons at C3 and C1 are close to each other and assignment of pK_a values for amino groups in the ring B would not be well justified.

4. Conclusions

The results of this study complete and clarify the protonation pattern of kanamycin A in the following aspects. The increase in ionic strength and sulfate concentration brings about increase in pK_a values due to stabilization of positively charged ammonium groups. The association constants of differently protonated forms of kanamycin A with sulfate increase from log K = 1.28 for monoprotonated to log K = 2.71 for tetraprotonated form. Protonation of all amino groups is exothermic, but the protonation enthalpy is relatively low and does not correlate with basicity of amino groups. A reliable NMR assignment of all protonation sites of kanamycin A has been obtained from the analysis of pH effect on chemical shifts of all carbon and proton atoms of the antibiotic, which shows that consecutive protonation steps involve amino groups at C6′ (ring A), C1 (ring B), C3″ (ring C) and C3 (ring B).

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Appendix A. Supplementary material

One-dimensional ^{1}H and ^{13}C spectra of kanamycin A recorded in the interval of pH from 5 to 10, Figs. S1 and S2, respectively. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2010.04.003.

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