Using Capillary Electrophoresis To Study the Electrostatic Interactions Involved in the Association of D-Ala-D-Ala with Vancomycin

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Abstract: This work examines the electrostatic interactions involved in the recognition of D-Ala-D-Ala (**DADA**) by vancomycin (**Van**) by using capillary electrophoresis (CE) and affinity capillary electrophoresis (ACE). Acetylation of the N-terminal amine of **Van** decreases its affinity for Di-Ac-L-Lys-D-Ala-D-Ala (**Ac₂KDADA**) by a factor of 11 at pH 7.1 (from 4.3 μM to 48 μM). Succinylation of the N-terminus of **Van** introduces a pendant negative charge that further decreases its affinity for **Ac₂KDADA** about 2-fold at pH 7.1. The association of Ac-D-Ala-D-Ala (**AcDADA**) with **Van** shifts the pK_a of the N-terminal amine of **Van** by 1.7 units from 7.1 to 8.8, and thus changes its net charge in the range of values of pH between 6 and 10. The electrostatic interaction between the $-CO_2^-$ group of the **DADA** moiety and the $-NH_2^+CH_3$ group of **Van** contributes approximately 5.9 kJ/mol to the free energy of binding of these species. In addition to establishing or confirming these thermodynamic parameters, this paper illustrates the use of CE as a physical-organic tool in examining electrostatic interactions in biomolecular recognition.
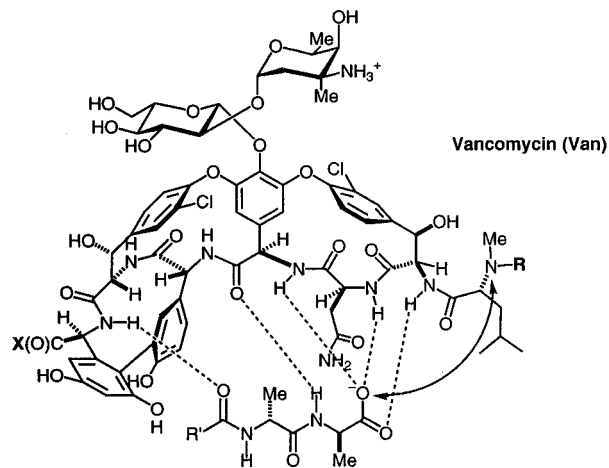
Introduction

This paper describes a study of the electrostatic interactions involved in the association of vancomycin (**Van**) and D-Ala-D-Ala-terminating peptide (**DADA**) by capillary electrophoresis (CE) and affinity capillary electrophoresis (ACE). **Van** and **DADA** comprise one of the best defined and most extensively studied[1–5] model systems for receptor–ligand interactions in aqueous solution. Electrostatic interactions—especially between the N-terminal amine on **Van** and the carboxylate of **DADA**—play an important role in the binding of **DADA** by **Van**.[6] CE is a powerful tool for separating molecules based on their net charge; ACE uses the resolving power of CE to measure binding constants, and can be used here to define the electrostatic interactions between **Van** and **DADA** by combining measurements of binding constants and charge.

Results and Discussion

We modified the C-terminus of **Van** to generate two derivatives having charges different from that of unmodified **Van**: AspNHCO**Van** and C₃H₇NHCO**Van** (Chart 1).[7] We estimated the binding constants of **Van** and these two derivatives to Ac-D-Ala-D-Ala (**AcDADA**) at pH 7.1 and 8.4 by ACE (Table 1), using procedures reported previously.[8] The C₃H₇NHCO**Van** has one more positive charge than **Van**, and the AspNHCO**Van**

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(7) We synthesized both compounds by coupling of the C-terminal carboxylate of **Van** with L-aspartic acid and 1-aminopropane mediated by HBTU. For details of this method, see reference: Sundram, U. N.; Griffin, J. H. *J. Org. Chem.* **1995**, *60*, 1102–1103.

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Chart 1. Model[a] of the Interaction between Vancomycin and Its Ligands[6]

R' = CH₃ :
N-Ac-D-Ala-D-Ala (**AcDADA**)

R' = N^{α,ε}-diacetyl-L-Lys :
N^{α,ε}-diacetyl-L-Lys-D-Ala-D-Ala (**Ac₂KDADA**)

	X	R
Van	O^-	H_2^+
VanN(CH₃)Ac	O^-	$C(O)CH_3$
C₃H₇NHCO**Van**	$CH_3(CH_2)_2NH$	H_2^+
VanN(CH₃)Suc	O^-	$C(O)CH_2CH_2CO_2^-$
AspNHCO**Van**	$^-{}_2OC-CH(NH)-CO_2^-$ (H)	H_2^+

[a] The dotted lines represent the intermolecular hydrogen bonds; the double-headed arrow indicates the electrostatic interaction between the N-terminal ammonium on **Van** and carboxylate on the ligand.

Table 1. Dissociation Constants (K_d) of **Van** and Its Derivatives to Ligands **Ac₂KDADA** and **AcDADA**

	K_d, μM (pH)	
receptor/ligand	this study[a]	literature
Van/Ac₂KDADA	2.3 (5.2)	0.7 (5.1),[b,c] 21 (5.1)[d]
	4.3 (7.1)	4 (7.1),[e] 1 (7.0)[f]
VanN(Me)Ac/Ac₂KDADA	42 (4.7)	13 (5.1)[b]
	42 (5.2)	
	48 (7.1)	
VanN(Me)Suc/Ac₂KDADA	64 (4.7)	
	76 (5.3)	
	105 (6.9)	
Van/AcDADA	99 (7.1)	115 (7.1),[e] 208 (7.5),[f]
	192 (8.4)	63 (7.0),[g] 91 (8.3)[h]
AspNHCO**Van/AcDADA**	95 (7.1)	
	167 (8.4)	
C₃H₇NHCO**Van/AcDADA**	76 (7.1)	
	107 (8.4)	

[a] ACE binding assay in this study. For pH 4.7, 5.2, and 5.3, 18 mM sodium acetate buffer was used; 20 mM sodium phosphate buffer was used for pH 6.9 and 7.1; and 25 mM Tris−192 mM Gly buffer was used for pH 8.4. [b] UV difference binding assay in 20 mM sodium citrate buffer.[6] [c] UV difference binding assay in 20 mM citrate.[3] [d] UV difference binding assay in 20 mM sodium citrate.[17] [e] ACE binding assay in 10 mM sodium phosphate buffer.[8] [f] ACE binding assay in 20 mM sodium phosphate buffer.[8] [g] Fluorescence binding assay in 100 mM phosphate buffer.[5] [h] CE binding assay in 50 mM Tris HCl.[18]

has one more negative charge. The C₃H₇NHCO**Van** bound **AcDADA** more tightly than did **Van** by a factor of 1.3 at pH 7.1 and 1.8 at pH 8.4 (Table 1). We conclude that changing the charge at the C-terminus of **Van** did not greatly affect its affinity for **AcDADA**. This result is consistent with those of Griffin *et al.*;[9] it is also physically reasonable, since X-ray crystallography[10] and ^{1}H-NMR spectroscopy[6,11] suggest that the C-terminus is approximately 12 Å away from the C-terminal carboxylate of the **DADA** group.

To study the electrostatic interactions between the N-terminal ammonium group and **DADA**, we acetylated the N-terminus of **Van**. In capillary electrophoresis, the mobility of an analyte correlates directly with its charge, Z, and inversely with its molecular weight, *M* (eq 1).[1] We assigned the acetylated

$$\mu = C_p(Z/M^\alpha) \qquad (1)$$

derivatives of **Van** to peaks in the electropherogram based on their different mobilities at pH 6.9. Vancomycin has two amino groups: an N-terminal secondary amine (p$K_a \sim 7.2^3$) and a sugar amino group (p$K_a \sim 8.6^3$). The acetylation of **Van** at pH 8.9 resulted in three derivatives that were resolved by CE at pH 6.9 (Figure 1). The difference in mobilities of **Van** and one of its acetylated derivatives is directly related to the amount of charge neutralized upon its modification; at pH 6.9, acetylation of the N-terminal (−NHCH₃) and sugar amino (−NH₂) groups (Chart 1) will neutralize approximately 0.5 and 1 unit of charge respectively. We, therefore, assigned peak 1 (Figure 1A) as the derivative of **Van** in which the N-terminal amino group has been acetylated, peak 2 as the derivative with the sugar amino group modified, and peak 3 as the derivative that has both amino groups acetylated. The mobilities of these peaks at different values of pH changed in a manner that was consistent with our assignment (Figure 1B).

By allowing **Van** to react with acetic anhydride at pH 6.8, we obtained a single acetylated derivative, **Van**N(CH₃)Ac, for

the binding study. The binding of **Van** and **Van**N(CH₃)Ac to Di-Ac-L-Lys-D-Ala-D-Ala (**Ac₂KDADA**) was carried out by ACE in both pH 5.2 acetate buffer and pH 7.1 phosphate buffer; these studies indicated that the affinity of **Van**N(CH₃)Ac for **Ac₂KDADA** was a factor of 11 less than that of **Van**NH₂⁺CH₃ (Table 1). Our estimated values of K_d were approximately the same as the literature values measured by ACE, but approximately 3−4-fold larger than the literature values estimated by other methods. This change in the value of K_d is equivalent to a loss of 5.9 kJ/mol in the free energy of binding of **Ac₂KDADA**, a value that is comparable with the results from other studies.[6] We propose that this decrease in affinity is due to the loss of positive charge at the N-terminal amine following its acetylation.[12] The close proximity between the N-terminal amine of **Van** and the carboxylate of **Ac₂KDADA** (approximately 5 Å) suggests a structural basis for a strong electrostatic interaction between these two residues.[13]

To confirm the importance of electrostatic interactions in the binding of **DADA** to **Van**, we determined the K_d of a derivative, **Van**N(CH₃)Suc, having a pendant negative charge in close proximity to its N-terminus (Chart 1) and thus to the C-terminus of the bound **Ac₂KDADA**. An ACE binding study of **Van**N-(CH₃)Suc indicated that its affinity for **Ac₂KDADA** was approximately one-half of that of **Van**N(CH₃)Ac at pH 7.1 (Table 1). To demonstrate that the decrease of the affinity of **Van**N-(CH₃)Suc for **Ac₂KDADA** was due to an electrostatic contribution and not an unfavorable steric effect from the longer succinate group, we carried out ACE experiments at different values of pH. When we decreased the pH of the ACE experiments, the affinity of **Van**N(CH₃)Suc for **Ac₂KDADA** increased (Table 1); at pH 4.7, it approached the affinity of **Van**N(CH₃)Ac. These observations suggest that the difference in the affinities of **Van**N(CH₃)Suc and **Van**N(CH₃)Ac for **Ac₂KDADA** is related to an unfavorable electrostatic interaction involving **Van**N(CH₃)Suc, and that any steric effect is probably small.

The effect of this electrostatic interaction prompted us to investigate the net charge of **Van**; we found that the net charge on **Van** changed on binding **Ac₂KDADA**. We determined the charge on **Van** experimentally using a technique based on charge ladders.[2] The method is based on the relationship between the electrophoretic mobilities of peaks in a charge ladder, μ_n, and their charges relative to unmodified **Van** ($\Delta Z_n = Z_n - Z_o$), where Z_o is the net charge on **Van** and Z_n is the net charge of derivatives of **Van** (eq 2).

$$\mu_n - \left(\frac{C_p}{M^\alpha}\right)Z_o = \left(\frac{C_p}{M^\alpha}\right)\Delta Z_n \qquad (2)$$

AspNHCO**Van**, **Van**, and C₃H₇NHCO**Van** constituted a series of three compounds (Figure 2A), each of which differs from the next by one unit of charge as a result of the different number of CO₂⁻ groups they contain. Since the carboxylate groups that are responsible for these differences are far from the carboxylate terminus of the **AcDADA** ligand, we expected this difference in charge to be preserved over the range of pH studied here. We treated *M* in eq 2 as a constant; the maximal change of *M* is not more than 8% in this charge ladder. A plot of μ_n vs ΔZ_n

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(10) Loll, P. J. B.; A. E.; Korty, B. D.; Axelsen, P. H. *J. Am. Chem. Soc.* **1997**, *109*, 1516−1522.
(11) Harris, C. M.; Harris, T. M. *J. Am. Chem. Soc.* **1982**, *104*, 4293−4295.

(12) Williams et al. postulated that there might be other interactions involved on binding (for example, a conformational change of the side chain of *N*-methyl leucine). Other studies, however, have shown that the side chain of *N*-methyl leucine maintains the same conformation on binding. (See: Molinari, H.; Pastore, A.; Lian, L.; Hawkes, G. E.; Sales, K. *Biochemistry* **1990**, *29*, 2271−2277.)
(13) At this distance, electrostatic interactions between the positive charge on the secondary ammonium group and the negative charge on the carboxylate ion would be expected to be large (for a distance of 0.5 nm and a dielectric constant of $\epsilon \approx 50$, the enthalpy of this interaction would be 5.4 kJ/mol).
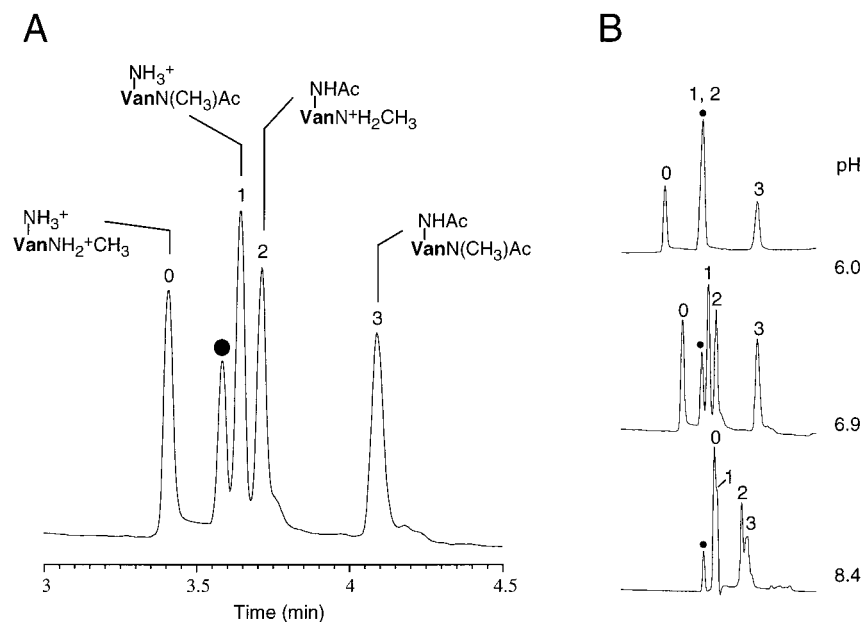
Figure 1. (A) Electropherogram of the charge ladder of **Van**, obtained by reaction with *N*-hydroxysuccinimidyl acetate at pH 8.9. PMBA (*p*-methoxybenzyl alcohol, ●) was used as the neutral marker. The running buffer was 20 mM phosphate (pH 6.9). The $-NH_3^+$ is on the disaccharide moiety; the $-NH_2^+CH_3$ is the N-terminating group. The state of charge shown for the unacylated groups corresponds to the major species that is present; at this pH, significant concentrations of protonated species are present for both amino groups. (B) The charge ladder of **Van** at different values of pH.
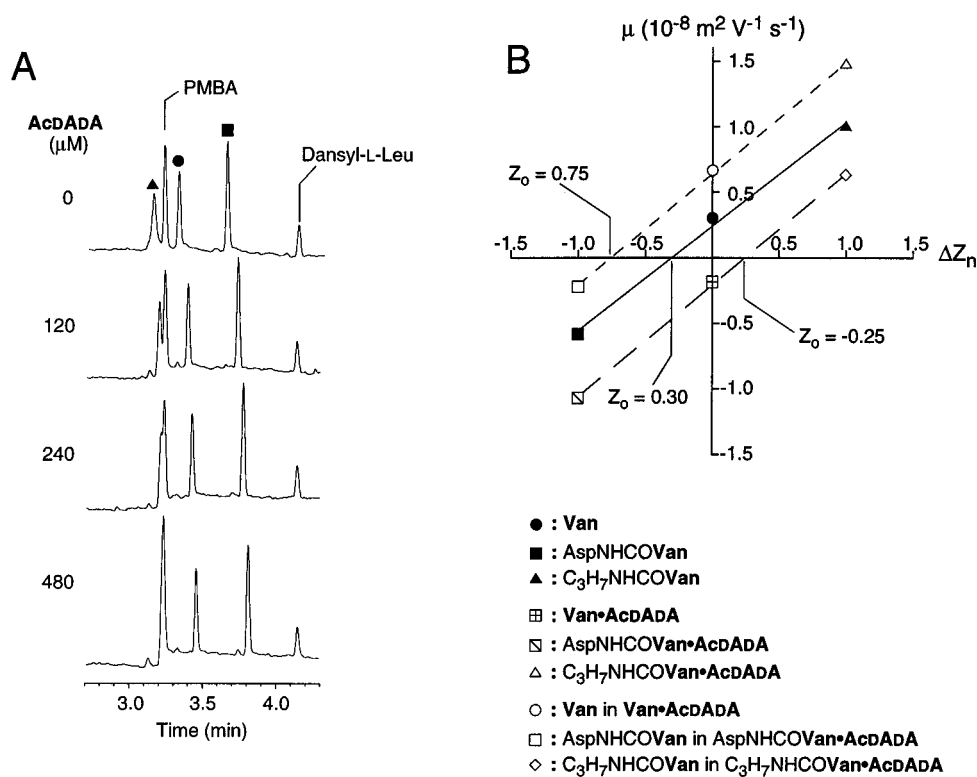

Figure 2. (A) Electropherograms of AspNHCO**Van** (■), **Van** (●), and C_3H_7NHCO**Van** (▲) at different concentrations of **AcDADA** in 25 mM Tris−192 mM Gly, pH 8.4, running buffer. Dansyl-L-Leu was used as a secondary marker in this assay. (B) Estimation of the values of Z_0 of **Van**, **Van·AcDADA**, and **Van** in the **Van·AcDADA** complex at pH 7.1. The values of mobility of free AspNHCO**Van**, **Van**, and C_3H_7NHCO**Van**, as well as their respective complexes with **AcDADA** at pH 7.1, were plotted as a function of ΔZ_n and the data were fit by the method of linear least squares. Extrapolation to the *x*-intercept of these fit lines gives values of Z_0 of 0.30 for free **Van** and of −0.25 for the **Van·AcDADA** complex. A similar analysis of the values of mobility of AspNHCO**Van**, **Van**, and C_3H_7NHCO**Van** in their respective complexes yields the value of $Z_0 = 0..75$ for **Van** in the **Van·AcDADA** complex at pH 7.1.

is linear, has a slope of C_p/M^α, and has an *x*-intercept equal to $-Z_0$.[2] We estimated the value of Z_0 of free **Van** to be +0.30 at pH 7.1 (where $\mu_n = 0$) (Figure 2B). A corresponding examination of the mobilities of AspNHCO**Van**, **Van**, and C_3H_7-NHCO**Van** complexed with **AcDADA** ([**AcDADA**] = 0.82 mM)

at pH 7.1 indicated that the Z_0 of the complex **Van·AcDADA** was −0.25 (Figure 2B).

The mobility of a receptor−ligand complex, **R·L**, can be expressed as the product of (C_p/M^α) of the complex and the sum of the constituent charges of the receptor **R**, Z_R, and of

complexed ligand **L**, Z_L (eq 3). If the charge of the ligand in the complex is equal to -1, as for **AcDADA** at pH 7.1, then we can estimate the mobility of the receptor in the complex, μ_R^*, by adding the value of (C_p/M^α) to $\mu_{R \cdot L}$ (eq 4). The values of

$$\mu_{R \cdot L} \cong \left(\frac{C_p}{M^\alpha}\right) Z_{R \cdot L} \cong \left(\frac{C_p}{M^\alpha}\right)(Z_R + Z_L) \qquad (3)$$

$$\cong \left(\frac{C_p}{M^\alpha}\right)(Z_R - 1) \cong \mu_R^* - \left(\frac{C_p}{M^\alpha}\right) \qquad (4)$$

mobility of AspNHCO**Van**, **Van**, and C_3H_7NHCO**Van** in their respective complexes with **AcDADA** were estimated in this manner, and analyzed to yield the value of Z_o of **Van** in the **Van·AcDADA** complex of 0.75, at pH 7.1. There was thus an increase in the value of Z_o of **Van** of $+0.45$ when it bound **AcDADA** at pH 7.1. A similar analysis of the association of the three compounds with **AcDADA** at pH 8.4 indicated an increase in Z_o of $+0.62$ on binding.

These results establish that the values of pK_a of functional groups on **Van** changed on complexation with **AcDADA**. The results from acetylation of the $-$NHCH$_3$ group, as well as the proximity of this group to the $-$CO$_2^-$ group of **AcDADA** in the complex, suggested that the value of pK_a of this N-terminal ammonium group would undergo the largest change on complexation with **AcDADA**. Further pH titration of the complex of **Van** with **Ac₂KDADA** supported this hypothesis.

For a systematic comparison, we first titrated **Van** and **Van**N-(CH$_3$)Ac by CE to determine the pK_a of the N-terminal amine: this titration consisted of measuring the electrophoretic mobility of these derivatives as a function of pH. In analyzing these mobilities, we assumed that the values of pK_a of the unmodified functional groups on **Van** were not affected by the acetylation of the N-terminal amino group. With this assumption, the difference between the curves of mobility vs pH for **Van** and **Van**N(CH$_3$)Ac reflected the contribution of the $-$NHCH$_3$/$-$NH$_2^+$CH$_3$ group to the mobility of **Van** (Figure 3). The change in electrophoretic mobility due to protonation of a basic residue, $\Delta\mu$, is related to its pK_a and the pH of the running buffer, described by eq 5,[14] where μ_{BH^+} and μ_B are the mobilities of

$$\Delta\mu = (\mu_{BH^+} - \mu_B)\frac{1}{1 + 10^{pH-pK_a}} \qquad (5)$$

the completely protonated and completely ionized forms of the residue, respectively. The resulting data were fit to eq 5 by using the method of nonlinear least squares; this analysis indicated a value of $pK_a = 7.1$ for the N-terminal $-$NH$_2$CH$_3^+$ group. This experimental value agrees well with the literature value of 7.2, determined by spectroscopic titration.[3]

We performed a similar pH titration of **Van** from pH 5.7 to 8.5 in the presence of 2.7 mM **Ac₂KDADA** in the running buffer. The value of K_d of **Van** for **Ac₂KDADA** at pH 7.1 is 4.3 μM, and **Van** was therefore present as a 1:1 complex with **Ac₂KDADA** under these conditions. The complex of **Van** with **Ac₂KDADA** was stable between pH 3 and 8.5;[3] our pH titration of the complex was limited to values of pH ≤ 8.5. The titration yielded the mobilities of the complex **Van•Ac₂KDADA**, $\mu_{Van \cdot Ac_2KDADA}$, as a function of pH. In our analysis, we assume that the value of C_p/M^α of the complex is approximately the same as that for
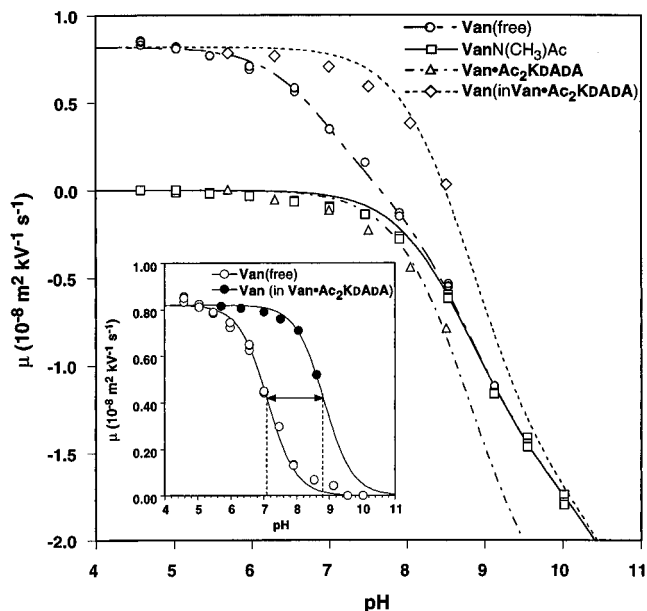

Figure 3. Electrophoretic mobilities of **Van**, **Van**N(CH$_3$)Ac, and the complex **Van·Ac₂KDADA** as a function of the pH of the buffer. The four lines are the nonlinear least-squares fit of the data to eq 5; the pK_a values of the functional groups on **Van** used in the fits are from the literature values (for the carboxylate, 2.9; for the sugar amino group, 8.6; for three phenolic groups, 9.6, 10.5, and 11.7),[3] except the value of the pK_a of the N-terminal $-$NH$_2^+$CH$_3$ group that was determined from the plot shown in the inset. This inset compares plots of the titration curves of the N-terminal $-$NH$_2^+$CH$_3$ group when **Van** is free and in the complex with **Ac₂KDADA**. The least-squares fit of both curves to eq 5 gives values of $pK_a = 7.1$ for this group when **Van** is free and of 8.8 when it is in the complex with **Ac₂KDADA**.

free **Van**.[15] At pH 4.6 the Z_o of **Van** is $+1$, therefore, C_p/M^α equals the mobility of **Van** (eq 1) and has a value of 8.2×10^{-9} m^2 V^{-1} s^{-1}. The mobilities of **Van** in the complex, μ_{Van}^*, were estimated according to eq 4 by adding the value of C_p/M^α to the mobilities of **Van·Ac₂KDADA**. Finally, the titration curve of the N-terminal amine on **Van** in **Van·Ac₂KDADA** was obtained by subtracting the mobilities of **Van**N(CH$_3$)Ac from the values of μ_{Van}^*. Here, the pK_a of the N-terminal ammonium group was 8.8—a surprisingly large shift of 1.7 units (Figure 3).

We propose that complexation of **Van** with **Ac₂KDADA** brings the carboxylate anion of the ligand into close proximity to the N-terminal amino group of **Van**. The carboxylate anion stabilizes the protonated ammonium ion ($-$NH$_2^+$CH$_3$) through an electrostatic interaction and thus shifts its pK_a to a higher value. Brown *et al.* have similarly reported that at values of pH above the pK_a of the N-terminal $-$NH$_2^+$CH$_3$ group, the binding of **DADA**$-$peptide with **Van** was accompanied by proton uptake.[16]

Conclusions

The combination of ACE/CE and charge ladders provides an extraordinary capability to investigate electrostatic effects

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(15) The calculated values of C_p/M^α, from the plots of mobilities vs ΔZ_n for **Van** and its C-modified derivatives (Figure 2), in the absence and presence of **AcDADA** were 7.9 $\times 10^{-9}$ and 8.5 $\times 10^{-9}$ m^2 V^{-1} s^{-1}, respectively. These results indicate that the value of C_p/M^α does not change appreciably (7%) whether **Van** is in the free or bound state.

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(17) Bugg, T. D. H.; Wright, G. D.; Dutka-Malen, S.; Arthur, M.; Courvalin, P.; Walsh, C. T. *Biochemistry* **1991**, *30*, 10408$-$10415.

(18) Carpenter, J. L.; Camilleri, P.; Dhanak, D.; Goodall, D. *J. Chem. Soc., Chem. Commun.* **1992**, 804$-$806.

in physiologically relevant media; electrostatic charges and binding constants can be measured independently as a function of pH. The electrostatic interaction between the $-NH_2^+CH_3$ group of **Van** and the $-CO_2^-$ group of **dADA** plays an important role in the recognition of **dADA** by **Van**: it contributes ~5.9 kJ/mol to the free energy of binding, changes the value of pK_a of the N-terminal amino group of **Van**, and thus influences the net charge on **Van**. The shift in the value of pK_a of the N-terminal amino group may have an important biological consequence; it extends the effective range within which **Van** binds **dADA** and may thereby enhance its bactericidal activity.

Experimental Section

General Procedure. Vancomycin hydrochloride and the peptide ligands **AcdADA** and **Ac₂KdADA** were purchased from Sigma. Reverse-phase HPLC was carried out with a Waters Model 600E chromatography system with use of Vydac C18 columns. A 4.6 mm i.d. column was used for analytical separations and a 21.4 mm i.d. column was used for preparative purifications. Linear gradients of 0.1% trifluoroacetic acid (TFA) in acetonitrile and 0.1% TFA in water were used in HPLC elutions. CE and ACE studies were performed on either an ISCO Model 3140 or a Beckman Model P/ACE 5500 capillary electrophoresis system. The ^{1}H-NMR spectra were recorded at 400 MHz on a Bruker spectrometer. Chemical shifts are reported in parts per million downfield of tetramethylsilane.

Synthesis of C₃H₇NHCOVan. The C₃H₇NHCO**Van** was prepared according to a literature procedure[7] by coupling of the C-terminal carboxylate of **Van** with 1-aminopropane mediated by 2-(1-hydroxy-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The ^{1}H-NMR spectrum of C₃H₇NHCO**Van** showed the inclusion of the aminopropane in C₃H₇NHCO**Van** and correlated well with that reported in the literature.[7] The FAB-MS showed an M + Na$^+$ ion at m/z 1511 (calcd for $C_{69}H_{82}N_{10}O_{23}Cl_2Na^+$, m/z 1511.5).

Synthesis of AspNHCOVan. The AspNHCO**Van** was synthesized by a modified literature procedure. The solution of vancomycin hydrochloride (50 mg, 34 μmol) in 0.5 mL of dry dimethyl sulfoxide (DMSO) and 0.5 mL of dry dimethylformamide (DMF) was cooled down to 0 °C, and 13 mg (1 equiv) of HBTU was added. The solution was allowed to warm to room temperature and stirred for 3.5 h, then a suspension of L-aspartic acid (10 mg, 2 equiv) and 24 mg of diisopropylethylamine (DIEA) in 0.4 mL of dry DMSO was added. After 3.5 h, CE showed only a small peak of vancomycin and a product with a higher negative charge. Analytical reverse-phase HPLC showed a more polar product. Fifteen milligrams of the 50 mg crude product was purified by preparative reverse-phase HPLC and lyophilized to afford 6 mg (38%) of AspNHCO**Van**. The ^{1}H-NMR (400 MHz)

spectroscopy (in DMSO-d_6) showed all the resonances attributable to vancomycin and new resonances of aspartic acid (δ 8.15 (b, $-NH-$) 3.92 (b, $-CH-$) 2.76 (m, $-CH_2-$)). The ESI-MS exhibited an M + H$^+$ ion at m/z 1564.7 (calcd for $C_{70}H_{83}N_{10}O_{27}Cl_2$, m/z 1564.5).

CE and ACE Studies. The uncoated fused silica capillaries with an internal diameter of 50 μm were purchased from Polymicro Technologies (Phoenix, AZ). For CE experiments conducted on a Beckman P/ACE 5500, reaction products were typically analyzed on an uncoated capillary of fused silica with a total length (L_{tot}) of 47 cm and a length from the inlet to the detector (L_{det}) of 40 cm, using 20 mM sodium phosphate buffer, pH 7.0, at 15 kV, 25 °C; for CE experiments performed on the ISCO Model 3140 system, samples were typically analyzed on an uncoated capillary of fused silica ($L_{tot} = 74$ cm, $L_{det} = 40$ cm), using 20 mM phosphate buffer, pH 7.0 at 30 kV, 28 ± 2 °C. The samples were detected at 214 nm. A neutral marker—p-methoxybenzyl alcohol (PMBA)—was used to indicate the electroosmotic flow (typically 80 μM). Electrophoresis running buffers having different values of pH were prepared from three different stock buffers [20 mM sodium acetate, pH 4.6 (for running buffers of pH 4.6, 5.0, and 5.5); 20 mM sodium phosphate, pH 7.0 (for running buffers of pH 5.7, 6.0, 6.3, 6.5, 7.0, 7.5, and 7.9) and 20 mM sodium borate, pH 9.1 (for running buffers of pH 8.5, 9.1, 9.6, and 10)] by adjusting the pH of each of the stock buffers with the corresponding conjugate acid or 1 N NaOH, as necessary.

Generation of VanN(CH₃)Ac, VanN(CH₃)Suc, and the Charge Ladder of Van. To a solution of **Van** (100 μL, 3 mg/mL) in phosphate buffer (pH 6.8, 100 mM) was added acetic anhydride (80 μL, 100 mM). The reaction mixture was mixed by vortexing, diluted with 20 mM phosphate buffer, and analyzed by CE. The characterization of **Van**N-(CH₃)Ac was described in the text. The ESI-MS exhibited an M + H$^+$ ion at m/z 1491.9 (calcd for $C_{68}H_{79}N_9O_{25}Cl_2$, m/z 1493). The same procedure was used to generate **Van**N(CH₃)Suc. The ESI-MS exhibited an M + H$^+$ ion at m/z 1549.9 (calcd for $C_{70}H_{81}N_9O_{27}Cl_2$, m/z 1551). The charge ladder of **Van** was generated by allowing **Van** (100 μL, 3 mg/mL) in borate buffer (pH 8.9, 80 mM) to react with N-hydroxy-succinimidyl acetate (20 μL, 100 mM).