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 (AcADA) with Van shifts the pK_s 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 −CO2 group of the dADA moiety and the −NH_2 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 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. 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: AspNHCOVan and C3H7NHCOVan (Chart 1). We estimated the binding constants for Van and these two derivatives to Ac-D-Ala-D-Ala (AcADA) at pH 7.1 and 8.4 by ACE (Table 1), using procedures reported previously. The C3H7NHCOVan has one positive charge greater than Van, and the AspNHCOVan

\[\text{AspNHCOVan} \quad O^- \quad \text{H}_2^+ \]

\[\text{VanN(CH3)Ac} \quad O^- \quad \text{C(O)CH}_3 \]

\[\text{C3H7NHCOVan} \quad \text{CH}_2(\text{CH}_3)\text{NH} \quad \text{H}_2^+ \]

\[\text{VanN(CH3)Suc} \quad O^- \quad \text{C(O)CH}_3\text{CH}_2\text{CO}_2^- \]

\[\text{AspNHCOVan} \quad \text{CO}_2^- \]

\[\text{X} \quad \text{R} \]

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.

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Table 1. dissociation Constants (Kd) of Van and its Derivatives to Ligands Ac-DADAD and AdoAdA

<table>
<thead>
<tr>
<th>receptor/ligand</th>
<th>this study</th>
<th>literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van/Ac-DADAD</td>
<td>2.3 (5.2)</td>
<td>0.7 (5.1)</td>
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<tr>
<td></td>
<td>4.3 (7.1)</td>
<td>4 (7.1)</td>
</tr>
<tr>
<td>Van(N-Me)Ac/Ac-DADAD</td>
<td>42 (4.7)</td>
<td>13 (5.1)</td>
</tr>
<tr>
<td></td>
<td>42 (5.2)</td>
<td>42 (5.2)</td>
</tr>
<tr>
<td>Van(N-Me)Suc/Ac-DADAD</td>
<td>64 (4.7)</td>
<td>76 (5.3)</td>
</tr>
<tr>
<td></td>
<td>105 (6.9)</td>
<td>105 (6.9)</td>
</tr>
<tr>
<td>Van/AdoAdA</td>
<td>99 (7.1)</td>
<td>115 (7.1)</td>
</tr>
<tr>
<td></td>
<td>192 (8.4)</td>
<td>63 (7.0)</td>
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<td></td>
<td>167 (8.4)</td>
<td>91 (8.3)</td>
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<tr>
<td></td>
<td>76 (7.1)</td>
<td>76 (7.1)</td>
</tr>
<tr>
<td></td>
<td>107 (8.4)</td>
<td>107 (8.4)</td>
</tr>
</tbody>
</table>

*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·HCl buffer was used for pH 8.4. *b* UV difference binding assay in 20 mM sodium citrate buffer. *c* UV difference binding assay in 20 mM sodium citrate. *d* UV difference binding assay in 20 mM sodium citrate. *e* ACE binding assay in 10 mM sodium phosphate buffer. *f* ACE binding assay in 20 mM sodium phosphate buffer. *g* Fluorescence binding assay in 100 mM phosphate buffer. *h* CE binding assay in 50 mM Tris HCl.

has one more negative charge. The C8H13NHCOAc bound Ac-DADAD 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-termis of Van did not greatly affect its affinity for Ac-DADAD. This result is consistent with those of Griffin et al. it is also physically reasonable, since X-ray crystallography and 1H-NMR spectroscopy suggest that the C-termis is approximately 12 Å away from the C-terminal carboxylate of the AdoAdA group.

To study the electrostatic interactions between the N-terminal ammonium group and AdoAdA, we acetylated the N-termis 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). We assigned the acetylated 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 (pKa ~ 7.2) and a sugar amino group (pKb ~ 8.6). 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(NCH3)Ac, for the binding study. The binding of Van and Van(NCH3)Ac to Di-Asp-L-Lys-d-Ala-d-Ala (Ac-DKADAD) 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(NCH3)Ac for Ac-DKADAD was a factor of 11 less than that of Van(NH2)CH3 (Table 1). Our estimated values of Kd 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 Kd is equivalent to a loss of 5.9 kJ/mol in the free energy of binding of Ac-DKADAD, a value that is comparable with the results from other studies. We propose that this decrease in affinity is due to the loss of positive charge at the N-terminal amine following its acetylation. The close proximity between the N-terminal amine of Van and the carboxylate of Ac-DKADAD (approximately 5 Å) suggests a structural basis for a strong electrostatic interaction between these two residues.

To confirm the importance of electrostatic interactions in the binding of AdoAdA to Van, we determined the Kd of a derivative, Van(NH2)Suc, having a pendant negative charge in close proximity to its N-terminus (Chart 1) and thus to the C-terminus of the bound Ac-DKADAD. An ACE binding study of Van(NH2)Suc indicated that its affinity for Ac-DKADAD was approximately one-half of that of Van(NH2)Ac at pH 7.1 (Table 1). To demonstrate that the decrease of the affinity of Van(NH2)Suc for Ac-DKADAD 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(NH2)Suc for Ac-DKADAD increased (Table 1); at pH 4.7, it approached the affinity of Van(NH2)Ac. These observations suggest that the difference in the affinities of Van(NH2)Suc and Van(NH2)Ac for Ac-DKADAD is related to an unfavorable electrostatic interaction involving Van(NH2)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 Ac-DKADAD. We determined the charge on Van experimentally using a technique based on charge ladders. The method is based on the relationship between the electrophoretic mobilities of peaks in a charge ladder, μn, and their charges relative to unmodified Van (ΔZn = Zn - Z0), where Z0 is the net charge on Van and Zn is the net charge of derivatives of Van (eq 2).

\[ \mu_n = \frac{C_p}{M^p} Z_n = \frac{C_p}{M^p} (13) \]

AstHPHNCOVan, Van, and C8H13NHCOVan 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 AdoAdA 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 μn vs ΔZn (12) Williams et al. postulated that there might be other interactions involved in 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 ε = 50, the enthalpy of this interaction would be 5.4 kJ/mol).
is linear, has a slope of $C_p/M_R$, and has an $x$-intercept equal to $-Z_o$. We estimated the value of $Z_o$ of free Van to be +0.30 at pH 7.1 (where $\mu_n = 0$) (Figure 2B). A corresponding examination of the mobilities of AspNHCOVan, Van, and C$_3$H$_7$-NHCOVan complexed with AcDAdA ($[\text{AcDAdA}] = 0.82$ mM) at pH 7.1 indicated that the $Z_o$ 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 ($C_p/M_R$) 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 AcDA DA at pH 7.1, then we can estimate the mobility of the receptor in the complex, $\mu_R^*$, by adding the value of $(C/\mu^0)$ to $\mu_{R-L}$ (eq 4). The values of

$$
\mu_{R-L} \approx \left( \frac{C}{\mu^0} \right) Z_{R-L} \approx \left( \frac{C}{\mu^0} \right) (Z_R + Z_L)
$$

(3)

$\Delta\mu$ mobility of AspNHCOVan, Van, and C_2H_5NHCOVan in their respective complexes with AcDA DA were estimated in this manner, and analyzed to yield the value of $Z_o$ of Van in the Van·AcDA DA 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 AcDA DA at pH 7.1. A similar analysis of the association of the three compounds with AcDA DA 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 AcDA DA. The results from acetylation of the NHCH_3 group, as well as the proximity of this group to the CO_2 group of AcDA DA in the complex, suggested that the value of $pK_a$ of this N-terminal ammonium group would undergo the largest change on complexation with AcDA DA. Further pH titration of the complex of Van with AcKoDA DA supported this hypothesis.

For a systematic comparison, we first titrated Van and VanN(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 VanN(CH_3)Ac reflected the contribution of the NH_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, where $\mu_{BH^+}$ and $\mu_B$ are the mobilities of 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_2CH_3 group. This experimental value agrees well with the literature value of 7.2, determined by spectrophotometric titration.

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

(5)

We performed a similar pH titration of Van from pH 5.7 to 8.5 in the presence of 2.7 mM AcKoDA DA in the running buffer. The value of $K_a$ of Van for AcKoDA DA at pH 7.1 is 4.3 $\mu$M, and Van was therefore present as a 1:1 complex with AcKoDA DA under these conditions. The complex of Van with AcKoDA DA was stable between pH 3 and 8.5, as our pH titration of the complex was limited to values of pH ≤8.5. The titration yielded the mobilities of the complex Van·AcKoDA DA, $\mu_{Van·AcKoDA DA}$, as a function of pH. In our analysis, we assume that the value of $C/\mu^0$ of the complex is approximately the same as that for the free Van. At pH 4.6 the $Z_o$ of Van is +1, therefore, $C/\mu^0$ equals the mobility of Van (eq 1) and has a value of $8.2 \times 10^{-9}$ m^2 V^{-1} s^{-1}. The mobilities of Van in the complex, $\mu_{Van}$, were estimated according to eq 4 by adding the value of $C/\mu^0$ to the mobilities of Van·AcKoDA DA. Finally, the titration curve of the N-terminal amine on Van in Van·AcKoDA DA was obtained by subtracting the mobilities of VanN(CH_3)Ac from the values of $\mu_{Van}$. 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 AcKoDA DA 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_2CH_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_2CH_3 group, the binding of DADA—peptide with Van was accompanied by proton uptake.

Conclusions

The combination of ACE/CE and charge ladders provides an extraordinary capability to investigate electrostatic effects.
in physiologically relevant media; electrostatic charges and binding constants can be measured independently as a function of pH. The electrostatic interaction between the \(-\text{NH}_2\)CH\(_2\) group of \(\text{Van}\) and the \(-\text{CO}_2^-\) group of \(\text{DADA}\) plays an important role in the recognition of \(\text{DADA}\) by \(\text{Van}\): 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 \(\text{Van}\), and thus influences the net charge on \(\text{Van}\). 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 \(\text{Van}\) binds \(\text{DADA}\) and may thereby enhance its bactericidal activity.

**Experimental Section**

**General Procedure.** Vancomycin hydrochloride and the peptide ligands \(\text{AcDADA}\) and \(\text{AcKdADA}\) were purchased from Sigma. Reverse-phase HPLC was carried out with a Waters Model 600E chromatography system with use of Vydes 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 \(\text{C}_3\text{H}_7\text{NHCOVan}\).** The \(\text{C}_3\text{H}_7\text{NHCOVan}\) was prepared according to a literature procedure\(^7\) by coupling of the C-terminal carboxylate of \(\text{Van}\) with 1-aminopropane mediated by 2-(1-hydroxybenzotriazol-1-yi)-1,1,3,3-tetramethyleironium hexafluorophosphate (HBTU). The \(^1\)H-NMR spectrum of \(\text{C}_3\text{H}_7\text{NHCOVan}\) showed the inclusion of the aminopropane in \(\text{C}_3\text{H}_7\text{NHCOVan}\) and correlated well with that reported in the literature.\(^7\) The FAB-MS showed an \(\text{M}^+\) ion at \(m/z\) 1511 (calcd for \(\text{C}_{69}\text{H}_{82}\text{N}_{10}\text{O}_{23}\text{Cl}_2\text{Na}\)).

**Synthesis of \(\text{AspNHCOVan}\).** The \(\text{AspNHCOVan}\) was synthesized by a modified literature procedure. The solution of vancomycin hydrochloride (50 mg, 34 \(\mu\)mol) 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 \(\text{AspNHCOVan}\). The \(^1\)H-NMR (400 MHz) spectroscopy (in DMSO-\(d_6\)) showed all the resonances attributable to vancomycin and new resonances of aspartic acid (\(\delta 8.15 (b, \text{-NH}^-)\), 3.92 (b, \(-\text{CH}^-\)) 2.76 (m, \(-\text{CH}_2^-\))). The ESI-MS exhibited an \(\text{M}^+\) ion at \(m/z\) 1564.7 (calcd for \(\text{C}_{72}\text{H}_{84}\text{N}_{10}\text{O}_{25}\text{Cl}_2\text{Na}\), \(m/z\) 1564.5).

**CE and ACE Studies.** The uncoated fused silica capillaries with an internal diameter of 50 \(\mu\)m 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 \((L_{tot}) = 74 \text{ cm}, L_{det} = 40 \text{ cm})\), 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 (PMB)—was used to indicate the electroosmotic flow (typically 80 \(\mu\)m). Electroosmosis 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 \(\text{Van}(\text{CH}_3)\text{Ac}\), \(\text{Van}(\text{CH}_3)\text{Suc}\), and the Charge Ladder of \(\text{Van}\).** To a solution of \(\text{Van}\) (100 \(\mu\)L, 3 mg/mL) in phosphate buffer (pH 6.8, 100 mM) was added acetic anhydride (80 \(\mu\)L, 100 mM). The reaction mixture was mixed by vortexing, diluted with 20 mM phosphate buffer, and analyzed by CE. The characterization of \(\text{Van}(\text{CH}_3)\text{Ac}\) was described in the text. The ESI-MS exhibited an \(\text{M}^+\) ion at \(m/z\) 1491.9 (calcd for \(\text{C}_{68}\text{H}_{79}\text{N}_9\text{O}_{25}\text{Cl}_2\)).

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