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Factors affecting the deamidation of vancomycin in aqueous solutions

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Abstract

The degradation kinetics of vancomycin were studied in aqueous solutions at 50°C. The deamidation of 2 mM vancomycin solutions maintained between pH 3 and 9.8 followed pseudo-first order kinetics over several half-lives. The amine pK_a values of vancomycin were titrated by ^{1}H-NMR at 50°C. These values were calculated to be 6.84 and 7.82 for pK_{a_2} and pK_{a_3}, respectively. These are predictably lower than the values 7.75 and 8.89 which were reported in the literature for the amine pK_a values at 25°C. Rate constants obtained from curve fitting the pH-rate profile for vancomycin to the experimentally observed data indicate that the reactivity of vancomycin toward deamidation in the region of pH 6–9 is influenced by its ionic state. The sites of ionization appear to be well removed from the reaction center, suggesting that the ionic state is influencing the solution conformation of vancomycin and subsequently affecting its degradation rate.

Key words: Vancomycin; Stability; Deamidation; pH-rate profile; Conformation; Ionization constant

1. Introduction

Vancomycin is a glycopeptide antibiotic which has become an important agent in the treatment of serious infections caused by methicillin-resistant gram-positive bacteria because of its high potency against these organisms and limited development of bacterial resistance (Cheung and DiPiro, 1986). The deamidation of vancomycin in aqueous solutions and formulations results in considerable instability, further complicated by the formation of a zwitterion degradation product which is sparingly water soluble (Mallet et al., 1982). Therefore, in order to fully understand the role that deamidation plays in the instability of vancomycin, a detailed study of the degradation of vancomycin was undertaken.

Although vancomycin was isolated in the 1950's, it was not until 1983 that the complete structure was reported (Fig. 1) (Harris et al., 1983). The structure of vancomycin consists of a heptapeptide backbone with one residue being asparagine. The deamidation, hydrolysis of the side chain amide linkage of the asparagine residue to form a free carboxylic acid, of vancomycin results in the formation of crystalline degradation product-I (CDP-I) which exists as two rotamers, CDP-I major (CDP-IM) and CDP-I minor (CDP-Im), in which the orientation of the chlorine on

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ring 2 differs by 180° (Fig. 2) (Sheldrick et al., 1978; Williamson and Williams, 1981). It has been proposed that in the neutral to basic pH region the process involves intramolecular attack by the succeeding peptide nitrogen at the side chain carbonyl carbon to form a cyclic imide. The cyclic imide is then spontaneously hydrolyzed into a mixture of components in which the backbone residues are joined via an α-carboxyl or a β-carboxyl linkage (Bornstein and Balian, 1977; Geiger and Clarke, 1987; Manning et al., 1989). It has been suggested that the conformation of proteins can influence the incidence and rate of deamidation of asparagine residues (Clarke, 1987). Since vancomycin is a relatively conformationally rigid glycopeptide, the deamidation of vancomycin and more specifically the relative deamidation of its various ionic species should provide some valuable insight into the reactivity of this interesting molecule.

2. Materials and methods

2.1. Materials

Vancomycin HCl was generously donated by Adria Laboratories. All chemicals were analytical grade and were used as received from the commercial suppliers. HPLC grade acetonitrile and tetrahydrofuran were obtained from Fisher Scientific (Fair Lawn, NJ). Deuterium oxide (99.9 atom% D), acetic-d_3 acid-d (99.9 atom% D) and 3-(trimethylsilyl)propionic acid, sodium salt, 99 + % were obtained from Aldrich Chemical Co. (Milwaukee, WI).

2.2. Apparatus

High-performance liquid chromatography (HPLC) was performed using a system consisting of two Shimadzu LC-6A pumps, a Shimadzu SCL-6A system controller, a Shimadzu SPD-6A variable-wavelength UV detector operating at 280 nm, a Perkin-Elmer ISS-100 autosampler equipped with a 20 μl injection loop, a Shimadzu C-R6A Chromatopac integrator and a Jones Chromatography block column heater (series 7960). The pH readings were recorded using a Corning pH/ion meter 155.

2.3. Kinetic measurements

The degradation of vancomycin was studied in aqueous solutions at 50°C. The following buffers

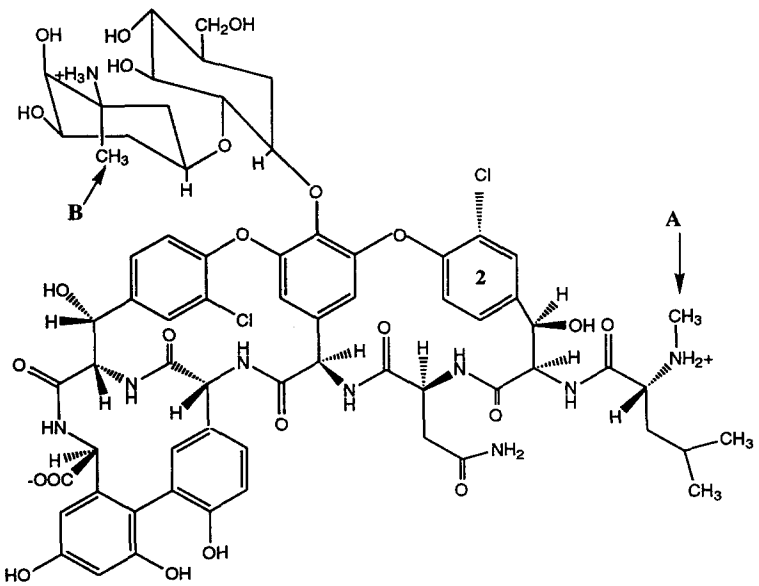

Fig. 1. Structure of vancomycin.

were used: HCl (pH 1.0 and 2.0), formate (pH 3.0), acetate (pH 4.0, 5.0 and 5.5), phosphate (pH 6.5, 7.0 and 7.4), Tris-HCl (pH 8.0), borate (pH 8.5 and 9.0) and bicarbonate (pH 9.8). The buffer concentrations were 0.01–0.04 M except for pH 8.5 (0.10–0.13 M), pH 9.0 (0.03–0.06 M) and pH 9.8 (0.08–0.14 M). A constant ionic strength of 0.15 M was maintained for each buffer by adding an appropriate amount of NaCl, except for pH 1.0–3.0 where KCl was used to avoid the precipitation effects reported in acidic media. The pH of the buffer solutions was adjusted at the experimental temperature (50°C).

Vancomycin was dissolved in a sufficient volume of buffer solution to make a bulk solution with a resulting concentration of 2.0×10^{-3} M. Aliquots (0.5 ml) of this bulk solution were added to ampules which were then sealed and stored in a 50°C oven. At various times the ampules were removed, cooled and diluted with triethylamine phosphate buffer (pH 3.20) prior to being analyzed by HPLC. The pH of the solution in an ampule of each concentration was measured at the termination of each kinetic experiment.

2.4. HPLC analysis

Analysis of vancomycin and its degradation products was performed on an ODS Hypersil C_{18} column (5 μm, 4.6 × 250 mm) maintained at 30°C using a modification of an assay reported by Inman (1987). This is a gradient elution method which consists of two mobile phases, A (91% triethylamine phosphate pH 3.20, 8% acetonitrile and 1% tetrahydrofuran) and B (70% triethylamine phosphate pH 3.20, 29% acetonitrile and 1% tetrahydrofuran). Elution is accomplished with 100% mobile phase A at 1 ml/min for the

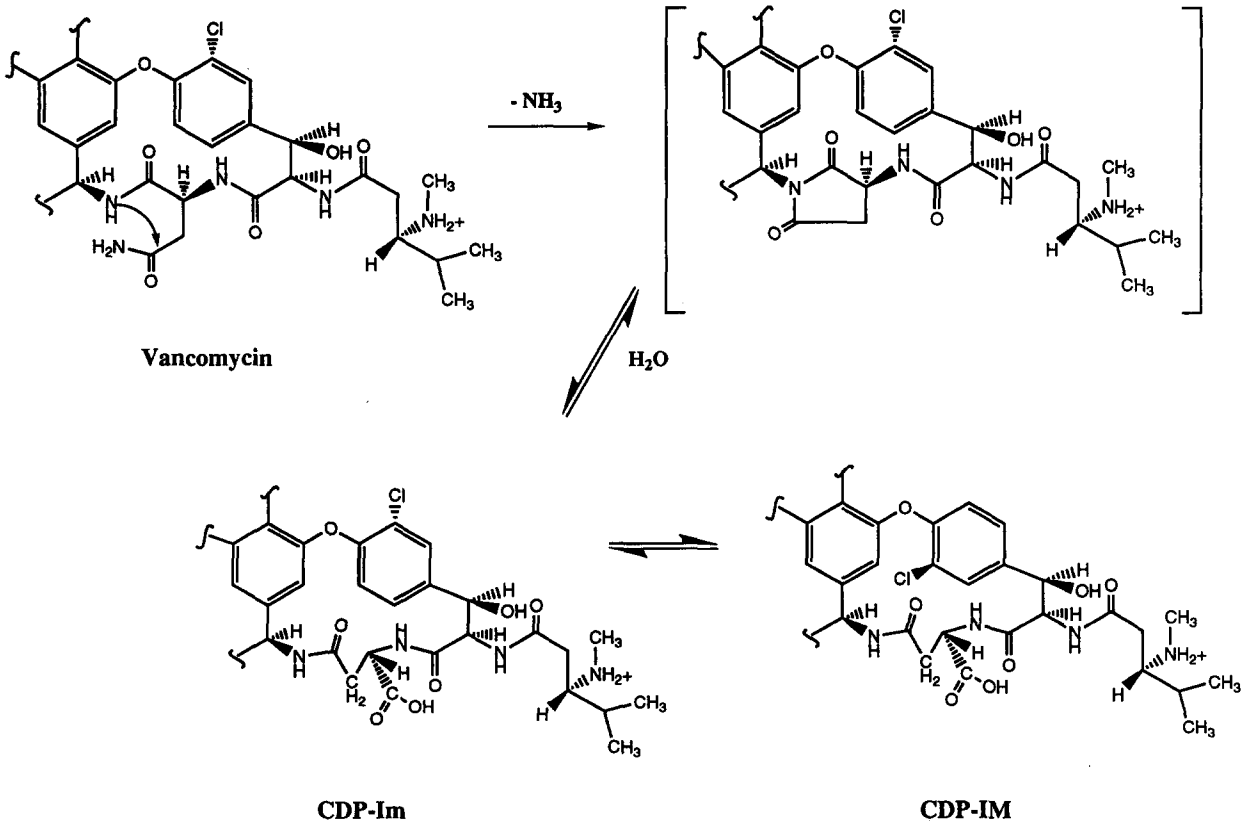

Fig. 2. Deamidation scheme for vancomycin.

first 8 min, followed by a 20 min linear gradient from 0 to 100% mobile phase B. The concentration of mobile phase B is held at 100% for 2 min before the process is reversed and the column is re-equilibrated before the next injection. Quantification of vancomycin was by peak area. The peak area was linearly related to the concentration of vancomycin over the range 0.006–0.100 mg/ml. Typical elution times (min) were as follows: 6.5 for vancomycin and 5.1 and 13.0 for the two rotamers of CDP-I.

2.5. Identification of degradation products

CDP-I was prepared using the method described by Marshall (1965). The two rotamers of CDP-I were separated by HPLC and collected. This was accomplished by isocratic elution of the reaction mixture at room temperature through a Whatman Partisil 10 ODS-2 column (10 μm, 9.4 $\times$ 500 mm) using the following mobile phase: 88% triethylamine phosphate, pH 3.20, 11% acetonitrile and 1% tetrahydrofuran. The flow rate was 3 ml/min and the detection wavelength was 280 nm. After collection of the separate rotamers, initial interconversion kinetics were followed. These rotamers of CDP-I were also analyzed by the previously described kinetic assay to verify their relative positions.

2.6. NMR analysis

The amine pK_a values for vancomycin were titrated at 50°C using ^{1}H-NMR. The following buffers were used: pH 4.86–5.57 (0.05 M acetic-d_3 acid-d/NaOH), pH 6.10–7.90 (0.01 M phosphate), pH 8.13–9.82 (0.05 M sodium borate/NaOH or 0.10 M boric acid/NaOH). All of the buffer solutions were prepared with a solution of 95% H_2O/5% D_2O. A 0.15 M NaOH solution was used to adjust the pH. A constant ionic strength of 0.15 M was maintained for each buffer by adding the appropriate amount of NaCl. The solutions were prepared at the experimental temperature (50°C). The NMR spectra were recorded at 50°C on a Bruker AM-500 instrument operating at 500.14 MHz for ^{1}H.

3. Results and discussion

3.1. Titration of amine pK_a values

The pK_a values for vancomycin at 25°C were reported by Takács-Novák and co-workers (1993) to be 2.18 for the carboxyl group, 7.75 and 8.89 for the amines and 9.59, 10.40, and 12.0 for the phenols. Since the kinetic experiments were performed at 50°C and it is known that nitrogenous bases are highly temperature sensitive and become weaker as the temperature is increased (Albert and Serjeant, 1971), the amine pK_a values were evaluated at this temperature using ^{1}H-NMR. The chemical shift changes for protons close to the site of ionization were recorded as a function of pH. The methyl protons on the *N*-methylleucine amine, A in Fig. 1, were used to follow the ionization of the first amine pK_a (pK_{a_2}) while the methyl protons on the vancosamine sugar, B in Fig. 1, were used to follow the ionization of the second amine pK_a (pK_{a_3}). Fig. 3 illustrates a simplified ionization scheme which accounts for the macroscopic ionization of vancomycin in the pH range 1–9 but ignores the microscopic ionization constants. The relationship between the chemical shift and the ioniza-

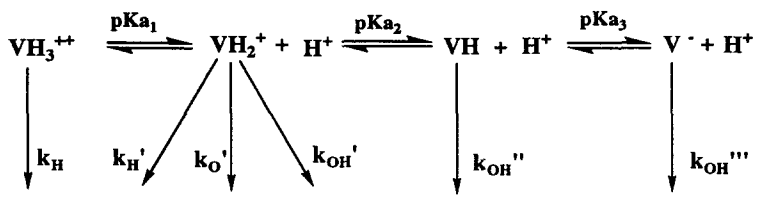

Fig. 3. Ionization scheme for vancomycin at pH 1–9.

tion of vancomycin near pK_{a_2} can be described by the following equations:

$$\delta_{obs} = f_{VH_2^+} \times \delta_{VH_2^+} + f_{VH} \times \delta_{VH} \quad (1)$$

where δ_{obs} is the observed chemical shift, and $\delta_{VH_2^+}$ and δ_{VH} denote the intrinsic chemical shifts for those ionic species. The ionic fractions of vancomycin may be described by the following equations:

$$f_{VH_2^+} = \frac{[H^+]}{[H^+] + K_{a_2}} \quad (2)$$

$$f_{VH} = \frac{K_{a_2}}{[H^+] + K_{a_2}} \quad (3)$$

If Eq. 2 and 3 are substituted into Eq. 1, the result is as follows:

$$\delta_{obs} = \frac{[H^+] \times \delta_{VH_2^+} + K_{a_2} \times \delta_{VH}}{[H^+] + K_{a_2}} \quad (4)$$

The data were plotted as chemical shift (δ) vs pH and were fitted to Eq. 4 using the SigmaPlot 4.14 curve fitter which utilizes the Marquardt-Levenberg algorithm. This method uses a least-squares procedure to minimize the sum of squares of the differences between the equation values and the experimental values. The results reported are the parameter and the asymptotic standard error. K_{a_2} was estimated from the fit to Eq. 4. The same equation was applied for the determination of K_{a_3}. The results are shown in Fig. 4. pK_{a_2} was calculated to be 6.84 ± 0.05 and pK_{a_3} was determined as 7.82 ± 0.05.

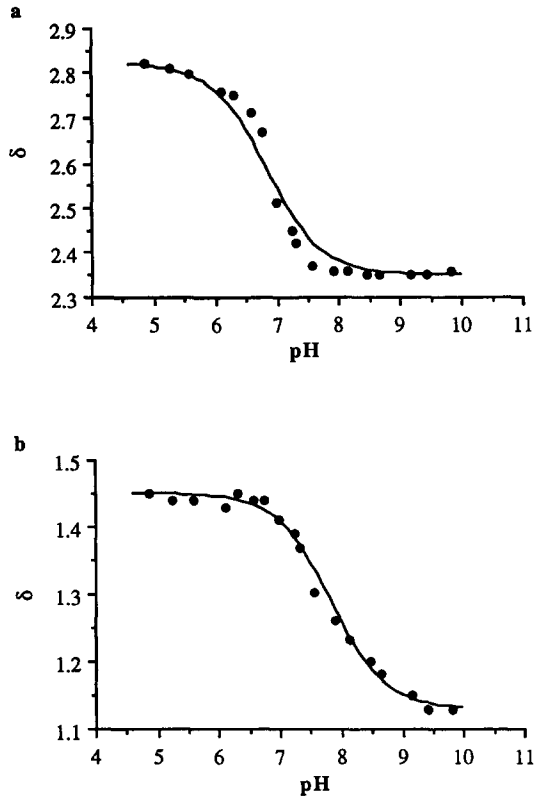

Fig. 4. Titration of (a) pK_{a_2} and (b) pK_{a_3} by ^{1}H-NMR at 50°C. The chemical shift changes (in ppm) for protons close to the site of ionization were recorded as a function of pH. The methyl protons on the *N*-methylleucine amine, A in Fig. 1, were used to follow the ionization of the first amine pK_a (pK_{a_2}) while the methyl protons on the vancosamine sugar, B in Fig. 1, were used to follow the ionization of the second amine pK_a (pK_{a_3}). (●) Observed chemical shift data; (solid line) theoretical curve obtained by fitting the observed data to Eq. 4.

3.2. Effect of pH and buffer concentration on the degradation of vancomycin

The aqueous stability of vancomycin was studied at various pH values. The results indicate that deamidation is the major degradation process occurring to vancomycin at pH values greater than 2 and less than 10. Fig. 5 illustrates the typical time course for the disappearance of vancomycin and the appearance of CDP-I at pH 7.0 and 50°C. The relative ratio of the two rotamers, CDP-Im and CDP-IM, remained constant at approx. 64:36 over the pH range 2–10. This ratio was reported in the literature by Harris and coworkers (1983). The degradation of vancomycin appears to follow pseudo-first order kinetics. The first order rate constants were obtained from plots of log concentration vs time.

The catalytic effects of the buffers were determined by measuring the rate of degradation of vancomycin at constant pH, ionic strength and temperature while varying the buffer concentration. The results indicate that the degradation of vancomycin appears to be subject to some buffer catalysis in the pH range 3–10. Table 1 summa-

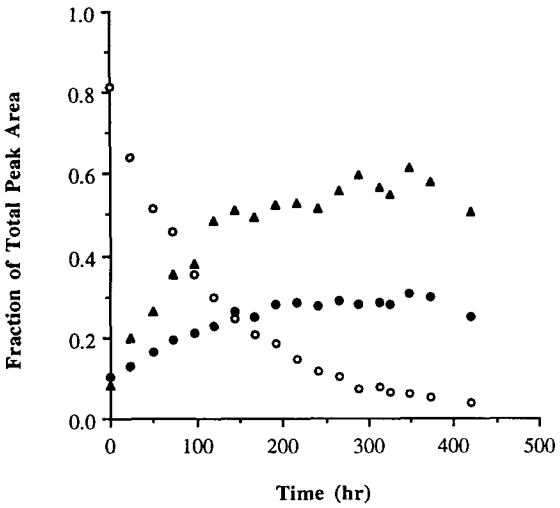

Fig. 5. Time profile for the disappearance of vancomycin (○) and appearance of CDP-IM (▲) and CDP-Im (●) at pH 7.0, 0.02 M phosphate buffer and 50°C.

rizes the dependence of the rate constant on buffer concentration. The rate constants at zero buffer concentration were obtained from the intercepts of the k_{obs} vs total buffer concentration graphs. The intercepts were calculated using a linear regression method on the StatWorks program. The rate constants are reported as the constant and the standard error of the estimate. No microscopic analysis of the buffer data was attempted because of the relatively small amount of catalysis observed and the fact that the fractional species of vancomycin were also changing in this pH range.

The pH dependence of the rate of degradation of vancomycin at 50°C is shown in Fig. 6. The pH of maximum stability is found to be approx. pH 5.5. This is slightly higher than the values (pH 3–5) reported for the pH of maximum stability for the deamidation of asparagine residues in small, linear peptides which deamidate through cyclic imide formation (Capasso et al., 1989; Patel and Borchardt, 1990). Kinetic results reported by both of these groups indicate that the peptides studied, Boc-Asn-Gly-Gly-NH_2 and Val-Tyr-Pro-Asn-Gly-Ala, deamidated exclusively through cyclic imide formation in the region of pH 5–12. It was reported that the rate constants in this region increased dramatically with increases in pH, however, there was no simple correlation with hydroxide ion concentration. The slopes of their pH-rate profiles in the region where base catalysis was presumed were reported to be between 0.5 and 0.8. The pH-rate profile for vancomycin (Fig. 6) contains a relatively pH-independent region between pH 5 and 8 and illustrates a shift in the strongly pH-dependent region from the expected initial value of pH 6 to pH approx. 8.5.

3.3. Analysis of the pH-rate profile

Vancomycin contains six ionizable groups; however, in the pH range studied only the carboxyl and the two amine groups appear to influence the kinetics significantly. The first phenolic pK_a, which may fall into the pH range studied, was omitted to simplify the rate equation calculations. A simplified ionization scheme for vancomycin in the pH range 1–9 is shown in Fig. 3. The shape of the overall pH-rate profile suggests that the rate of degradation of vancomycin may be expressed by the following equation:

$$k_{obs} = f_{VH_3^{2+}} k_H[H^+] + f_{VH_2^+} k'_H[H^+] + f_{VH_2^+} k'_O + f_{VH_2^+} k'_{OH}[OH^-] + f_{VH} k''_{OH}[OH^-] + f_V k'''_{OH}[OH^-] \quad (5)$$

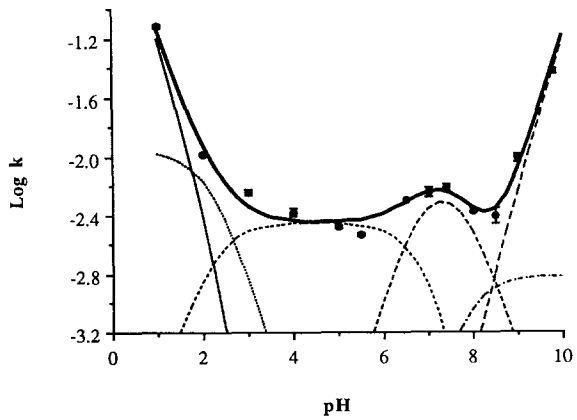

Fig. 6. pH-rate profile for vancomycin at 50°C. (●) Observed data at zero buffer concentration; (bold solid line) theoretical curve obtained by curve fitting the observed data to Eq. 6. The plain solid line and the broken lines represent each of the six components in Eq. 5.

Table 1
Observed rate constants for the degradation of vancomycin in aqueous buffer solutions at 50°C

pH	[Buffer] (M)	k_{obs} (S.E.) (h^{-1})
1.0 (HCl)	0.1	7.65×10^{-2} (2.10×10^{-3})
2.0 (HCl)	0.01	1.05×10^{-2} (2.87×10^{-4})
3.0 (formate)	0	5.71×10^{-3} (2.77×10^{-4})
	0.01	5.81×10^{-3} (2.56×10^{-4})
	0.02	6.06×10^{-3} (3.40×10^{-4})
	0.03	5.78×10^{-3} (4.38×10^{-4})
	0.04	6.27×10^{-3} (3.49×10^{-4})
4.0 (acetate)	0	4.17×10^{-3} (2.69×10^{-4})
	0.01	4.28×10^{-3} (8.98×10^{-5})
	0.02	4.64×10^{-3} (8.43×10^{-5})
	0.03	5.05×10^{-3} (2.72×10^{-4})
	0.04	4.86×10^{-3} (7.37×10^{-5})
5.0 (acetate)	0	3.34×10^{-3} (1.13×10^{-4})
	0.01	3.49×10^{-3} (1.06×10^{-4})
	0.02	3.89×10^{-3} (1.10×10^{-4})
	0.03	4.01×10^{-3} (1.21×10^{-4})
	0.04	4.20×10^{-3} (1.25×10^{-4})
5.5 (acetate)	0	2.94×10^{-3} (1.03×10^{-4})
	0.01	3.21×10^{-3} (1.16×10^{-4})
	0.02	3.49×10^{-3} (1.24×10^{-4})
	0.03	3.90×10^{-3} (1.40×10^{-4})
	0.04	4.03×10^{-3} (1.02×10^{-4})
6.5 (phosphate)	0	5.14×10^{-3} (4.07×10^{-4})
	0.01	5.91×10^{-3} (1.70×10^{-4})
	0.02	7.48×10^{-3} (1.22×10^{-4})
	0.03	8.37×10^{-3} (1.42×10^{-4})
	0.04	9.02×10^{-3} (1.41×10^{-4})
7.0 (phosphate)	0	5.76×10^{-3} (4.14×10^{-4})
	0.01	6.57×10^{-3} (1.56×10^{-4})
	0.02	7.23×10^{-3} (1.43×10^{-4})
	0.03	8.62×10^{-3} (2.82×10^{-4})
	0.04	8.84×10^{-3} (2.81×10^{-4})
7.4 (phosphate)	0	6.18×10^{-3} (3.61×10^{-4})
	0.01	6.73×10^{-3} (1.48×10^{-4})
	0.02	7.70×10^{-3} (1.52×10^{-4})
	0.03	8.61×10^{-3} (2.56×10^{-4})
	0.04	8.80×10^{-3} (2.73×10^{-4})
8.0 (Tris)	0	4.34×10^{-3} (1.52×10^{-4})
	0.01	4.90×10^{-3} (1.31×10^{-4})
	0.02	5.74×10^{-3} (1.20×10^{-4})
	0.03	6.37×10^{-3} (2.10×10^{-4})
	0.04	6.86×10^{-3} (1.47×10^{-4})
8.5 (borate)	0	3.95×10^{-3} (4.85×10^{-4})
	0.10	6.78×10^{-3} (2.69×10^{-4})
	0.12	7.44×10^{-3} (4.46×10^{-4})
	0.13	7.61×10^{-3} (4.64×10^{-4})
9.0 (borate)	0	9.91×10^{-3} (6.17×10^{-4})
	0.03	1.15×10^{-2} (7.74×10^{-4})
	0.04	1.25×10^{-2} (6.19×10^{-4})
	0.05	1.32×10^{-2} (7.76×10^{-4})
9.8 (bicarbonate)	0	3.76×10^{-2} (1.43×10^{-3})
	0.08	4.67×10^{-2} (1.06×10^{-3})
	0.10	4.80×10^{-2} (1.18×10^{-3})
	0.12	5.03×10^{-2} (1.12×10^{-3})
	0.14	5.32×10^{-2} (1.21×10^{-3})

If the fraction of each ionic species of vancomycin is substituted into Eq. 5 then the following equation is obtained:

$$k_{obs} = \Big\{ k_H[H^+]^4 + k'_H K_{a_1}[H^+]^3 + k'_O K_{a_1}[H^+]^2 + k'_{OH} K_{a_1} K_w [H^+] + k''_{OH} K_{a_1} K_{a_2} K_w + \frac{k'''_{OH} K_{a_1} K_{a_2} K_{a_3}}{[H^+]} \Big\} / \Big\{ [H^+]^3 + K_{a_1}[H^+]^2 + K_{a_1} K_{a_2}[H^+] + K_{a_1} K_{a_2} K_{a_3} \Big\} \quad (6)$$

The pH-independent term, $f_{VH_2^{\pm}} k'_O$, is kinetically equivalent to a base-catalyzed term $f^{2+}_{VH3} k_{OH}[OH^-]$. The base-catalyzed terms, k'_{OH}, k''_{OH} and k'''_{OH}, were chosen over their kinetically equivalent pH-independent terms because of the tendency for deamidation to be a base-catalyzed reaction. The experimental data were fitted to Eq. 6 using the SigmaPlot 4.14 curve fitting program. The pK_a values derived from NMR were used for pK_{a_2} and pK_{a_3}. Since the pK_a of the carboxyl group was not expected to be dramatically affected by the increase in temperature from 25 to 50°C, 2.18 was used for the value of pK_{a_1}. The K_w value, adjusted for the increase in temperature, was 5.495×10^{-14} M. The values for the individual rate constants are given in Table 2. As seen in Table 2, there is a difference of two orders of magnitude in the reactivity of the base-catalyzed rate constants, k'_{OH}, k''_{OH} and k'''_{OH}. Since the mechanism of deamidation is assumed to be the same for all three regions, namely, pH 6–8, 8–8.5 and 8.5–9.8, the disparity in reactivity is an unexpected observation. In Fig. 6, the bold line represents the theoretical curve calculated by substituting the rate constants from Table 2 into Eq. 6 and the dotted lines indicate the influence

Table 2
Calculated rate constants, according to Eq. 6, for the deamidation of vancomycin at 50°C

	Rate constant	S.E.
k_H	$0.66\ M^{-1}\ h^{-1}$	$0.15\ M^{-1}\ h^{-1}$
k'_H	$1.70\ M^{-1}\ h^{-1}$	$0.56\ M^{-1}\ h^{-1}$
k'_O	$3.52\times10^{-3}\ h^{-1}$	$3.73\times10^{-4}\ h^{-1}$
k'_{OH}	$2.13\times10^{4}\ M^{-1}\ h^{-1}$	$0.33\times10^{4}\ M^{-1}\ h^{-1}$
k''_{OH}	$4.24\times10^{2}\ M^{-1}\ h^{-1}$	$2.84\times10^{2}\ M^{-1}\ h^{-1}$
k'''_{OH}	$1.19\times10^{2}\ M^{-1}\ h^{-1}$	$0.21\times10^{2}\ M^{-1}\ h^{-1}$

that each individual rate constant and ionic state of vancomycin has on the overall rate of deamidation of vancomycin. An interesting question is why should there be two orders of magnitude difference in reactivity for the three ionic forms of vancomycin when the ionic sites seem to be well removed from the deamidation reaction site. One possible explanation for the observed results may be that the three-dimensional positions of the ionizable sites in the solution structure of vancomycin are not as remote from the deamidation reaction center as they appear in the two-dimensional representations. Another possibility is that ionization of the amine sites on vancomycin may cause a change in the solution conformation, thus altering the rate of the intramolecular deamidation reaction. The role played by changing pH and ionic state on the conformation of vancomycin is the basis of ongoing studies. These factors appear to strongly influence the rate of deamidation of vancomycin yet are not adequately discussed in the literature. Previous conformational analyses have focused primarily on the solution structure and binding properties of vancomycin with mucopeptide precursors in dimethyl sulfoxide (Williams, 1884; Williamson, 1984; Waltho, 1988; Molinari and Pastore, 1990)

In conclusion, the results indicate that the deamidation of vancomycin deviates from the expected behavior in the neutral to basic pH region. That is, apparent base catalysis does not appear to occur until pH values greater than 8.5. However, analysis of the data shows that this may be due to the altered reactivity of vancomycin as it changes ionic states, the deamidation being slower for vancomycin mono-anion (V^-) than its zwitterion (VH) and its mono-cation (VH^+).

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