Kinetics and Mechanism of Binding of Specific Peptides to Vancomycin and Other Glycopeptide Antibiotics

Paul H. Popieniek and R. F. Pratt*

Contribution from the Department of Chemistry, Wesleyan University, Middletown, Connecticut 06457. *Received May 29, 1990*

Abstract: Measurement of the rates and equilibria of peptide binding to glycopeptide antibiotics of the vancomycin class has been facilitated by the fluorescent reporter peptide ϵ-*N*-acetyl-α-*N*-dansyl-L-lysyl-D-alanyl-D-alanine (ADLAA). The pH dependence of both rates and equilibrium constants of the binding of ADLAA to vancomycin suggested that the protonated N-terminus of the latter is not essential to strong binding. The apparent second-order rate constants of binding of ADLAA, and of the nonfluorescent analogue, α,ϵ-*N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine, to vancomycin, ristocetin A, and α-avoparcin were very similar, and too small, by at least 2 orders of magnitude, to reflect a simple diffusion-controlled association mechanism. This conclusion was supported by the absence of a viscosity effect on the rate of association of ADLAA with vancomycin. These reactions appear to proceed through slow rearrangement of a loose initial complex, whose formation would presumably be diffusion controlled. Although fast conformational preequilibria of the free antibiotics may also contribute to these small rate constants, some other process must define the transition states. The likely possibilities are a slow conformational rearrangement of the initial complex, a slow desolvation/solvent rearrangement event, or a concerted combination of the two. Analysis of the effects of the organic cosolvents acetonitrile, methanol, and dimethyl sulfoxide on the rates and equilibria of these reactions suggested that the rates in water may be significantly limited by a solvent rearrangement process, probably involving hydrogen-bond breaking. These solvent effects, and the results of experiments with aglucovancomycin, suggest that both the final complex and the dominant transition state leading to it are stabilized by hydrophobic interaction between the bound peptide and the pendant sugar residues of vancomycin. The effects of the organic cosolvents on antibiotic structure were assessed through consideration of CD spectra.

The specific, noncovalent binding of ligands to proteins is one of the most fundamental processes in biochemistry. Examples include the interactions of substrates and allosteric effectors with enzymes, antigens with antibodies, hormones and drugs with receptors, nucleic acids with proteins, and proteins with each other. A complete understanding of these binding interactions is fundamental to the understanding of biology at a molecular level. Much of course has been accomplished toward reaching this goal. The physical forces involved in holding the ligand and protein together are largely understood; i.e., the thermodynamics of the binding process are, in principle at least, clear. What is not so well understood at present is the molecular mechanism of binding, i.e., the pathway from initial contact between ligand and protein, through recognition, to the final specific complex. Conceptual thinking about this process has progressed from rigid lock-and-key models to a point where, in the general case, the flexibility of both ligand and protein is recognized as important to the final result. The final structure seems to result from mutual induction of structure between the two partners. It is the details of this process that need further attention.

Both experimental and theoretical methods of investigating these mechanistic details in proteins are hampered by the sheer size of the molecular system involved, and the large number of variables thus present. It seems likely therefore that much could be learned from appropriate smaller model systems. There appear to be lower limits however to the size of effective ligand-binding sites. Small linear peptides, for example, self-associate only very weakly in aqueous solution.[1] In order to achieve tight (say, micromolar dissociation constants) binding, the entropy loss undergone by flexible components in forming the complex must be reduced. This may be done by the incorporation of structural rigidity into one or more of the components.

The glycopeptide antibiotics of the vancomycin class represent a naturally occurring peptide-binding system, whose size and flexibility appear to be carefully balanced to achieve complexes of micromolar dissociation constants with specific peptides. These antibiotics kill bacteria by their binding to the D-alanyl-D-alanine-terminating peptides that are metabolic intermediates in cell wall (peptidoglycan) biosynthesis.[2] They have enjoyed considerable clinical application in recent years against infections caused by β-lactam-resistant Gram-positive bacteria.[3] Recently, we introduced a fluorescent peptide ligand, ϵ-*N*-acetyl-α-*N*-dansyl-L-lysyl-D-alanyl-D-alanine (ADLAA), for glycopeptide antibiotics and demonstrated its advantages in both thermodynamic[4] and kinetic[5] studies of the binding process. In this paper we employ this compound to explore further the mechanism of association of specific peptides with the glycopeptide antibiotics.

Experimental Section

Instrumentation. Absorption and fluorescence spectra were obtained from Cary 219 and Perkin-Elmer MPF-44A spectrophotometers, respectively. The rates of binding were obtained from a Durrum D110 stopped-flow spectrophotometer, operating in the fluorescence mode. CD spectra were recorded on an Aviv 60 ds CD spectrophotometer located in the Instrument Center of the Chemistry Department of Yale University, New Haven, CT. Titrations with base were carried out in a Metrohm titrator in the laboratory of Dr. Norma Allewell.

Materials. Pure vancomycin was generously provided by Eli Lilly and Co. (Dr. C. F. Murphy). Ristocetin A and α-avoparcin were gifts from Lundbeck A/C (Dr. A. Jorgensen) and American Cyanamid Co., Lederle Laboratories (Dr. M. Kunstmann), respectively. Aglucovancomycin, where both the vancosamine and glucose residues have been removed, was prepared by the method of Marshall.[6]

ϵ-*N*-Acetyl-α-*N*-dansyl-L-lysyl-D-alanyl-D-alanine (ADLAA) was prepared as previously described.[4] α,ϵ-*N*,*N*′-Diacetyl-L-lysyl-D-alanyl-D-alanine (AALAA) was purchased from Sigma Chemical Co.

Glycerol and methanol were analytical grade reagents (Baker) and used as supplied. Reagent grade acetonitrile and dimethyl sulfoxide were distilled, the latter under reduced pressure, from P_2O_5 and NaH, respectively. Buffer materials were of reagent grade. Mixed solvents were prepared by weighing the appropriate amount of organic solvent and adjusting it to volume by addition of an aqueous solution containing the required buffer components.

(1) Blackburn, G. M.; Lilley, T. H.; Walmsley, E. *J. Chem. Soc., Faraday Trans. 1* **1982**, *78*, 1641-1655.

(2) (a) Perkins, H. R. *Pharmcol. Ther.* **1982**, *16*, 181-197. (b) Barna, J. C. J.; Williams, D. H. *Annu. Rev. Microbiol.* **1984**, 339-357.

(3) (a) *Symposium on Vancomycin Therapy*; Wise, R., Reeves, D., Eds. *J. Antimicrob. Chemother.* **1984**, *14* (Suppl. D). (b) *Symposium on Teicoplanain—Clinical use and laboratory correlation*; Speller, D., Greenwood, D., Eds. *J. Antimicrob. Chemother.* **1988**, *21* (Suppl. A).

(4) Popieniek, P. H.; Pratt, R. F. *Anal. Biochem.* **1987**, *165*, 108-113.

(5) Popieniek, P. H.; Pratt, R. F. *J. Am. Chem. Soc.* **1988**, *110*, 1285-1286.

(6) Marshall, F. *J. Med. Chem.* **1965**, *8*, 18-22.

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Table I. Rate and Equilibrium Constants for the Binding of ADLAA to the Glycopeptide Antibiotics[a]

antibiotic	$k_f \times 10^{-6}$, s^{-1} M^{-1}	k_r, s^{-1}	$K \times 10^{-5}$, M^{-1}
vancomycin	9.3 ± 1.6	31 ± 5	3.0
ristocetin A	7.2 ± 2.1	24 ± 7	3.0
α-avoparcin	4.1 ± 1.3	28 ± 9	1.5
aglucovancomycin	2.7	135	0.2

[a] 0.1 M phosphate buffer, 25 °C, pH 7.0 (vancomycin and aglucovancomycin), 6.5 (ristocetin), 6.0 (avoparcin); the rate and equilibrium constants refer to Scheme I.

Equilibrium and Rate Constant Determinations. Equilibrium and rate constants of binding of ADLAA to vancomycin, aglucovancomycin, ristocetin A and α-avoparcin were determined fluorometrically as previously described.[4,5] Equilibrium constants for binding of AALAA and *N*-acetyl-D-alanyl-D-alanine to the glycopeptides were also determined fluorometrically by competition against ADLAA.[4] Attempts to determine rate constants for the binding of these nonfluorescent peptides were also made by competitive measurements. In these experiments, mixtures of ADLAA and the nonfluorescent peptide were mixed in the stopped-flow apparatus with appropriate concentrations of glycopeptide. Recordings of fluorescence intensity with time were made, from which, under certain conditions described below, the required rate constants could be obtained, given equilibrium dissociation constants of both peptides and the rate constant of association of ADLAA. In order to do this, simultaneous differential equations, derived from Scheme III, were solved numerically, yielding fluorescence vs time curves.[7] These could be fitted to experimental data by a Simplex optimization procedure,[7] where the rate constant of association of the nonfluorescent peptide and the relative quantum yield of the ADLAA/vancomycin complex were adjustable parameters.

All rate and equilibrium measurements were made at 25 °C unless otherwise specified. The buffers used for determinations of the variation of the rate and equilibrium constant with pH were phosphate (pH 5.9–7.7), pyrophosphate (pH 7.9–8.5), and carbonate (pH 9.0–9.6); the ionic strength of these solutions was adjusted to 0.20 with potassium chloride. For reasons of solubility, the buffer of pH 7.5 used in the mixed solvents was 6.4 mM Tris, prepared by dissolving appropriate amounts of the free base and Tris hydrochloride in the solvent.

pK_a Determinations. Solutions of the glycopeptides (50 μM) in 0.2 M aqueous potassium chloride were prepared. Samples of these solutions (3 mL) were titrated at 25 °C with 11.5 mM sodium hydroxide under nitrogen. Data obtained from the addition of at least 0.5 molar equiv of base were used to determine the pK_as. Up to this point the pK_a calculated from the pH readings did not change significantly with pH, but on addition of further base, systematic deviations occurred, presumably because of dissociation of other functional groups (see Results and Discussion). These titrations were repeated in the presence of 50 μM AALAA.

Results and Discussion

Association of ADLAA with the Glycopeptides. We have previously reported the equilibrium and rate constants for the binding of ADLAA to vancomycin, ristocetin A, and α-avoparcin at pH 7.0,[4,5] in terms of Scheme I. These data are collected in Table I for reference. Also included are the values for aglucovancomycin, determined as part of the present work. Our previous measurements on vancomycin showed that the binding reaction to 1.0 μM ADLAA was kinetically a second-order process, to a concentration of vancomycin of 30 μM.[5] We have been able to extend the concentration range by employing a lower temperature (5 °C) in order to maintain rates slow enough to be measured with the available stopped-flow apparatus. The resulting pseudo-first-order rate constants were linear in vancomycin concentration to 100 μM (Figure 1). Thus, under these conditions, any fast preequilibrium complex could not have an association constant greater than 10^4 M^{-1}; this would probably be true at 25 °C also. The apparent second-order rate constant for the association at 5 °C, from the slope of the line in Figure 1, was (3.8 ± 0.4) × 10^6 s^{-1} M^{-1}. This, with the value at 25 °C (Table I), yields $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values of (6.8 ± 1.7) kcal/mol and (−4.0 ± 8.0) cal/(deg·mol), respectively.

Scheme I

$$G + D \underset{k_r}{\overset{k_f}{\rightleftharpoons}} GD \qquad K = k_f/k_r$$

G = glycopeptide antibiotic D = ADLAA

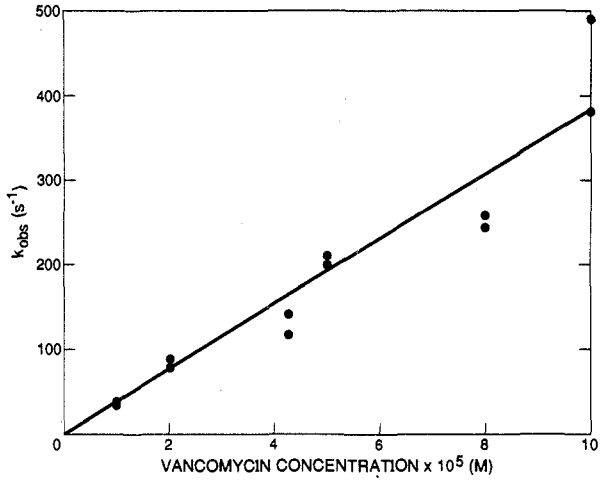


Figure 1. Variation of the pseudo-first-order rate constant at 5 °C of association of ADLAA (1.0 μM) with vancomycin as a function of vancomycin concentration. The solid line represents a linear least-squares fit to the data.

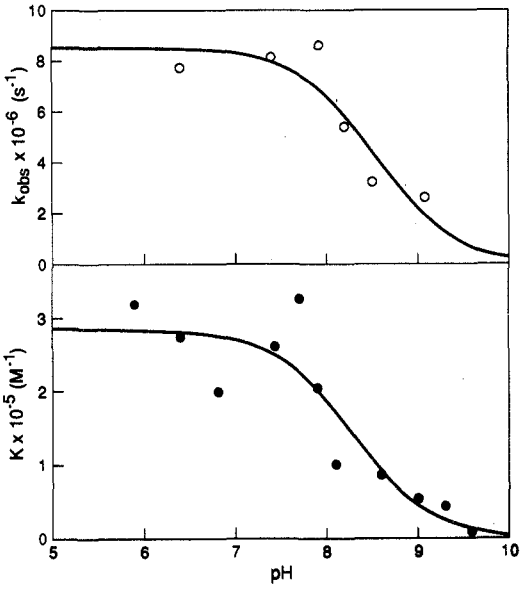


Figure 2. Variation with pH of the rate constant (○) and equilibrium constant (●) of association of ADLAA with vancomycin. The solid lines represent nonlinear least-squares fits to the data, using an equation derived from a reaction scheme where the binding reaction requires the acidic form of a single functional group.

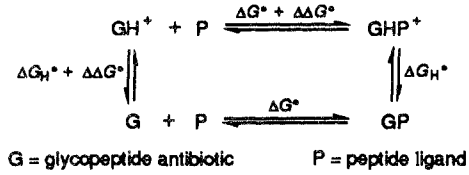
Both the association equilibrium constant and second-order rate constant for the binding of ADLAA to vancomycin decrease at higher pH (Figure 2). Although, in the past, this has been interpreted in terms of dissociation of the N-terminal ammonium group of the glycopeptides,[8] more recent research has suggested that the N-terminal ammonium ion is not essential for strong binding.[9] The results shown in Figure 2 support the latter position. The fitting of the equation for a single dissociation to the data of Figure 2 (solid lines) yields pK_a values of 8.3 ± 0.3 and 8.5 ± 0.3 for the thermodynamics and kinetics of binding, respectively. Titration with base yielded a pK_a of (7.7 ± 0.1) for vancomycin, which is somewhat higher than some earlier determinations,[10,11]

(7) Anderson, E. G.; Pratt, R. F. *J. Biol. Chem.* **1983**, *258*, 13120–13126.

(8) Brown, J. P.; Feeney, J.; Burgen, A. S. V. *Mol. Pharmacol.* **1975**, *11*, 119–125.

(9) (a) Herrin, T. R.; Thomas, A. M.; Perun, T. J.; Mayo, J. L.; Fesik, S. W. *J. Med. Chem.* **1985**, *28*, 1371–1375. (b) Trani, A.; Ferrari, P.; Pallanza, R.; Tarzia, G. *J. Med. Chem.* **1989**, *32*, 310–314.

Scheme II



but not all.[8] This first dissociation has been shown[10] to largely reflect dissociation of the N-terminal ammonium ion. Thus, the loss of binding strength appears to more closely reflect the dissociation of a group of higher pK_a than the ammonium group. The next highest of the reported[10,11] pK_as, 8.7 and 9.6, are thought to mainly reflect phenolic dissociation.[10] The data of Figure 2 therefore suggest that changes more deleterious to binding occur on dissociation of a phenol than dissociation of the ammonium ion. Nieto and Perkins also came to this conclusion during their pioneering studies of vancomycin.[10] A similar situation probably occurs with ristocetin; the association equilibrium constant did not decrease between pH 5.5 and 7.0 although the titrimetric first pK_a of ristocetin was determined to be 7.05 ± 0.1.

On titration of the glycopeptide antibiotics (50 μM) in the presence of AALAA (50 μM), the first dissociation constants were perturbed slightly toward higher values. The perturbations (ΔpK_a) were 0.3 ± 0.2, 0.2 ± 0.2 and 0.3 ± 0.2 units for vancomycin, ristocetin A, and α-avoparcin, respectively [the pK_a of free α-avoparcin was determined to be (6.9 ± 0.1)]. These values can be interpreted as measurements of the difference in free energy of interaction ($\Delta\Delta G°$) between AALAA and the N-terminal protonated and free amine species (Scheme II). With the assumption that no structural changes accompany these dissociations (an assumption that has yet to be tested), these free energy differences are quantitative measures of the electrostatic interaction ($\Delta\Delta G°$) between AALAA and each of the protonated glycopeptides. The thus derived values are therefore 0.41, 0.29, and 0.44 kcal/mol for vancomycin, ristocetin, and α-avoparcin, respectively. This appears to be a relatively small component of the total binding energies, 7.47, 7.47, and 7.06 kcal/mol, respectively (calculated from the equilibrium constants of Table I).

The much weaker binding of *N,N'*-diacetyl-L-lysyl-D-alanyl-D-alanine methyl ester[12] and amide[4,13] to vancomycin and to ristocetin A and α-avoparcin,[14] although understandable in terms of the importance of electrostatic stabilization, must presumably be seen as more reflecting losses in hydrogen-bonding interactions and the appearance of unfavorable steric interactions. The significant decreases in binding strength thus brought about must attest to the fragile specificity of the carboxylate binding pocket in glycopeptide antibiotics.

The pK_a perturbations on ligand binding, small as they are, do permit use of an indicator method to measure binding rates, as previously described.[5] For example, rate constants of association of ADLAA to vancomycin and ristocetin were found to be 1.4×10^7 and 8.5×10^6 s^{-1} M^{-1}, respectively, by this method. The analogous rates for AALAA were 2.8×10^7 and 1.5×10^7 s^{-1} M^{-1}.

Rates of Association of Other Ligands. The fluorescent peptide ADLAA can also, in principle at least, be used in competition experiments to obtain the rate constants of binding of nonfluorescent ligands. In order to do this, a solution containing both ADLAA and the nonfluorescent peptide was mixed with a vancomycin solution in the stopped-flow apparatus. Under these circumstances, the trace of fluorescence with time is sensitive to the rate of association of the nonfluorescent ligand with the vancomycin under a rather narrow set of conditions. The desired sensitivity requires first that the common component, V, of the two competing reactions of Scheme III, be depleted by at least one of the reactions; in this way the reactions will be significantly coupled. It was convenient in these experiments to maintain ADLAA, as a reporter ligand, at a low concentration (1.0 μM). The requisite coupling could then be achieved by the thermodynamic requirement that $[P]_0 \geq K_2^{-1} + [V]_0$.

Scheme III

$$D + V \underset{k_{r1}}{\overset{k_{f1}}{\rightleftharpoons}} DV \qquad K_1 = k_{f1}/k_{r1}$$

$$P + V \underset{k_{r2}}{\overset{k_{f2}}{\rightleftharpoons}} PV \qquad K_2 = k_{f2}/k_{r2}$$

V = vancomycin D = ADLAA

P = nonfluorescent peptide

Table II. Effects of Glycerol and Ethanol on the Rate and Equilibrium Constants of ADLAA Binding to Vancomycin[a]

solvent	η/η (H_2O)	ϵ	$k_f \times 10^{-6}$, s^{-1} M^{-1}	k_r, s^{-1}	$K \times 10^{-5}$, M^{-1}
H_2O	1.0	78.5[d]	9.3	31	3.0
30% (w/v) glycerol	3.1[b]	71.3[e]	1.77	16.5	1.07
15% (w/v) ethanol	1.7[c]	70.3[d]	2.14	15.0	1.43
60% (w/v) glycerol	8.8[b]	61.5[e]	2.11	30.0	0.71
30% (w/v) ethanol	2.4[c]	61.6[d]	4.30	26.0	1.66

[a] 0.1 M phosphate buffer, pH 7.0, 25 °C; the rate and equilibrium constants refer to Scheme I. [b] Determined by means of an Ostwald viscometer by B. George in this laboratory; 25 °C. [c] Reference 41; 25 °C, in the absence of buffer. [d] Reference 42; 25 °C, in the absence of buffer. [e] Reference 43; 20 °C, in the absence of buffer.

A kinetic requirement also applies, viz. that the pseudo-first-order rate constant for formation of DV is at least as great as that of PV, i.e., $(k_{f1}[V]_0 + k_{r1}) \geq (k_{f2}[V]_0 + k_{r2})$; if this were not so, the rates of fluorescence change would reflect only the formation of DV. Finally, in our hands, the method was restricted by the stopped-flow apparatus to first-order rate constants of ≤500 s^{-1}. Led by these considerations, we were able to find conditions ($[V]_0$ = 10.0 μM, $[P]_0$ = 10.0 μM) whereby the rate constant for association of vancomycin and AALAA could be determined; this procedure yielded a value of 3.4×10^7 s^{-1} M^{-1}, which is in good agreement with the value quoted above from the indicator/proton uptake method.[5] Numerical simulation showed that the observed fluorescence changes should indeed be sensitive to the k_{f2} value, in the range of 10^6–10^8 s^{-1} M^{-1}, under the above concentration conditions.

The method was not useful however for *N*-acetyl-D-alanyl-D-alanine, which, at concentrations of 100 μM and above (needed to satisfy the thermodynamic requirement), generated data from which a unique and robust value of k_{f2} could not be obtained. This suggests, from the kinetic condition, a k_{f2} value of $\geq 10^7$ s^{-1} M^{-1}. Numerical simulation again showed the self-consistency of this conclusion. For weaker binding ligands in general, a method of following faster reactions, e.g., temperature jump, would therefore be needed.

A Kinetic Mechanism of Binding. The second-order rate constants for association of ADLAA (and AALAA) with the glycopeptide antibiotics (Table I) are much smaller than would be expected for a diffusion-controlled reaction. For example, the Smoluchowski equation[15] yields a rate constant of 6.9×10^9 s^{-1} M^{-1} for association of ADLAA and vancomycin at 25 °C (assuming charges of +1 and −1, radii of 6.3 and 2.3 Å, and diffusion coefficients of 15×10^7 and 40×10^7 cm^2 s^{-1}, for vancomycin and ADLAA, respectively). Although this rate constant estimate should be decreased somewhat to take account of the fact that the binding site on vancomycin does not cover its entire surface, the correction factor involved, taken to be the linear extension of the target site,[16] and estimated from models of the vancomy-

(10) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, 773–788.
(11) Sitrin, R. D.; Chan, G. W.; Dingerdissen, J. J.; Holl, W.; Hoover, J. R. E.; Valenta, J.; Webb, L.; Snader, K. M. *J. Antibiot.* **1985**, *38*, 561–571.
(12) Perkins, H. R. *Biochem. J.* **1969**, *111*, 195–205.
(13) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *123*, 789–803.
(14) Popieniek, P. H. Ph.D. Thesis, Wesleyan University, 1988.

(15) (a) von Smoluchowski, M. *Z. Phys. Chem. (Leipzig)* **1917**, *92*, 129–168. (b) Hiromi, K. In *Kinetics of Fast Enzyme Reactions*; Halsted Press: New York, 1979; pp 256–261.

cin/AALAA complex to be approximately 0.7, does not reduce the calculated rate constant significantly; the corrected value (4.8×10^9 s^{-1} M^{-1}) is still at least 2 orders of magnitude above the observed values. The enthalpy of activation for ADLAA binding to vancomycin (given above) appears to be slightly too large, but not impossibly so, for a diffusion-controlled reaction.[17,18]

Further evidence for the unimportance of diffusion processes in these association rates is demonstrated by the data of Table II. Although the rate constant for association decreases in 30% glycerol, which has a viscosity some 3 times that of water, no additional decrease in rate is seen in 60% glycerol with a viscosity nearly 9 times that of water. The rate decreases observed here largely reflect decreases in the association equilibrium constant since the dissociation rate constant is largely unchanged. These rate constants are also similar to those in 15% and 30% ethanol solutions, whose viscosities are less than those of the glycerol solutions, but which have bulk dielectric constants similar to those of 30% and 60% glycerol, respectively. It seems likely from these data that viscosity per se has little effect on the observed rate constants, and thus the association reaction of the glycopeptide antibiotics cannot be either diffusion or pseudodiffusion[19] controlled. Solvent effects on the rates and equilibria are discussed in detail below.

The association reaction of vancomycin and ADLAA must therefore consist of at least two distinct steps, a diffusion together of the glycopeptide and ligand and another, rate-controlling, step. The two likely possibilities are represented by mechanisms 1 and 2. In the first, the rate-determining step is seen as the rearrangement of an initial "loose" complex, whose formation is diffusion controlled. In the second, the slow step is the rearrangement of the free glycopeptide in solution to a less stable form, which then binds the peptide ligand at diffusion-controlled rates.

mechanism 1

$$D + V \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DV_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DV_2$$

mechanism 2

$$V \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} V' + D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DV'$$

Since only a single pseudo-first-order phase of reaction is observed under conditions of $[V]_0 \gg [D]_0$, either one step is much faster than the other, or a steady-state situation, where the intermediate species DV_1 or V′ remains at low concentration, obtains. These possibilities are considered in turn with an expression for the observed pseudo-first-order rate constant, k_{obs} ($k_f[V]_0$), given in each case.

1. Mechanism 1. (a) Step 1 as a fast equilibrium

$$k_{obs} = \frac{k_2[V]_0}{[V]_0 + K_1^{-1}} + k_{-2}$$

Since no saturation in $[V]_0$ is observed, we must have $K_1^{-1} \gg [V]_0$ and thus

$$k_{obs} = k_2K_1[V]_0 + k_{-2} \quad (1)$$

(b) Step 2 as a fast equilibrium

$$k_{obs} = k_1[V]_0 + \frac{k_{-1}}{1 + K_2} \quad (2)$$

(c) PV_1 as a steady-state intermediate

$$k_{obs} = \frac{k_1(k_2 + k_{-2})[V]_0}{k_{-1} + k_2 + k_1[V]_0} + \frac{k_{-1}k_{-2}}{k_{-1} + k_2 + k_1[V]_0}$$

A reaction first order in $[V]_0$ can only be obtained by the assumption that $(k_{-1} + k_2) \gg k_1[V]_0$, which leads to eq 3.

$$k_{obs} = \frac{k_1(k_{-2} + k_2)[V]_0}{k_{-1} + k_2} + \frac{k_{-1}k_{-2}}{k_{-1} + k_2} \quad (3)$$

2. Mechanism 2. (a) Step 1 as a fast equilibrium

$$\frac{k_2[V]_0}{1 + K_1^{-1}} + k_{-2} \quad (4)$$

(b) Step 2 as a fast equilibrium

$$k_{obs} = k_1 + k_{-1}K_2^{-1} \quad (5)$$

This assumes tight ($K_2[D] \gg 1$) binding in the second step, which is required in order to achieve first-order kinetics.

(c) V′ as a steady-state intermediate

$$k_{obs} = \frac{k_1k_2[V]_0}{k_{-1} + k_1 + k_2[V]_0} + \frac{k_{-2}(k_1 + k_{-1})}{k_{-1} + k_1 + k_2[V]_0} \quad (6)$$

For first-order behavior in $[V]_0$, $(k_1 + k_{-1}) \gg k_2[V]_0$, and hence eq 6 reduces to eq 4.

The requirement for first-order dependence in $[V]_0$ and the absence of a viscosity effect eliminates all possibilities but situation 1a (eq 1). Thus, the likely reaction pathway (mechanism 1) involves a rapid, diffusion-controlled preequilibrium association of glycopeptide and ligand, followed by a slower, rate-controlling rearrangement of the initial encounter complex.

Since no saturation in $[V]_0$ is seen up to 100 μM, it follows from eq 1 and the data of Table I that $K_1 \leq 10^4$ M^{-1}, and hence $k_2 \geq (1.86 \pm 0.32) \times 10^3$ s^{-1}, and that $k_{-2} = (31 \pm 5)$ s^{-1}, and hence $K_2 \geq 60$. Further, assuming that k_1 has achieved the diffusion-controlled limit (see above), it follows that $k_{-1} \geq 4.8 \times 10^5$ s^{-1}.

Nature of the Rate-Determining Step. The rearrangement of DV_1 to DV_2 (mechanism 1) would most likely involve either a conformational change or changes, a desolvation or solvent rearrangement process, or some combination of the two. Since the rate constant for ADLAA binding to vancomycin is very similar to that of AALAA, the process is unlikely to specifically involve the dansyl group of ADLAA.[5] Further, since the conformation of the bound ligand seems, from modeling based on NMR studies, to be a low-energy extended form,[2] any slow conformational change would likely mainly involve the glycopeptide.

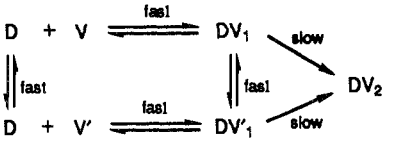
There is much NMR evidence for the existence in solution of more than one conformer of several of the glycopeptide antibiotics. For example, there seems good evidence that the N-terminus of vancomycin is mobile in solution.[20] The mobility of ristocetin seems less because of the cross-linking of the N-terminal residues, but the ring thus formed is mobile.[21,22] The sugar residues are also likely to be in motion.[23] Conformational changes on ligand binding have also been indicated from NMR evidence, especially in vancomycin, where the N-terminus becomes organized into a carboxylate binding site;[20,24] the N-terminus of ristocetin may also lose flexibility on ligand binding.[22] These results certainly suggest that the binding of a ligand to glycopeptide antibiotics might

(16) Berg, O. G.; von Hippel, P. H. *Annu. Rev. Biophys. Biophys. Chem.* **1985**, *11*, 131–160.
(17) Fenichel, I. R.; Horowitz, S. B. *Ann. N.Y. Acad. Sci.* **1965**, *125*, 290–297.
(18) Eftink, M. R.; Ghiron, C. A. *Biochemistry* **1977**, *16*, 5546–5551.
(19) Garst, J. F. *J. Chem. Soc., Chem. Commun.* **1987**, 589–590.
(20) Waltho, J. P.; Williams, D. H.; Stone, D. J. M.; Skelton, N. J. *J. Am. Chem. Soc.* **1988**, *110*, 5638–5643.
(21) (a) Williamson, M. P.; Williams, D. H.; Hammond, S. J. *Tetrahedron* **1984**, *40*, 569–577. (b) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332–1339. (c) Fesik, S. W.; O'Donnell, T. J.; Gampe, R. T., Jr.; Olejniczak, E. T. *J. Am. Chem. Soc.* **1986**, *108*, 3165–3170.
(22) Williamson, M. P.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 1* **1985**, 949–956.
(23) Kannan, R.; Harris, C. M.; Harris, T. M.; Waltho, J. P.; Skelton, N. J.; Williams, D. H. *J. Am. Chem. Soc.* **1988**, *110*, 2946–2953.
(24) (a) Convert, O.; Bongini, A.; Feeney, J. *J. Chem. Soc., Perkin Trans. 2* **1980**, 1262–1270. (b) Williams, D. H.; Butcher, D. W. *J. Am. Chem. Soc.* **1981**, *103*, 5697–5700. (c) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332–1339. (d) Waltho, J. P.; Cavanagh, J.; Williams, D. H. *J. Chem. Soc., Chem. Commun.* **1988**, 707–709. (e) Waltho, J. P.; Williams, D. H. *J. Am. Chem. Soc.* **1989**, *111*, 2475–2480.

involve more than a rigid lock-and-key process. However, it should be noted that isomerism of free glycopeptide cannot, from the above analysis, be rate limiting, at least in the binding of ADLAA to vancomycin; this might not be true in all cases, of course.

It is certainly possible however, in view of the NMR evidence, that rapid conformational preequilibria do precede the rate-determining process, and that these are partially responsible for the apparently slow binding. Nonetheless, subsequent binding cannot be diffusion controlled, as demonstrated by the viscosity experiments. Weak binding to more than one conformer may also lead, via conformational changes concerted with the slow-binding step, to the same final complex, e.g., mechanism 3.

mechanism 3



If it is assumed that the slow binding of ADAA to vancomycin, ristocetin A, and α-avoparcin all derives from the same source, it seems unlikely that this source is solely a conformational change, since such a common conformational change has yet to be identified from structural considerations. In particular, the structural differences between the N-termini of these three antibiotics, where the greatest flexibility of these structures seems to lie (except for that of the pendant sugars perhaps), do not predict a significant common conformational event. Nonetheless, there is no direct evidence at this time against some conformational component to the energy barrier to binding.

Solvent Effects on the Kinetics and Thermodynamics of Binding. The binding of peptide ligands to the glycopeptide antibiotics is thought to involve hydrogen-bonding, electrostatic interactions and hydrophobic interactions.[2] A change in solvent would, in general, perturb all of these. Organic solvents tend to decrease the bulk dielectric constant of the medium and thus enhance electrostatic interactions and hydrogen bonding.[25] Cosolvents may also be competitive hydrogen bond donors or acceptors, of greater or lesser effectiveness than water. Finally, organic solvents may affect the stability of hydrophobic interactions. The latter can occur either by direct interaction of the hydrophobic surfaces with the organic solvent or by a change in the solvent structure. The stability of a complex of glycopeptide antibiotic and peptide ligand might well therefore be affected by the addition of organic solvents.

It is also possible that the kinetics of the binding process would be affected by solvent perturbation. Two extremes can be imagined for the main contribution to the activation energy of binding two flexible polar molecules in water. The first is that the dominant energy barrier arises from conformational rearrangement of the reactants, as considered above, and the second is that desolvation or solvent rearrangement is the dominant barrier. In cases of binding a strongly solvated molecule, such as a metal ion, to a rigid ligand, it is likely that the second case applies.[26,27] With less organized ligands and less polar solvents, the contribution of ligand reorganization increases.[28] In many cases it is thought likely that the two are concerted to a greater or lesser extent.[27,29-32] It is impossible, for example, that complete desolvation of a metal

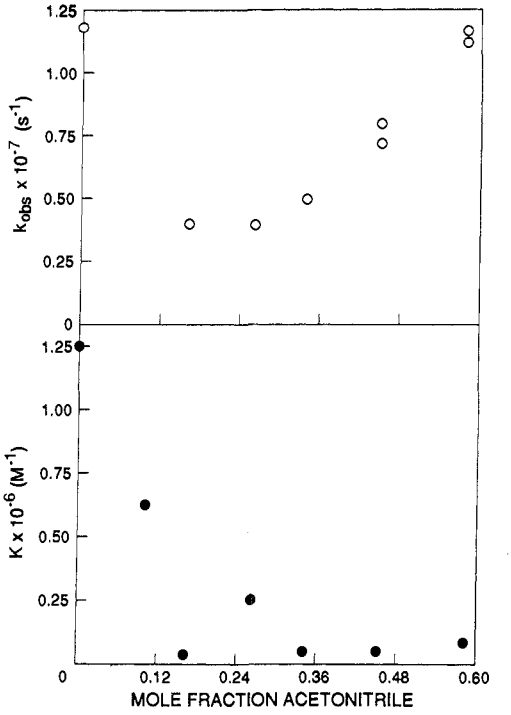


Figure 3. Effect of increasing mole fractions of acetonitrile on the rate (○) and equilibrium (●) constants of association of ADLAA with vancomycin.

ion occurs prior to some binding to the ligand to provide energy compensation.[30] Any asynchrony however between desolvation and binding, derived for example from the structure and dynamics of the ligand, would likely lead to significant energy barriers.[33,34] The situation with respect to peptide–peptide interactions in aqueous solution, where charge–charge, hydrogen-bonding and hydrophobic interactions may all be involved, is probably complex. Desolvation is required as part of all three types of interaction. It seems likely that, as with the metal ions, and particularly with extended peptides, complete desolvation cannot occur prior to any direct interaction between the two components of the complex. These considerations have given rise to concerted schemes, often referred to as "zippering", where desolvation and complex formation occur in a stepwise fashion, e.g., one hydrogen bond at a time.[35] Williams and co-workers[21a] have proposed such a zipperlike mechanism for the binding of peptides to the glycopeptide antibiotics. In such a process it is clear that desolvation of the components is an important requirement but it is not clear at present, at least in this case, and probably in most, including the binding of peptides to proteins, to what extent desolvation per se contributes to the energy barrier for binding. With these considerations in mind, we undertook some experiments in mixed organic aqueous solvents.

The solvents chosen were methanol, dimethyl sulfoxide, and acetonitrile. They have similar dielectric constants. Methanol, like water, is a hydrogen-bond donor and acceptor, dimethyl sulfoxide is a strong hydrogen-bond acceptor, while acetonitrile is a very weak hydrogen-bond acceptor. All form mixtures of complex structure with water, such that the effects of changes in solvent composition must be interpreted very carefully.[36]

Figure 3 shows the effect of increasing acetonitrile concentrations on the equilibrium and rate constants of formation of the complex between vancomycin and ADLAA. It is important first to distinguish thermodynamic and kinetic effects, i.e., effects predominantly on ground-state structures from those on transition states. It seems, for example, that the decrease in rate of formation

(25) Williamson, M. P.; Williams, D. H. *Eur. J. Biochem.* **1984**, *138*, 345–348.
(26) Cox, B. G.; Garcia-Rosas, J.; Schneider, H. *J. Am. Chem. Soc.* **1981**, *103*, 1054–1059.
(27) Cram. D. J. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1039–1057.
(28) Rebek, J., Jr.; Luis, S. V.; Marshall, L. R. *J. Am. Chem. Soc.* **1986**, *108*, 5012–5014.
(29) Diebler, H.; Eigen, M.; Ilgenfritz, G.; Maas, G.; Winkler, R. *Pure Appl. Chem.* **1969**, *20*, 93–115.
(30) Chock, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1939–1942.
(31) Fux, P.; Lagrange, J.; Lagrange, P. *J. Am. Chem. Soc.* **1985**, *107*, 5927–5931.
(32) Maynard, K. J.; Irish, D. E.; Eyring, E. M.; Petrucci, S. *J. Phys. Chem.* **1984**, *88*, 729–736.
(33) Bernasconi, C. F. *Acc. Chem. Res.* **1987**, *20*, 301–308.
(34) Kurz, J. L. *J. Am. Chem. Soc.* **1989**, *111*, 8631–8639.
(35) Burgen, A. S. V.; Roberts, C. G. K.; Feeney, J. *Nature* **1975**, *253*, 753–755.
(36) Blandamer, M. J. *Adv. Phys. Org. Chem.* **1977**, *14*, 203–352.

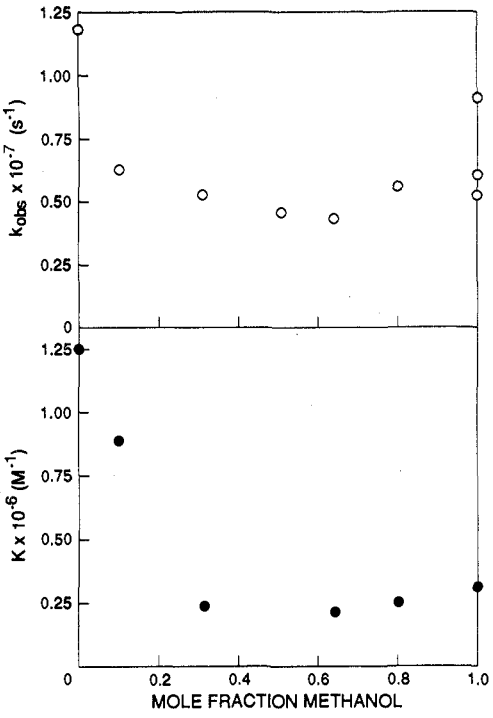


Figure 4. Effect of increasing mole fractions of methanol on the rate (○) and equilibrium (●) constants of association of ADLAA with vancomycin.

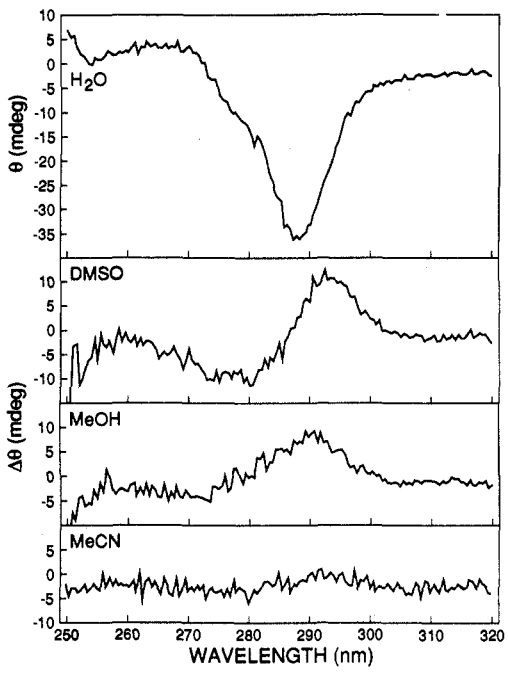


Figure 5. CD spectrum of vancomycin (0.2 mM; 1 cm cell path length) in aqueous solution between 250 and 320 nm (upper panel) and difference spectra (aqueous − mixed solvent) in 0.5 mol fraction acetonitrile, 0.5 mol fraction methanol, and 0.3 mol fraction dimethyl sulfoxide.

of the complex on addition of acetonitrile to a mole fraction, X_s, of 0.2 reflects transmission to the transition state of part of the greater decrease in equilibrium constant in this solvent region. The cause of this change will be discussed below. Of greater kinetic significance is the observation that k_f increases significantly as X_s increases from 0.2 to 0.6 although K shows little change; k_r must therefore also increase with X_s. The solvent change in this region of X_s therefore stabilizes the transition state with respect to reactants and products. Since the bulk polarity of the solvent does not change significantly in this region,[37] it seems possible that the kinetic effect arises largely from the greater ease of desolvation of the peptides as acetonitrile replaces water. Although the peptides will presumably remain selectively hydrated, at least at hydrogen-bonding sites, this situation will become entropically more unfavorable, and the water perhaps more easily displaced. This seems to be a reasonably firm indication that desolvation is at least partly rate determining to formation of the final complex.

The response of ristocetin to acetonitrile (not shown) was essentially identical with that of vancomycin, with a decrease in both K and k_f at low X_s, and an increase in k_f, but not in K, at high X_s. This is further evidence that a similar process is rate determining to the binding of substrate to both antibiotics, and again therefore that an N-terminal conformational change is probably not the dominant contributor to the activation energy.

Figure 4 shows the response of vancomycin to methanol. Again, a decrease in both k_f and K, greater in the latter, occurs at low X_s. This change is followed in the region of $0.4 < X_s \leq 1.0$ by very little change, perhaps a very slight increase, in both k_f and K. If desolvation represents a significant part of the activation energy, then the ease of desolvation of methanol, in the regions of $X_s > 0.4$ at least, must be comparable to that of water. This would suggest that the breaking of hydrogen bonds might be an important part of the desolvation process, which seems not unreasonable for peptides.

Measurements in dimethyl sulfoxide were limited to $X_s < 0.4$ because of the weakness of the binding at higher X_s, and probably also by a decrease in the fluorescence change on binding. Both K and k_f decreased sharply with X_s to an X_s of 0.1, and then more slowly at higher values (not shown). No sign of any increase in k_f was observed at the higher X_s. This could be interpreted in terms of the difficulty of desolvation of dimethyl sulfoxide from peptides, but might also relate to changes in the vancomycin structure (see below).

The striking decrease in binding strength at low X_s, observed with all three cosolvents, probably correlates with their common destructive effect on the structure of water[36,38,39] and, presumably, therefore on the hydrophobic effect. The hydrophobic effect has been implicated in the peptide-binding reactions of glycopeptide antibiotics, both in the interaction of the alanyl methyl groups of D-alanyl-D-alanine ligands with the glycopeptides[21,25] and, more recently,[23] in the interactions of the pendant sugar residues with the ligands. Loss at low X_s of the favorable contributions to the binding energy created by these interactions would give rise to the observed results. Partial transmission of this change in the stability of the complex to the transition state, i.e., to k_f, presumably indicates the partial formation of hydrophobic structure in the transition state and the positive contribution of this process to the association rate. Further evidence for the role of the sugar residues in the changes in rate and equilibrium constants at low X_s comes from consideration of the case of aglucovancomycin. The removal of the sugar residues from vancomycin gives rise to a significant decrease in both K and k_f in water (Table I) with respect to those of vancomycin itself, and one of a magnitude comparable to the change in these parameters for vancomycin brought about by small amounts of the organic cosolvents. Further, no sharp decrease in either k_f or K occurred with aglucovancomycin on addition of small amounts, $X_s \leq 0.2$, of acetonitrile.

Solvent Effects on Structure. It was of course possible that the effect of organic cosolvents on the kinetics and thermodynamics of complex formation by the glycopeptide antibiotics reflected the effects of these solvents on the conformation of the free or bound peptides, with the most likely candidates being the free glycopeptides. Therefore CD spectra of vancomycin and ristocetin were taken in water, in 0.15 and 0.50 mol fraction acetonitrile, 0.5 mol fraction methanol, and 0.3 mol fraction dimethyl sulfoxide. Essentially no difference was observed between the spectra (210–300 nm) of vancomycin and ristocetin in water and in

(37) Balakrishnan, S.; Easteal, A. J. *Aust. J. Chem.* **1981**, *34*, 943–947.

(38) Cox, B. G. *J. Chem. Soc., Perkin Trans. 2* **1973**, 607–610.
(39) Ben-Naim, A. *J. Phys. Chem.* **1967**, *71*, 4002–4006.

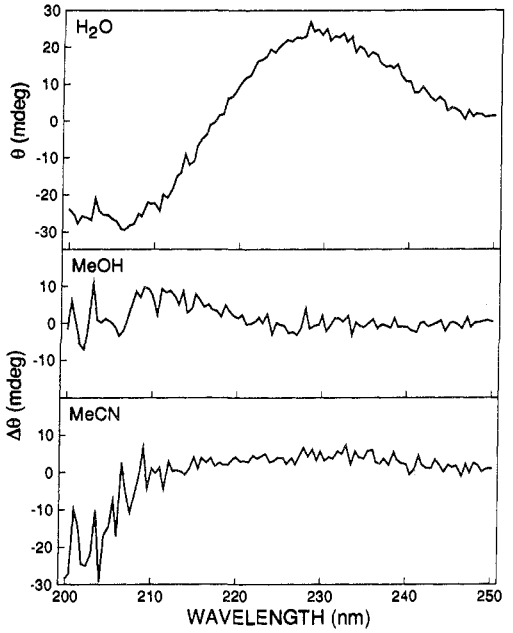


Figure 6. CD spectrum of vancomycin (10 μM; 1 cm cell path length) in aqueous solution between 200 and 250 nm (upper panel) and difference spectra (aqueous − mixed solvent) in 0.5 mol fraction acetonitrile and 0.5 mol fraction methanol.

aqueous acetonitrile. Figures 5 and 6, for example, show the CD spectrum of vancomycin in water and the difference spectrum between that and the spectrum in 0.5 mol fraction acetonitrile. Although addition of AALAA induced spectral changes in the CD spectra of these antibiotics in the aromatic region (270–300 nm), very similar changes were induced in ristocetin in 0.6 mol fraction acetonitrile as in water; a comparable spectrum of the vancomycin complex could not be taken due to its lesser solubility in this solvent mixture. These observations give no indication of substantial conformational changes in vancomycin and ristocetin on addition of acetonitrile to aqueous solutions. Methanol appeared to induce a small change in intensity in the aromatic absorption region of the spectrum (Figure 5), which may reflect some change in position of the aromatic rings. Any such change did not seriously affect the strength or rate of binding however, as discussed above. In aqueous DMSO, the aromatic CD peak changed its position rather significantly to a higher wavelength (Figure 5). This change may relate to the weaker and slower binding in this solvent mixture. Spectra at lower wavelength in aqueous DMSO could not be obtained because of strong solvent absorption.

Concluding Discussion. Measurement of the rates and equilibria of binding of peptides to the glycopeptide antibiotics has been facilitated by employment of ADLAA. Although the binding reaction appears to be kinetically second order under all conditions employed in this study, the rate-determining step does not appear to be diffusion of peptide and antibiotic together. This follows from consideration of the magnitude of the second-order rate constants of the binding process, which are too small for a diffusion-controlled process, and from the lack of significant effect of solvent viscosity on the rate of one such reaction, the association of vancomycin with ADLAA. Since similar slow rates were observed for vancomycin, aglucovancomycin, ristocetin A, and α-avoparcin, it seems likely that a comparable process is rate determining to all glycopeptide antibiotics of the vancomycin class. Analysis of the kinetics suggests that a two (at least) step binding process must occur, consisting of rapid, loose preequilibrium binding followed by slow rearrangement of the initial complex. Although fast conformational preequilibria may also be present, and contribute to the apparent slow binding, these processes cannot be solely rate determining.

There seems no clear evidence at present that conformational rearrangement of the initial complex is cleanly rate determining to the binding of peptides by the glycopeptide antibiotics. In order to achieve sufficient binding strength to be effective antibiotics, the glycopeptides have to be significantly rigid, a situation that is achieved by oxidative cross-linking of aromatic amino acid substituents. Such flexibility as remains to them appears to be centered at the N-terminus, where the apparent flexibility increases in the order ristocetin A < α-avoparcin < vancomycin. It is difficult to see a conformational event common to all three and yielding rate constants as similar as they are (Table I). The latter situation could perhaps be achieved by a fortuitous combination of several pre- and post-peptide-contact conformational events, but this could only be demonstrated by further experiments. Since a comparable low rate of association is also seen with aglucovancomycin (Table I), the pendant sugars cannot be a limiting factor in the rate-controlling step.

The results in aqueous acetonitrile, for both vancomycin and ristocetin A, do however support a common desolvation or solvent rearrangement process as being important to the rate-determining step, and the results in methanol suggest that rearrangement of hydrogen bonds may be involved. The aglucovancomycin result also focuses attention on desolvation of the peptide backbone of the antibiotics rather than the substituent sugar residues, although the latter probably do make contact with the ligand in the final complex.[23] The above is not to say that conformational changes do not accompany the binding process, since it is clear that they must, particularly in vancomycin,[20,24] but the dominant contribution to the energy barrier of binding seems more likely to be desolvation or solvent rearrangement. The latter might well be concerted with facile conformational changes, giving rise to the "zippering" mechanism discussed by others.[21,35]

This result may also apply in many examples of the general phenomenon of ligands, particularly peptides or proteins, binding to proteins. In these situations, rate constants comparable to those found here are often observed.[40] Although such rates have usually been interpreted in terms of conformational events,[40] and indeed there is good evidence in many specific cases that such events do occur, some contribution from desolvation of protein and/or ligand also seems likely. It may be that in cases where tight binding is the goal, for example, in the binding of certain hormones to receptors, or antigens to antibodies, desolvation of a rather rigid template will be largely rate determining, whereas in enzymes, where tight binding of substrates is not normally an important criterion, and where protein flexibility may be important to catalysis, conformational events may be more likely to control the rate.

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Registry No. ADLAA, 111290-37-0; AALAA, 24570-39-6; vancomycin, 1404-90-6; ristocetin A, 11021-66-2; α-avoparcin, 73957-86-5; aglucovancomycin, 55598-74-8.

(40) Reference 15b, pp 262–271.
(41) Barr, G. In *International Critical Tables of Numerical Data; Physics, Chemistry, Technology*; Washburn, E. W., Ed.; McGraw-Hill: New York, 1929; Vol. 5, p 22.
(42) Hall, J. L.; Phillips, H. O. *Proc. W. Va. Acad. Sci.* **1954**, *26*, 26–27.
(43) Douzou, P. In *Cryobiochemistry. An Introduction*; Academic Press: London, 1977; p 37.