Dimerization of A82846B, Vancomycin and Ristocetin: Influence on Antibiotic Complexation with Cell Wall Model Peptides

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The thermodynamics of glycopeptide antibiotic dimerization have been studied by means of sedimentation equilibrium, using A82846B, vancomycin, ristocetin and complexes formed with several cell wall model peptides. These results indicate that vancomycin dimerization can be strongly promoted in two ways: i) stabilization of the antibiotic conformation in which the carbonyl group of residue three is on the back face of the molecule and ii) preferential interaction of the dimer with the lysine residue of *N,N'*-diacetyl-lysyl-D-alanyl-D-alanine. This effect was not found in ristocetin. A82846B forms stable dimers at very low antibiotic concentration. Two conformational forms have been found for complexed A82846B by ^{1}H NMR. However, calorimetric binding experiments have shown that all its binding sites are thermodynamically equivalent. The affinity of the A82846B dimer for the tripeptide has been estimated to be about $3\,kJ\cdot mol^{-1}$ higher than that of the vancomycin monomer and about $-2.6\,kJ\cdot mol^{-1}$ lower than that of dimeric vancomycin. The possible role of dimerization in the biological activity of glycopeptide antibiotics[1] is discussed further on the basis of present thermodynamic data.

The glycopeptide group of antibiotics is becoming increasingly important in the treatment of infections caused by Gram-positive organisms, especially those which are β-lactam resistant. Although most of these organisms are susceptible to vancomycin and teicoplanin (both under clinical use), tolerance of, and resistance to these agents has been reported[2~4]. Therefore, alternative agents with potent activity against Gram-positive organisms are of considerable interest. Recently, dimerization of glycopeptide antibiotics and their peptide complexes have been reported and the possibility of a role in the *in vivo* action of these antibiotics has been postulated[5~7].

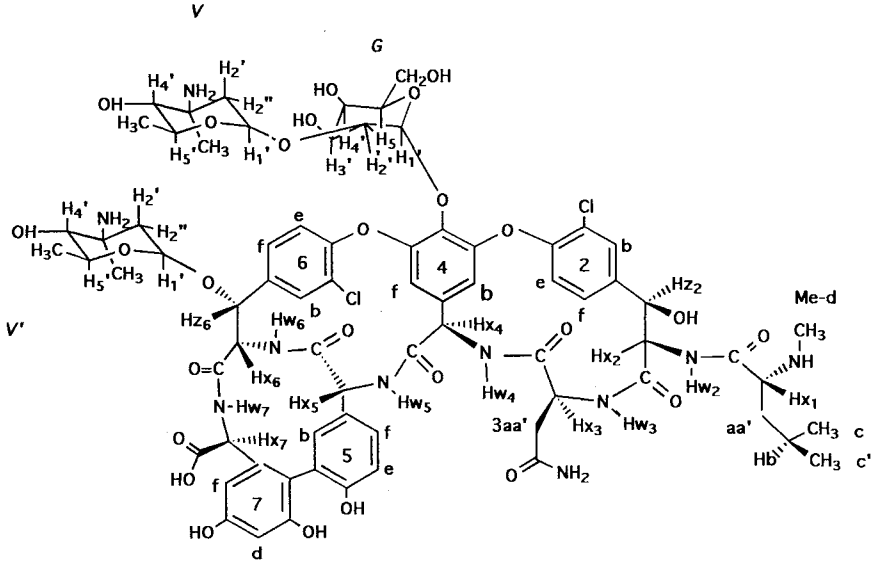
The model peptides *N*-Acetyl-D-Ala-D-Ala and di-*N*-acetyl-L-Lys-D-Ala-D-Ala have been used extensively to study the intermolecular interactions that characterize complex formation by NMR.[8~12] The thermodynamics of glycopeptide-peptide complexation have been studied using vancomycin, ristocetin and teicoplanin antibiotics[13,14]. Dimerization of glycopeptide antibiotics involves hydrogen bonding between the non binding faces of two glycopeptide molecules[5~7]. Recently, quantitative studies of eremomycin dimerization by NMR have shown the effect of attached sugars on the stabilization of these dimers[7].

A82846B is a naturally occurring glycopeptide isolated from *Amycolatopsis orientalis*[15]. Its chemical structure (Fig. 1) is similar to that of vancomycin except that it contains two 4-L-epi-vancosamine moieties instead of one[16]. *In vitro* activity of A82846B is comparable to that of teicoplanin, but it is two to five times more active than vancomycin[16].

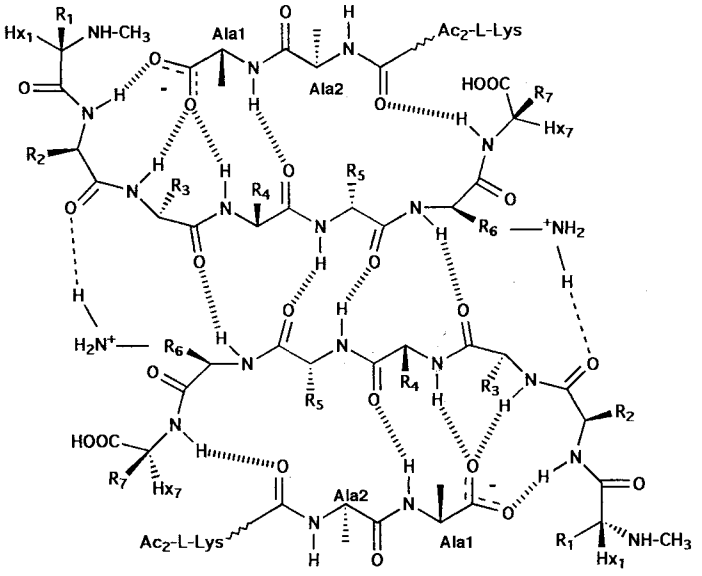
This paper deals with the thermodynamic characterization of the complexes formed between monomeric or dimeric glycopeptide antibiotics and cell wall model-peptides. This information is of major importance in order to determine, to what extent, dimerization may correlate with an enhancement of affinity toward bacterial cell wall precursors or biological activity. The dimerization equilibrium constants of A82846B, and free and bound ristocetin and vancomycin are reported, as well as the thermodynamic parameters for ligand binding to either monomeric and dimeric vancomycin and ristocetin. The thermodynamics of A82846B complexation with different peptides have been also determined and the partitioning of the energetic contributions (ΔG, ΔH, ΔS) to ligand binding is presented. In addition, the structures of free A82846B and its Ac_2-L-Lys-D-Ala-

Fig. 1

A. Structure of A82846B including the code used to designated ^1H NMR resonances.



B. Representation of the hydrogen bonding network of the dimer formed between two molecules of A82846B when bound to Ac_2-L-Lys-D-Ala-D-Ala, following the scheme employed in reference 25.



Ligand residues are labeled at the Hα proton. The broken lines indicate the position of intermolecular hydrogen bonds.

D-Ala complex have been characterized in aqueous solution. The reported data are discussed, in order to evaluate how the structural differences influence the energetics of dimerization and of peptide binding. Finally, the previously proposed role of dimerization in the biological activity of these antibiotics and eremomycin[1,7,17] is also discussed on the basis of present thermodynamic data.

Results

Sedimentation Equilibrium

The dimerization constants of the free antibiotics and their complexes were directly determined by sedimentation equilibrium. Dimerization of A82846B in 0.1 M phosphate buffer at pH 7.0 was quantitatively analyzed using antibiotic concentrations ranging from 1 μM to

1 mM. From the calculated dimerization constant of $7.5 \times 10^6\ M^{-1}$ (Table 1), it is evident that A82846B remains as a dimer at the antibiotic concentrations used in the present study. The dimerization constant of vancomycin is three orders of magnitude less (Table 1). Dimer formation is even less favorable at pH 5. This result suggests that protonation at the *N*-terminus reduces K_2 by a factor of 20. Tripeptide binding, at both neutral and acidic pH, promotes vancomycin dimerization ($K_2^L = 1.6 \times 10^5 \sim 4.6 \times 10^5\ M^{-1}$). In contrast, Ac-D-Ala-D-Ala binding to vancomycin, slightly modifies the antibiotic dimerization at neutral pH. In the case of ristocetin, the association constant is very similar to that obtained for vancomycin at neutral pH, but the presence of tripeptide has no significant effect on it (Table 1).

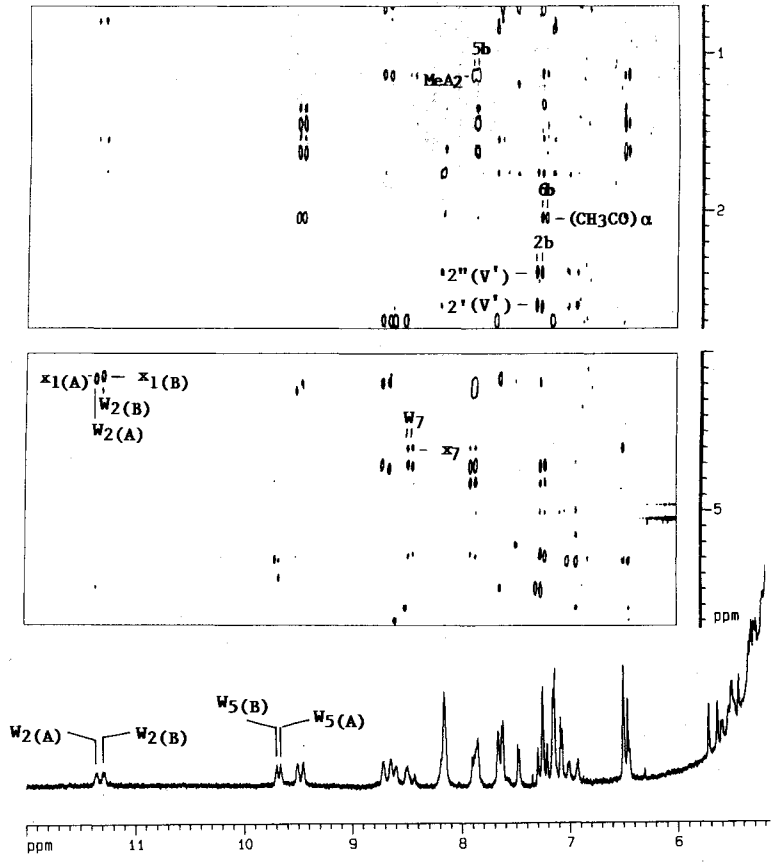
NMR Spectroscopy

The assignment of the ^1H NMR spectra of A82846B and A82846B/Ac_2-Lys-D-Ala-D-Ala complex was carried out mainly from data of two-dimensional experiments (COSY, TOCSY, NOESY and ROESY) at pH 4.3 in aqueous solution. The letter code used to identify each

Table 1. Equilibrium dimerization constants for A82846B, vancomycin and ristocetin by sedimentation equilibrium in 0.1 M sodium phosphate buffer pH 7.0.

Peptide	K_2 (M^{-1})[a]	95% Confidence limits
A82846B	7.5×10^6	$1.9 \times 10^6 \sim 4.6 \times 10^7$
Vancomycin	3888	3170 ~ 4750
Vancomycin/Ac_2-Lys-D-Ala-D-Ala	4.6×10^5	$2.7 \times 10^5 \sim 8.2 \times 10^5$
Vancomycin/Ac-D-Ala-D-Ala	8500	8064 ~ 9200
Vancomycin (pH 5)	192	148 ~ 238
Vancomycin/Ac_2-Lys-D-Ala-D-Ala (pH 5)	1.6×10^5	$1.1 \times 10^5 \sim 2.2 \times 10^5$
Ristocetin	4464	3888 ~ 5040
Ristocetin/Ac_2-Lys-D-Ala-D-Ala	4608	3600 ~ 5600

Fig. 2. 1D and 2D NOESY NMR spectrum (600 MHz) of the aromatic and amide proton region of A82846B-tripeptide complex in H_2O at 15°C.



Intraresidual, sequential and intermolecular NOEs are labeled.

Table 2. Proton chemical shifts (δ, ppm), coupling constants (J, Hz) and temperature coefficients of amide protons α(ppb/K) for A82846B-tripeptide complex (conformers A and B) and free antibiotic.

Proton	A82846B-Tripeptide complex B (δ, ppm)/(J, Hz) 298 K/pH 4.3	α(ppb/K) 278~298 K/pH 4.3	A82846B-Tripeptide complex B (δ, ppm)/(J, Hz) 298 K/pH 4.3	α(ppb/K) 278~298 K/pH 4.3	Free A82846B (δ, ppm)/(J, Hz) 298 K/pH 4.3	α(ppb/K) 278~298 K/pH 4.3
w_2	11.37/broad	0	11.31/broad	1.50	8.46	−2.67
w_5	9.70/broad	1.50	9.73/broad	1.0	9.52	−1.50
NH-Lys	9.25	−10.0	9.30	−10.5		
w_3	8.71	1.0	8.68	1.50	8.21	−3.72
w_4	8.69	4.0	8.57	3.0	7.95	−3.17
NH-Ala1	8.64	−4.0	8.59	−3.0		
w_7	8.47	−3.5	8.52	−2.5	9.01	−8.10
NHε-Lys	8.01	−7.0	8.01	−7.0		
5b	7.89		7.84		7.16	
NH-Ala2	7.76	−5.50	7.76	−5.0		
2f	7.65/d (7.8 Hz)		7.65/d (7.8 Hz)		7.67/d (8.9 Hz)	
NH_2(Asn)	7.52/broad		7.52/broad		7.45/broad	
NH_2(Asn)	6.96/broad		6.96/broad		6.65/broad	
w_6	7.41		7.53			
2e	7.25/d (7.8 Hz)		7.45/d (7.8 Hz)		7.48/broad	
2b	7.25/s		7.29/s		7.37/s	
6b	7.25/s		7.21/s		7.82/s	
5f	7.16/d (8.9 Hz)		7.16/d (8.9 Hz)		7.10/d (8.9 Hz)	
5e	7.07/d (8.6 Hz)		7.07/d (8.6 Hz)		7.03/d (8.7 Hz)	
6f	7.01/d (5.7 Hz)		6.93/d (5.7 Hz)		7.16/broad	
7f	6.52/s		6.52/s		6.52/s	
7d	6.47/s		6.47/s		6.57/s	
x_4	6.50		6.46		6.46	
4b	5.73/s		5.65/s		5.60/s	
1′(G)	5.58/d (6.5 Hz)		5.23/d (6.5 Hz)			
x_2	5.52				5.47	
z_2	5.50		5.50		5.58	
4f	5.44/s		5.33/s		5.40/s	
6e	5.36/d (5.7 Hz)		5.37/d (5.7 Hz)		5.36/broad	
1′(V)	5.36		5.30		5.34	
z_6	5.28		5.28		5.42	
1′(V′)	5.07 or 5.14		5.07 or 5.14		5.06	
x_3	5.03		5.03		4.96	
x_5	4.85		4.85		4.57	

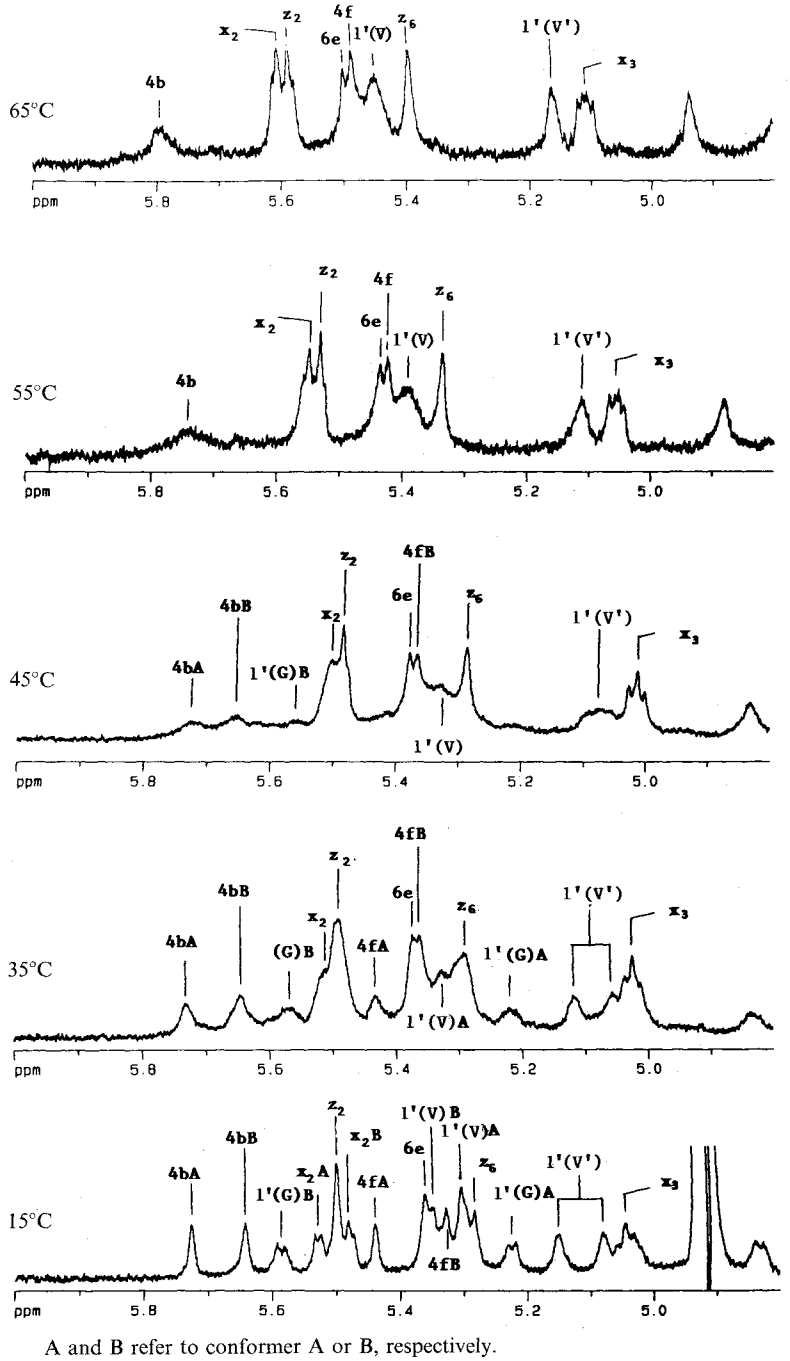
Proton	A82846B-Tripeptide complex A (δ, ppm)/(J, Hz) 298 K/pH 4.3	A82846B-Tripeptide complex B (δ, ppm)/(J, Hz) 298 K/pH 4.3	Free A82846B (δ, ppm)/(J, Hz) 298 K/pH 4.3
Hα-Ala2	4.71	4.75	
x_6	4.69	4.69	4.34
x_7	4.62	4.62	4.64
5′(V)	4.35	4.61	4.53
3′(G)	4.31	3.67	3.75 or 4.15
Hα-Lys	4.21	4.25	
2′(G)	3.77	4.23	3.75 or 4.15
Hα-Ala1	4.20	4.18	
x_1	4.18	4.18	4.26
5′(V′)	3.93	3.93	3.82
CH_2OH(G)	3.81, 3.32	3.89, 3.83	
4′(V′)	3.48	3.48	3.51
4′(V)	3.46	3.35	3.45
4′(G)		3.23	
CH_2ε-Lys	3.23	3.23	
Me-d	2.85/s	2.85/s	2.87/s
3aa′	2.68	2.68	2.60, 2.67
2′(V′)	2.59	2.59	2.51
2″(V′)	2.40	2.40	2.41
2′(V)	2.39	2.31	
2″(V)	2.13	2.08	
$(CH_3CO)\alpha$	2.05	2.05	
$(CH_3CO)\varepsilon$	2.02	2.02	
Me_3(V′)	1.76/s	1.76/s	1.65
aa′	1.74, 1.60	1.74, 1.60	1.85
$CH_2\beta$-Lys	1.65, 1.64	1.65, 1.64	
b	1.65	1.65	1.71
Me_5(V′)	1.52	1.52	1.41
Me_3(V)	1.48/s	1.20/s	1.10~1.50/broad
$CH_2\gamma$-Lys	1.45, 1.36	1.45, 1.36	
Me_5(V)	1.32	1.10	1.10~1.50/broad
Me-Ala2	1.12	1.13	
cc′	0.78, 0.84	0.78, 0.84	0.93
Me-Ala1	0.78, 0.84	0.78, 0.84	

proton in the antibiotic moiety is shown in Fig. 1. Ala1 (or A1) and Ala2 (or A2) refers to the alanine residues at the *C*-terminal and intermediate position of the ligand, respectively. An exhaustive analysis of data at pH 2.5 was also necessary to observe all exchangeable protons. The proton spectra of A82846B/Ac_2-L-Lys-D-Ala-D-Ala complex show the presence of two conformational forms, A and B, in an approximate ratio of 1 : 1 (see Fig. 2), which were completely identified on the basis of non-overlapping cross-peaks of the two species on the 2D-experiments.

Temperature coefficients of amide protons, α(ppb/K), were calculated in the range 5~25°C at pH 4.3 for A82846B/Ac_2-L-Lys-D-Ala-D-Ala complex and at pH 2.5 for free A82846B. Proton chemical shifts and temperature coefficients are summarized in Table 2.

In both free and complexed forms, the assignment of the peptide backbone protons in H_2O was achieved following the well-established sequence-specific methodology[18]. Spins systems were identified from phase-

Fig. 3. NMR spectra at variable temperature of the A82846B-Ac_2-L-Lys-D-Ala-D-Ala complex in the region 4.6~6.0 ppm showing the signals of coalescence of the protons 4b, 4f, 1′(G), 1′(V) and 1′(V′).



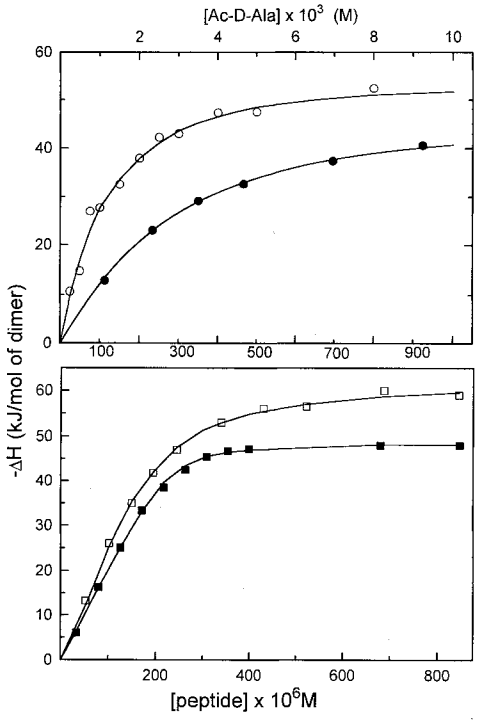
A and B refer to conformer A or B, respectively.

sensitive COSY and TOCSY experiments. The sequential assignment was carried out by identifying short-range NOEs involving NH(w), H_α(x) and H_β(z) protons in the NOESY spectrum. Some of these sequential NOEs are shown in the NOESY spectrum of Fig. 2. The five aromatic systems were identified from *J*-coupling correlations in the COSY and TOCSY spectra and were connected with the peptide backbone using long-range connectivities: 2b↔ z_2, 2f↔w_2, 5f↔w_5, 5b↔x_6, 6b↔x_6, 6f↔1′(V′) and 7f↔x_7. The spin system of ring 4, characterized by the unusual low chemical shifts of protons 4f and 4b (5.40 and 5.60, respectively)[19] was assigned on the basis of a medium NOE from x_5 to 4f. The three sugar spin systems were identified separately by successive *J*-coupling correlations starting from the anomeric protons 1′(V′), 1′(V) and 1′(G). Intersugar NOEs, characteristic of the disaccharide portion (1′(V)↔2′(G) and 1′(V)↔3′(G)), and long-range NOE connectivities involving protons of the vancosamine V′ and protons of the peptidic chain (1′(V′)↔z_6) allowed the assignment of the sugar moieties in positions 4 and 6. However, owing to the existence of two conformational forms of the complex, an additional study at variable temperatures (see Fig. 3) was necessary for the unambiguous assignment of some sugar and aromatic protons. Thus, protons 4b, 4f, 1′(V′), 1′(V) and 1′(G) were identified in each conformer by warming until coalescence of the couple of resonances (at about 45°C). Finally, information of ROESY experiments was used to assign the signals of 2f which exhibited chemical exchange cross-peaks corresponding to the aromatic proton 2e in each conformer.

Binding Studies

The titration of A82846B with Ac-D-Ala and the dipeptides was carried out using both calorimetric and spectrophotometric techniques. Fig. 4 shows the calorimetric titration curves, normalized to kJ per mol of antibiotic dimer. The experimental results were analyzed in order to derive both the enthalpy change and the binding constants, as previously described[13]. The saturation fraction is well described assuming two identical sites for peptide binding in the dimer. Solid lines in Fig. 4 correspond to the theoretical fits of the experimental data using the thermodynamic parameters reported in Table 3, expressed per mole of binding site. The association constants derived from spectrophotometric curves were in good agreement with those derived from calorimetric curves. The binding constant for the Ac_2-Lys-D-Ala-D-Ala-A82846B complex was determined from spectrophotometric titration curves and the enthalpy change of complexation was determined by using saturating Ac_2-Lys-D-Ala-D-Ala concentrations. The thermodynamic parameters for the tripeptide complex formation are presented in Table 3.

Fig. 4. Calorimetric titration of A82846B with Ac-D-Ala (○, upper scale), Ac-D-Ala-Gly (●), Ac-Gly-D-Ala (□), Ac-D-Ala-D-Ala (■).



The curves were calculated by the use of the thermodynamic parameters reported in Table 3.

Discussion

Structure of Dimeric A82846B

In agreement with the high dimerization constant calculated above, signals of monomeric A82846B in the ^{1}H NMR spectra were not observed.

In addition to NOEs which define the three-dimensional structure of A82846B backbone (free and complexed), the NOEs arising from the dimeric structure of the antibiotic were also observed (see Table 4 and Fig. 2 NOESY spectra). These NOEs, together with data provided by temperature coefficients (see Table 2), indicate that the dimer which was formed had the same hydrogen bond-network as that proposed for ristocetin and eremomycin (see Fig. 1)[6]. The low temperature coefficient of W_5 in both free and complexed A82846B confirmed its implication in dimer formation. Proton w_6 was also expected to be hydrogen bonded to the opposite $(CO)_3$ group, and a low temperature coefficient was found in both complexed forms. However, due to

Table 3. Thermodynamic parameters of peptide binding to A82846B and vancomycin[a] in 0.1 M-phosphate buffer, pH 7.0 at 25°C, expressed per mole of binding site.

Antibiotic	$-\Delta H_{app}$	$-\Delta H_M$	$-\Delta H_D$	$-\Delta G_{app}$	$-\Delta G_M$	$-\Delta G_D$	$-\Delta S_{app}$	$-\Delta S_M$	$-\Delta S_D$	Peptide
	kJ/mol			kJ/mol			J/mol·K			
A82846B	—	—	29	—	—	17.2	—	—	40	Ac-D-Ala
	—	—	34.6	—	—	26.3	—	—	28	Ac-Gly-D-Ala
	—	—	25.1	—	—	29.0	—	—	−14	Ac-D-Ala-D-Ala
	—	—	23.7	—	—	21.3	—	—	8	Ac-D-Ala-Gly
	—	—	32.3	—	—	33.9	—	—	−5	Ac_2-Lys-D-Ala-D-Ala
Vancomycin	32.0	29.0	30.5	14.0	11.5	12.5	60	59	60	Ac-D-Ala
	30.4	27.4	28.9	23.8	21.3	22.3	22	20	22	Ac-Gly-D-Ala
	31.7	28.7	30.2	25.4	22.9	23.9	21	19	21	Ac-D-Ala-D-Ala
	26.6	23.6	25.1	19.8	17.3	18.3	23	21	22	Ac-D-Ala-Gly
	45[b]	34.0	35.5	33.1[c]	30.7	36.5	40	11	−3	Ac_2-Lys-D-Ala-D-Ala

The subscripts M and D refers to monomeric or dimeric species, respectively, and ΔX_{app} is the experimental magnitude. [a] ΔX_{app} are from references 6d and 8; unless otherwise stated vancomycin concentration was 300 μM. [b,c] Experimental values determined at vancomycin concentrations of 127 and 22 μM, respectively.

Table 4. Selected NOEs inconsistent with a covalent monomeric structure.

Conformer A	NOE size	Conformer B	NOE size	Free Antibiotic	NOE size
2c↔Me_3(V′)	W				
2b↔2′(V′)	S	2b↔2′(V′)	S	2b↔2′(V′)	S
2b↔2″(V′)	S	2b↔2″(V′)	S	2b↔2″(V′)	S
2b↔Me_3(V′)	W	2b↔Me_3(V′)	W		
x_2↔2′(V′)	W	x_2↔2′(V′)	W		
x_3↔Me_3(V′)	S	x_3↔Me_3(V′)	S	x_3↔Me_3(V′)	S
x_3↔2′(V′)	W	x_3↔2′(V′)	W	x_3↔2′(V′)	W
x_3↔2″(V′)	W	x_3↔2″(V′)	W	x_3↔2″(V′)	W

Abbreviations: S, Strong; W, weak.

the lack of data of w_6 in the free antibiotic, it is not possible to demonstrate that this proton was involved in antibiotic dimerization rather than in peptide binding.

In addition to the NOEs reported for ristocetin and eremomycin, two weak NOEs between, 2b and Me_3(V′), and, x_2 and 2′(V′) were observed in both conformers of complexed A82846B. Moreover, a NOE between 2e and Me_3(V′) was found only in conformer A.

The NOEs which define the backbone structure of the *N*-terminal part of the antibiotic (in particular NOEs between NH_2(Asn) and cc′; 3aa′and aa and 3aa′and cc′) are the same for both free and complexed A82846B. These results and the low temperature coefficients found for w_2, w_3 and w_4 protons in the free antibiotic suggest a closed conformation for this part of the molecule.

Structure of the Complex A82846B/Ac_2-Lys-D-Ala-D-Ala

The binding of Ac_2-Lys-D-Ala-D-Ala caused extensive shielding effects on some parts of the A82846B molecule in comparison to the free antibiotic (see Table 2). The set of intermolecular NOEs, defining the structure of the complex in aqueous solutions (see Table 5), and the temperature coefficients observed for the NH proton of Ala 1, w_2, w_3, w_4 and w_7, show that the main structural features are very similar to those previously reported for vancomycin and ristocetin (Fig. 1)[8~11]. Nevertheless, some important differences are observed concerning the interactions established through the *N*-terminal *N*-methylleucine, the sugar moiety attached to residue 4 and the Ac_2-lysine residue of the tripeptide.

Qualitative evaluation of all NOEs involving Ala-1 protons confirm its proximity to residue 1 and the aromatic ring of 2. These NOEs are somewhat different from those found in vancomycin, but are coincident with the results reported for *N*,*N*′-diacetylvancomycin/Ac_2Lys-D-Ala-D-Ala.[20] They suggest that the *N*-methylleucine side chain folds in the direction of the Ala1 residue binding site, providing an extended hydrophobic wall to the carboxylic pocket. The location of the Ala2 residue is defined by a strong NOE between Me-Ala2 and the aromatic proton 5b (see NOESY spectra in Fig. 2) and other lower intensity NOEs with aromatic protons of residue 7, x_5 and x_6. No NOEs were found between this residue and the *N*-terminal region of the antibiotic.

The location of the Lys residue in A82846B complex is defined by the NOEs 6b↔NHα-Lys and 6b↔$(CH_3CO)\alpha$, and also several NOEs of $(CH_3CO)\alpha$ with

Table 5. Selected intermolecular NOEs observed within the antibiotic A82846B-tripeptide complex in both forms A and B.

Conformer A	NOE size	Conformer B	NOE size
5b↔Me-Ala2	S	5b↔Me-Ala2	S
2f↔Me-Ala1	S	2f↔Me-Ala1	S
2e↔Me-Ala1	S	2e↔Me-Ala1	S
6b↔NH-Lys	S	6b↔NH-Lys	S
6b↔$(CH_3CO)\alpha$-Lys	S	6b↔$(CH_3CO)\alpha$-Lys	S
6b↔Me-Ala2	W	6b↔Me-Ala2	W
7f↔$CH_2\beta$-Lys	S	7f↔$CH_2\beta$-Lys	S
7f↔$CH_2\gamma$-Lys	M	7f↔$CH_2\gamma$-Lys	M
7f↔$CH_2\gamma$-Lys	W	7f↔$CH_2\gamma$-Lys	W
7f↔Me-Ala2	W	7f↔Me-Ala2	W
7d↔$CH_2\beta$-Lys	W	7d↔$CH_2\beta$-Lys	W
7d↔$CH_2\gamma$-Lys	W	7d↔$CH_2\gamma$-Lys	W
7d↔Me-Ala2	M	7d↔Me-Ala2	M
4b↔Me-Ala1	M	4b↔Me-Ala1	M
		1′(G)↔Me-Ala1	S
z_6↔$(CH_3CO)\alpha$-Lys	S	z_6↔$(CH_3CO)\alpha$-Lys	S
1′(V′)↔$(CH_3CO)\alpha$-Lys	W	1′(V′)↔$(CH_3CO)\alpha$-Lys	W
x_5↔Me-Ala2	M	x_5↔Me-Ala2	M
x_6↔$(CH_3CO)\alpha$-Lys	W	x_6↔$(CH_3CO)\alpha$-Lys	W
x_6↔Me-Ala2	M	x_6↔Me-Ala2	M
x_7↔$CH_2\beta$-Lys	W	x_7↔$CH_2\beta$-Lys	W
Hα-Ala1↔cc′	S	Hα-Ala1↔cc′	S
5′(V′)↔$(CH_3CO)\alpha$-Lys	W	5′(V′)↔$(CH_3CO)\alpha$-Lys	W
$(CH_3CO)\alpha$-Lys↔4′(V′)	W	$(CH_3CO)\alpha$-Lys↔4′(V′)	W
$(CH_3CO)\alpha$-Lys↔Me_5(V′)	S	$(CH_3CO)\alpha$-Lys↔Me_5(V′)	S
Me_5(V)↔Me-Ala1	S		

Abbreviations: S, strong; M, medium; W, weak.

z_6 and protons of the epi-vancosamine sugar V′. In addition, Lys side chain protons $CH_2\beta$ and $CH_2\gamma$ presented several NOEs with the aromatic protons 7f and 7d and the x_7 proton, confirming the extension of the Lys residue over ring 7. The absence of NOEs between the remaining side chain protons ($CH_2\delta$, $CH_2\varepsilon$, NH_ε and $(CH_3CO)\varepsilon$) and the antibiotic portion, shows the flexibility of this part of the Lys residue. These results are consistent with the observations reported for other glycopeptide complexes,[10,21,22] but differ in the case of the ristocetin/Ac_2-Lys-D-Ala-D-Ala complex in D_2O[9]. This observation could be related with the dissimilarities of ristocetin and A82846B or vancomycin dimerization upon tripeptide binding.

Conformational Equilibrium of A82846B-tripeptide Complex

The NMR studies showed that, the three-dimensional structure of the antibiotic moiety and the interactions with the tripeptide, are very similar in both A and B conformations (see Table 5). The largest differences were observed in the sugar portion attached to ring 4 (V and G), the aromatic proton 2e and the Me-Ala1. In conformer A, the Me-Ala1 presented two strong NOEs with the aromatic proton 2e and the methyl group of epi-vancosamine, Me_5(V). Another NOE from 2e to Me_5(V) indicates the proximity of each of these protons, Me_5(V), 2e and Me-Ala1, to the others. In conformer B, it is the anomeric proton of glucose, 1′(G), which presented NOEs with 2e and Me-Ala1. A weak NOE is also observed between Me_3(V) and the aromatic proton 2e. The equal intensities of the two sets of resonances observed for complexed A82846B upon warming from 5 to 45°C suggest the existence of a single asymmetric dimer. The presence of two different symmetric dimers would implicate a transconformational $\Delta H \approx 0$ in order to explain the invariability of the equilibrium constant with temperature. It is likely that the major difference between the two conformers present in the asymmetric dimer results from a different orientation of the glucose moiety with respect to the aglycon portion of the antibiotic, specifically with respect to ring 4. These results agree with recent data reported for the eremomycin-pyrrole-2-carboxylate complex[23], for which a single dimer, with an asymmetric parallel alignment of the two disaccharide units has been identified.

Linkage between Peptide Binding and Antibiotic Dimerization

Sedimentation equilibrium experiments have shown the enhancement of vancomycin dimerization induced by increasing pH and ligand binding. Deprotonation of the *N*-terminal aminoleucine stabilizes the vancomycin structure in which the carbonyl $(CO)_3$ is at the back of the molecule[24]. This conformational change should be responsible for the enhancement of vancomycin

dimerization observed from acid to neutral pH, since the carbonyl $(CO)_3$ is involved in two of the four hydrogen bonds formed between the back faces of the two molecules in the dimer (Fig. 1). According to this, cell-wall analogues should also enhance dimerization since they bind selectively to this vancomycin conformer. At neutral pH, dimerization of Ac-D-Ala-D-Ala/vancomycin was just two times higher than the free antibiotic, but Ac_2-Lys-D-Ala-D-Ala binding increases vancomycin dimerization by two orders of magnitude. In contrast, at pH 5.0, vancomycin dimerization in the presence of the tripeptide increases by three orders of magnitude. These results suggest that, at neutral pH, most of the vancomycin molecules could already have adopted the right conformation for peptide binding. Furthermore, they also indicate either a preferential interaction of the lysine residue with dimeric vancomycin, or the induction of a conformational change in the antibiotic leading to a more suitable conformation for dimer formation. The stronger effect of Ac_2-Lys-D-Ala-D-Ala at pH 5.0 would reflect both the induction of the vancomycin conformer with the $(CO)_3$ group at the back of the molecule and the preferential interaction with lysine. These results would explain the observed aggregation of cell wall fragments induced by vancomycin[7]. The influence of tripeptide binding on vancomycin dimerization has not been described so far and was comparable to the reported effect of this ligand on eremomycin, MM4 7761 and A82846B dimerization at pD 3.7[1]. The dimerization constant of A82846B at pH 7.0 was of an order of greater magnitude than the value calculated by NMR, using hydrogen-deuterium exchange rates at pD 3.7[25]. This difference was consistent with the influence of pH on vancomycin dimerization (see Table 1).

In contrast with vancomycin, tripeptide binding does not modify the formation of ristocetin dimers. The identity of both K_2 and K_2^L values supports the conclusion that Ac_2-Lys-D-Ala-D-Ala binding is independent of ristocetin dimerization. The experimental difficulties found to determine K_2^L for ristocetin complexes using NMR[1], could explain the differences found between present values and previous estimations for ristocetin-ψ/tripeptide $(K_2^L/K_2 = 14)$[1] and ristocetin/tripeptide $(K_2^L/K_2 = 0.7)$[26] complexes by NMR. The different behavior of ristocetin suggests some differences in the interactions with the lysine chain, or the impossibility of the more rigid molecule of ristocetin to undergo a structural change similar to that induced in the antibiotics sharing the same heptapeptide core as vancomycin.

Using K_2 and K_2^L values from Table 1, at neutral pH, the apparent linkage free energy change, coupling vancomycin dimerization to peptide binding, can be estimated to be -11.8 ± 0.6 Kj·(mol of dimer)$^{-1}$, for Ac_2-Lys-D-Ala-D-Ala binding, and -1.9 ± 0.3 Kj·(mol of dimer)$^{-1}$ for Ac-D-Ala and the dipeptides†. Thus, ligand binding to dimeric vancomycin will be favored, on average, by -5.9 and -0.95 kJ per mol of binding site, respectively†.

Taking into account the experimental conditions employed for binding studies[13,14] and the dimerization constants of free and complexed vancomycin, the free energy change contributed by dimerization to the experimental ΔG values can be estimated to be -2.4 kJ·(mol monomer)$^{-1}$, for Ac_2-Lys-D-Ala-D-Ala binding and -2.5 kJ·(mol monomer)$^{-1}$ for Ac-D-Ala and the dipeptides. Analogously, using the reported dimerization enthalpies[1,7,27], ΔH_2 and ΔH_2^L, the contribution of vancomycin dimerization to the apparent enthalpy change was estimated in -11 kJ·mol monomer^{-1} for the tripeptide and -3 kJ·mol monomer^{-1} for the other ligands††. The estimated thermodynamic parameters for cell wall analogue binding to monomeric and dimeric vancomycin are shown in Table 3. The stabilization of Ac-D-Ala/ and dipeptide/vancomycin complexes by dimerization is of enthalpic origin. In the case of the tripeptide both the enthalpy and the entropy changes contribute to enhance its binding to the dimeric vancomycin.

Partitioning of Energetic Contributions to Ligand Binding

The complexes of A82846B with Ac-D-Ala or the dipeptides are, in general, more stable than those of

† The apparent linkage free energy change coupling ligand binding to antibiotic dimerization is $\Delta G_{linkage} = \Delta G_2^L - \Delta G_2$, where ΔG_2^L and ΔG_2 are the dimerization free energy changes of the bound and free antibiotics, respectively. The free energy change of ligand binding to the dimer is $\Delta G_D = \Delta G_M + 1/2\ \Delta G_{linkage}$.

†† The apparent binding constant is

$$K_{app} = \frac{K_M + 2K_D K_2 (1 + K_D[L])[A]}{1 + 2K_2[A](1 + K_D[L])}$$

where K_M and K_D are the binding constant to the monomer and dimer $(K_D = K_M(K_2^L/K_2)^{1/2})$. The free concentration of monomer is

$$[A] = \frac{-(1 + K_M[L]) + \sqrt{(1 + K_M[L])^2 + 8[A]_t K_2 (1 + K_D[L])^2}}{4K_2(1 + K_D[L])^2},$$

and $[A]_t$ is the total concentration of binding sites. Theoretical values of Kapp were calculated as a function of [L], the free ligand concentration, using the K_2 and K_2^L values shown in Table 1; K_M was varied until the best fit between the calculated and experimental values of K_{app} was found. The apparent enthalpy change is $\Delta H_{app} = \Delta H_M + 1/2(\Delta H_2^L \cdot f(A_2L_2)_{sat} - \Delta H_2 \cdot f(A_2)_0)$, where $f(A_2)_0$ and $f(A_2L_2)_{sat}$ are the fractions of dimer in the absence and under saturation by the tripeptide, respectively.

vancomycin (see Table 3). Their free energy changes are very close to those found for ristocetin[13,14] and about 2 kJ·mol^{-1} lower than those of teicoplanin[13]. The present thermodynamic results indicate that differences between peptide-antibiotics interactions in either A or B conformations of A82846B should be minor, since all the binding sites were found to be identical, within experimental error.

The comparison, between ΔG values for dimeric vancomycin and A82846B, shows that, the sugar moiety on residue 6 (V′) stabilizes the binding of Ac-D-Ala, Ac-Gly-D-Ala and Ac-D-Ala-D-Ala by about −4 kJ·mol^{-1}; Ac-D-Ala-Gly binding is stabilized only by −3 kJ·mol^{-1} and V′ opposes tripeptide binding by 2.6 kJ·mol^{-1}. Although the intrinsic affinity of the A82846B dimer for the tripeptide is about 3 kJ·mol^{-1} higher than that of the vancomycin monomer, tripeptide binding to dimeric vancomycin is more favorable than it is to the A82846B dimer.

Table 6 shows the variations observed in the thermodynamic parameters of peptide binding when one amino acid is added or changed in the bonding peptide. Taking into account the low influence of ligand binding on ristocetin dimerization, the reported values should be representative of peptide binding to both association states. The differences found in the energetics of peptide binding to A82846B by substitution of alanine for glycine, or peptide chain elongation, resemble more those found for ristocetin or teicoplanin (see Table 6), despite the closest structural relationship between A82846B and vancomycin.

The free energy of Ac-D-Ala binding to dimeric vancomycin is about 5 kJ·mol^{-1} less favorable than to A82846B. Half of this value is composed of contributions from differences in hydrophobic interactions with the antibiotic due to Me-Ala1 (Table 6). The energetic balances between the other binding forces are similar for all the antibiotics.

The interaction with the Me-Ala2 increases the free energy of binding to A82846B by 2.7 kJ·mol^{-1}. The enthalpy and entropy terms contributed by this group in A82846B resemble the contributions found for ristocetin and teicoplanin. These three antibiotics contain a sugar substituent in residue 6, but NOEs between Me-Ala2 and the sugar have not been found. Nevertheless, the NOEs of Me-Ala2 with aromatic protons of ring 6 and Hx_6, indicate the proximity of this residue in A82846B and ristocetin[10] complexes.

The substitution of the *C*-terminal alanine by glycine decreases the free energy of binding by 7.7 kJ·mol^{-1}. $\Delta\Delta S$ values of Me-Ala1 interaction account for about 80% of $\Delta\Delta G$ values in ristocetin and A82846B, while a similar percentage is contributed by $\Delta\Delta H$ in teicoplanin and vancomycin. In contrast with A82846B, dimeric vancomycin lacks the two hydrogen bonds between the amino group of the sugar on residue 6 and the carbonyl group of residue 2, proposed in A82846B and ristocetin[6] dimers. These additional hydrogen bonds could improve the interaction with the Me-Ala1 group by decreasing the entropy loss upon complexation, due to the stronger restriction of motion of the peptide backbone in the free dimer. This agrees with the higher entropy loss associated with vancomycin complexation with peptides terminating in D-Ala.

The increased stability on passing from Ac-D-Ala-D-Ala to Ac_2-Lys-D-Ala-D-Ala complex is of enthalpic

Table 6. Differences in the thermodynamics of antibiotic-peptide binding, generated by modification or addition of one amino acid in the bonding peptide[a].

Peptide change		$-\Delta\Delta G$ (kJ/mol)				$-\Delta\Delta H$ (kJ/mol)				$-\Delta\Delta S$ (J/mol·K)			
		$V_{M/D}$	A_D	$R_{M/D}$	T^b	$V_{M/D}$	A_D	$R_{M/D}$	T	$V_{M/D}$	A_D	$R_{M/D}$	T
Ac-D-Ala ↓ Ac-Gly-D-Ala		9.8	9.1	8.7	10.4	−1.6	5.6	4	12	−38	−12	−16	6
Ac-Gly-D-Ala ↓ Ac-D-Ala-D-Ala	Me2	1.6	2.7	0.2	2.8	1	−9.5	−7	−8	−1	−42	−26	−36
Ac-D-Ala-Gly ↓ Ac-D-Ala-D-Ala	Me1	5.6	7.7	8.9	7.8	5	1.4	2	6	−2	−22	−23	−7
Ac-D-Ala-D-Ala ↓ Ac_2-Lys-D-Ala-D-Ala	V_M	7.8	4.9	3.4	5.8	V_M 5.3	7	7	9	V_M −8	9	14	11
	V_D	12.6				V_D 5.3				V_D −24			
Ac-D-Ala minus Me1[c]	V_M	5.9	9.5	8.4	10.2	V_M 24	28	31	30	V_M 61	62	75	67
	V_D	6.9				V_D 25.5				V_D 62			

[a] The experimental values for vancomycin, ristocetin and teicoplanin are taken from RODRÍGUEZ-TÉBAR *et al.* (1986) and ARRIAGA *et al.* (1990). Abbreviations: V, vancomycin; A, A82846B; R, ristocetin: T, teicoplanin. The subscripts M and D refers to monomeric or dimeric species, respectively. A single value means that both association states are equivalent. [b] Teicoplanin does not form stable dimers[1,28]. [c] Me1 contributions in raw 3 were subtracted from Ac-D-Ala values.

origin, indicating that polar interactions, probably van der Waals forces, are mainly involved in contacts with the lysine residue in A82846B, ristocetin and teicoplanin. In contrast, both enthalpy and entropy changes favor lysine interaction with vancomycin, suggesting that the sugar substituent on aminoacid 6 opposes a more favorable lysine-antibiotic hydrophobic contact. The NOEs of $(CH_3CO\text{-}NH)\alpha$ of lysine with several protons of the epi-vancosamine sugar V', suggest the proximity between them. Taken into account the structural similarities between A82846B and vancomycin, the epi-vancosamine residue V' opposes lysine-A82846B interactions by $7.7\,kJ\cdot mol^{-1}$.

The different affinities of A82846B and eremomycin for Ac_2-Lys-D-Ala-D-Ala1,[25,29] could be attributed to the chlorine atom on ring 6. The NOEs of $(CH_3CO\text{-}NH)$ of lysine with protons 6b, z_6 and several protons of the epi-vancosamine sugar V', suggest the proximity between ring 6 and this residue.

Antibiotic Dimerization and Biological Activity

A82846B is two to five times more active than vancomycin. Eremomycin (the monochloride derivative on amino acid 2 of A82846B), is as active as A82846B *in vitro* and both have very high dimerization constants[25]. The affinity of eremomycin for Ac_2-Lys-D-Ala-D-Ala is about 20 fold lower ($\sim 7.4\,kJ\cdot mol^{-1}$,) in terms of the binding constant, than that of A82846B[29]. This lack of correlation between efficacy and affinity towards Ac_2-Lys-D-Ala-D-Ala of eremomycin and the effect of low Ac_2-Lys-D-Ala-D-Ala concentrations on its antibacterial activity, leads to the proposal that, the efficacy of this antibiotic may be derived from the formation of dimers[29]. In the present study, no cooperativity has been observed between the two binding sites of dimeric A82846B. The slopes of the Hill plots yield values very close to unity[30] for all the peptides tested. Nevertheless, "*in vivo*" and assuming that the cell wall precursors were locally concentrated, saturation of the second binding site would be an intramolecular process. This would increase its free energy change by at least $-10\,kJ\cdot mol^{-1}$ (the cratic term contributed by the entropy of mixing[31]). However, it remains unclear why antibacterial activities of A82846B and eremomycin became unaffected by their differences in affinity, since both form stable dimers at very low concentrations. Recently, the higher tendency of eremomycin, over A82846B, to form dimers has been considered as a plausible explanation for this anomalous behavior[1,25]. However, at neutral pH about 75% of free A82846B is a dimer at an antibiotic concentration as low as $1\,\mu M$ and this quantity increases up to 99% upon Ac_2-Lys-D-Ala-D-Ala binding[1,25]. These values, and the free energy change contributed by the linkage between the binding of Ac_2-Lys-D-Ala-D-Ala and A82846B dimerization (about $-5\,kJ\cdot(mol\ of\ site)^{-1}$ under these conditions) indicate that eremomycin and A82846B activities cannot result as a simple balance between differences in their dimerization constants and their ligand binding affinities.

Dimerization may play a more clear role in the biological action of those antibiotics for which precursor binding significantly enhanced dimer population. Vancomycin dimerization upon tripeptide binding constitutes an example. With a dimerization constant of $4.6\times10^5\ M^{-1}$ for the tripeptide-vancomycin complex, at a vancomycin concentration as low as $1\,\mu M$, the 37% of the antibiotic would become a dimer upon Ac_2-Lys-D-Ala-D-Ala binding, at neutral pH. Under these conditions, the difference between the free energy change accompanying the total process (binding plus dimerization) for A82846B and vancomycin could be estimated to be $-2\,kJ\cdot(mole\ of\ site)^{-1}$, or $-5\,kJ\cdot(mol\ of\ site)^{-1}$ if the mixing entropy change is taken into account. These values are in the range expected considering the antibacterial activities of both antibiotics. The possibility of a steric component in the inhibition mechanism, or a selective function of the sugars in binding cell wall precursors, could help to explain the anomalous behavior of eremomycin.

Experimental

Chemicals

N-Acetyl-D-Ala-D-Ala (Ac-D-Ala-D-Ala), *N*-acetyl-D-alanyl-Glycine (Ac-D-Ala-Gly) and *N*-acetyl-glycyl-D-alanine (Ac-Gly-D-Ala) were prepared by acetylation of their corresponding dipeptides[32] (from Sigma Chemical Co., St. Louis, MO, U.S.A.). *N*-Acetyl-D-alanine (Ac-D-Ala) and *N*,*N*'-Diacetyl-lysyl-D-alanyl-D-alanine (Ac_2-Lys-D-Ala-D-Ala) were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

A82846B (LY264826B) was a gift from Eli Lilly Co., Indianapolis, U.S.A., vancomycin was obtained from Sigma Chemical Co. and ristocetin A was from Lundbeck, Copenhagen, Denmark. All the antibiotics were used without further purification. Antibiotic concentrations were measured spectrophotometrically by employing the absorption coefficients reported elsewhere[32~34].

NMR Spectroscopy

Free A82846B and (1 : 1) A82846B-tripeptide complex were dissolved in 0.5 ml of either D_2O or 90% H_2O/10% D_2O leading to 1.1 mM solutions. ^1H NMR spectra were recorded at pH 4.3 and pH 2.5 on a BRUKER AMX-600 spectrometer at different temperatures from 5 to 65°C, at intervals of 10°C. Sodium 3-trimethylsilyl(2,2,3,3-$^2H_4$)propionate (TSP) was used as the internal reference. Two-dimensional spectra COSY,[35] TOCSY,[36] NOESY[38] and ROESY[37] were performed on both D_2O and H_2O samples. All spectra were recorded in the phase sensitive mode using the time proportional mode[39] and presaturation of the solvent signal. The carrier frequency was placed on the H_2O or HOD resonance frequency for each temperature, and typical 512 experiments were collected, each containing

2048 data points. NOESY and ROESY spectra were recorded with mixing times of 300 and 200 ms, respectively.

Microcalorimetric Measurements

Calorimetric titrations were carried out at 25°C with a LKB microcalorimeter equipped with a titration assembly (LKB 2107-350). The amplified output voltage-time curves were integrated by a micro-processor and monitored by a potentiometric recorder. The calorimeter was calibrated as described previously[40]. The results were corrected for small differential effects due to compression, mixing and dilution, which were determined in separate control experiments. At least three independents sets of measurements were performed for all the antibiotic-peptide complexes studied. The experiments were performed in 0.1 M sodium phosphate buffer at pH 7.0, using antibiotic concentrations of about 250 μM.

Spectrophotometric Measurements

U.V. measurements were carried out in a Cary 210 spectrophotometer equipped with thermostatically controlled cells. All the measurements were done in a differential form, with dilute solutions of the antibiotic (24 μM in 0.1 M-sodium phosphate buffer, pH 7.0 at 25°C) and matched cells with 1 cm pathlength. The temperature was kept constant at $\pm$0.1°C. The accuracy in absorbance was $\pm$0.0005 units. Difference absorption spectra were recorded from 360 to 230 nm. Equilibrium constants were calculated from the change in absorbance of the antibiotic spectrum generated by peptide binding[13]. At least two different wavelengths between 290 and 240 nm were employed.

Sedimentation Equilibrium Experiments

Sedimentation equilibrium measurements of A82846B (10^{-6} to 10^{-3} M), vancomycin (10^{-5} to 10^{-3} M, +/− Ac-D-Ala-D-Ala and tripeptide), and ristocetin (10^{-4} to 10^{-3} M, +/− tripeptide) in 0.1 M sodium phosphate buffer, were performed at 25°C by centrifuging 80 μl samples at 40,000 rpm in an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Inc.). 12 mm double sector centerpieces of epon-charcoal were employed. After equilibration, radial scans of the samples were taken at 1 hour intervals, at different wavelengths (240, 280, and 300 nm). Ligand concentrations were such that the glycopeptide remained more than 95% complexed.

In order to obtain the equilibrium dimerization constants (K_2 and K_2^L for free and bound antibiotics), the experimental data for each antibiotic were simultaneously analyzed using the ORIGIN version of the NONLIN algorithm[41], with the standard deviation of the absorbance readings as a weighing factor. The monomer molecular mass (in daltons) were constrained to the following: 1590 (A82846B), 1447 (vancomycin), 2066 (ristocetin). The partial specific volumes, calculated from aminoacid and sugar composition,[42] were 0.690 (A82846B, vancomycin) and 0.680 cm^3/g (ristocetin).

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References

1) MACKAY, J. P.; U. GERHARD, D. A. BEAUREGARD, M. S. WESTWELL, M. S. SEARLE & D. H. WILLIAMS: Glycopeptide antibiotic activity and the possible role of dimerization: A model for biological signaling. J. Amer. Chem. Soc. 116: 4581~4590, 1994
2) RUOFF, K. L.: Gram-positive vancomycin-resistant clinical isolates. Clin. Microbiol. Newslett. 11: 1~4 1987
3) MORONI, M. C.; C. GRANOZZI, F. PARENTI, M. SOSIO & M. DENARO: Cloning of a DNA region of *Actinoplanes teichomyceticus* confering teicoplanin resistance. FEBS Letters 253: 108~112, 1989
4) WALSH, CH. T.: Vancomycin resistance: Decoding the molecular logic. Science 261: 308~309, 1993
5) WILLIAMS, D. H.; M. S. SEARLE, J. P. MACKAY, U. GERHARD R. A. MAPLESTONE: Toward an estimation of binding constants in aqueous solution: Studies of association of vancomycin group antibiotics. Proc. Natl. Acad. Sci. U.S.A. 90: 1172~1178, 1993
6) WALTHO, J. P. & D. H. WILLIAMS: Aspects of molecular recognition: Solvent exclusion and dimerization of the antibiotic ristocetin when bound to a model bacterial cell-wall precursor. J. Amer. Chem. Soc. 11: 2475~2480, 1989
7) GERHARD, U.; J. P. MACKAY, R. A. MAPLESTONE & D. H. WILLIAMS: The role of the sugar and chlorine substituents in the dimerization of vancomycin antibiotics. J. Amer. Chem. Soc. 115: 232~237, 1993
8) WILLIAMS, D. H.; M. P. WILLIAMSON, D. W. BUTCHER & S. HAMMOND: Detailed binding sites of the antibiotics vancomycin and ristocetin A: Determination of intermolecular distances in antibiotic/substrate complexes by use of the time-dependent NOE. J. Am. Chem. Soc. 105: 1332~1339, 1983
9) WILLIAMSON, M. P. & D. H. WILLIAMS: ^{1}H NMR studies of the structure of ristocetin A and of its complexes with bacterial cell wall analogues in aqueous solution. J. Chem. Soc. Perkin I: 949~956, 1985
10) FESIK, S. W.; R. T. O'DONNEL, T. T. GAMPE & E. T. OLEJNICZAK: Determining the structure of a glycopeptide-Ac_2-Lys-D-Ala-D-Ala complex using NMR parameters and molecular modeling. J. Amer. Chem. Soc. 108: 3165~3170, 1986
11) MOLINARI, H.; A. PASTORE, L. LIAN, G. E. HAWKES & K. SALES: Structure of vancomycin and a vancomycin/D-Ala-D-Ala complex in solution. Biochemistry 29: 2271~2277, 1990
12) CONVERT, O.; A. BONGINI & J. FEENY: A ^{1}H Nuclear magnetic resonance study of the interactions of vancomycin with *N*-Actyl-D-Alanyl-D-Alanine and related peptides. J. Chem. Soc. Perkin II: 1262~1270, 1980

13) ARRIAGA, P.; J. LAYNEZ, M. MENÉNDEZ, J. CAÑADA & F. GARCÍA-BLANCO: Thermodynamic analysis of the interaction of the antibiotic teicoplanin ant its aglycone with cell-wall peptides. Biochemical J. 265: 69～77, 1990
14) RODRÍGUEZ-TÉBAR, A.; D. VÁZQUEZ, J. L. PÉREZ VELÁZQUEZ, J. LAYNEZ & I. WADSÖ: Thermochemistry of the interaction between peptides and vancomycin or ristocetin. J. Antibiotics 39: 1578～1583, 1986
15) YAO, R. C.; D. F. MAHONEY, D. K. BISDEN, F. P. MERTZ, J. A. MABE & W. M. NAKATSUKA: 28th Interscience Conference on Antimicrob. Agents and Chemother. Abstract 974, 1988
16) NAGARAJAN, R.: Structure-activity relationship of vancomycin-type glycopeptide antibiotics. J. Antibiotics 46: 1181～1195, 1993
17) BEAUREGARD, D. A.; D. H. WILLIAMS, M. N. GWYNN & D. J. C. KNOWLES: Dimerization and membrane anchors in extracellular targeting of vancomycin group antibiotics. Antimicrob. Agents. Chemother. 39: 781 785, 1995
18) WÜTHRICH, K.: *In* NMR of proteins and nucleic acids. *Eds.*, WILEY New York, 1986
19) SKELTON, N. J.; D. H. WILLIAMS, M. J. RANCE & J. C. RUDDOCK: Structure elucidation of a novel antibiotic of the vancomycin group. The influence of ion-dipole interactions on peptide backbone conformation. J. Amer. Chem. Soc. 113: 3757～3765, 1991
20) KANNAN, R.; C. M. HARRIS, T. M. HARRIS, J. P. WALTHO, N. J. SKELTON & D. H. WILLIAMS: Function of the amino sugar and *N*-terminal amino acid of the antibiotic vancomycin and its complexation with cell wall peptides. J. Amer. Chem. Soc. 110: 2946～2953, 1988
21) FESIK, S. W.; I. M. ARMITAGE, G. A. ELLESTAD & W. J. MCGAHRE: Nuclear magnetic resonance studies on the interaction of avoparcin with model receptors of bacterial cell wall. Mol. Phar. 25: 281～286, 1984
22) ANG, S.-G.; M. P. WILLIAMS & D. H. WILLIAMS: Structure elucidation of a glycopeptide antibiotic OA-7653. J. Chem. Soc. Perkin Trans. I. 1949～1956, 1988
23) GROVES P.; M. S. SEARLE, J. P. MACKAY & D. H. WILLIAMS: The structure of an asymmetric dimer relevant to the mode of action of the glycopeptide antibiotics. Structure 2: 747～754, 1994
24) BOOTH, P. M. & D. H. WILLIAMS: Preparation and conformational analysis of vancomycin hexapeptide and aglucovancomycin hexapeptide. J. Chem. Soc. Perkin Trans. I: 2335～2339, 1989
25) MACKAY, J. P.; U. GERHARD, D. A. BEAUREGARD, R. A. MAPLESTONE & D. H. WILLIAMS: Dissection of the contributions toward dimerization of glycopeptide antibiotics. J. Amer. Chem. Soc. 116: 4573～4580, 1994
26) GROVES, P.; M. S. SEARLE, I. CHICARELLI-ROBINSON & D. H. WILLIAMS: Recognition of the cell-wall binding site of the vancomycin-group antibiotics by unnatural structural motifs. ^{1}H NMR studies of the effects of ligand binding on antibiotic dimerization. J. Chem. Soc. Perkin Trans. I: 659～665, 1994
27) COOPER, A. & K. E. MCAULEY-HECHT: Microcalorimetry and the molecular recognition of peptides and proteins. Phil. Trans. R. Soc. Lond. 345: 23～35, 1993
28) CORTI, A.; A. SOFFIENTINI & G. CASSANI: Binding of the glycopeptide antibiotic teicoplanin to D-alanyl-D-alanine-agarose: the effect of micellal aggregates. J. Appl. Biochem. 7: 133～137, 1985
29) GOOD, V. M.; M. N. GWYNN & J. C. KNOWLES: MM45289. A potent glycopeptide antibiotic which interacts weakly with diacetyl-L-Lysyl-D-Alanyl-D-Alanine. J. Antibiotics 43: 550～555, 1990
30) WYMAN, J. & S. GILL: *In* Binding and linkage. pp. 46～49, University Science Books, Mill Valley, 1990
31) GOURNEY, R. W.: *In* Ionic processes in solution. pp. 90～104, Dover Publications, New York, 1962
32) NIETO, M. & H. R. PERKINS: Physicochemical properties of vancomycin and iodovancomycin and their complexes with diacetyl-L-Lysyl-D-Alanyl-D-Alanine. Biochemical J. 123: 773～787, 1971
33) HAMILL, R. L.; P. J. BAKER, D. M. BERRY, M. DEBONO, R. M. MOLLOY & D. S. MORELAND: Abstracts of 28th Interscience Conference on Antimicrob. Agents Chemother., No. 975, 1988
34) NIETO, M. & H. R. PERKINS: The specificity of combination between ristocetin and peptides related to bacterial cell wall mucopeptide precursors. Biochemical J. 124: 845～852, 1971
35) AUE, W. P.; E. BARTHOLDI & R. R. ERNST: Two dimensional spectroscopy application to nuclear magnetic resonance. J. Chem. Phys. 64: 229～2246, 1976
36) BAX, A. & D. G. DAVIS: MLEV-17-Based two-dimensional homonuclear magnetization transfer spectroscopy. J. Magn. Reson. 65: 355～360, 1985
37) KUMAR, A.; R. R. ERNST & K. A. WÜTHRICH: Two-dimensional nuclear overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. Biochem. Biophys. Res. Commun. 95: 1～6, 1980
38) BOTHNER-BY, A. A.; R. L. STEPHENS & J. M. LEE: Structure determination of a tetrasaccharide: Transient nuclear overhauser effects in the rotating frame. J. Amer. Chem. Soc. 106: 811～813, 1984
39) MARION, D. & K. WÜTHRICH: Application of phase-sensitive two dimensional correlated spectroscopy (COSY) for measurements of ^{1}H-^{1}H spin-spin coupling constants in proteins. Biochem. Biophys. Res. Commun. 113: 967～974, 1983
40) CHEN, A. & I. WADSÖ: Simultaneous determination of ΔG, ΔH and ΔS by an automatic microcalorimetric titration technique. Application to protein ligand binding. J. Biochem. Biophys. Methods. 6: 307～316, 1982
41) JOHNSON, M. L.; J. J. CORREIA, D. A. YPHANTIS & H. R. HALVORSON: Analysis of data from the analytical ultracentrifuge by nonlinear least-squares technique. Biophys. J. 36: 575～588, 1981
42) LAUE, T. M.; B. D. SHAH, T. M. RIDGEWAY & S. L. PELLETIER: Computer-aided interpretation of analytical sedimentation data for proteins. *In* Analytical ultracentrifugation in Biochemistry & Polymer Science. *Eds.*, S. E. HARDING, H. C. HORTON & A. J. ROWE, pp. 90～125, Royal Society of Chemistry, London, 1993