Thermodynamic analysis of the interaction of the antibiotic teicoplanin and its aglycone with cell-wall peptides

Paloma ARRIAGA, Jose LAYNEZ,* Margarita MENENDEZ, Javier CAÑADA and Francisco GARCIA-BLANCO

Instituto de Química-Física 'Rocasolano', Serrano 119, Madrid 28006, Spain

The thermodynamics of the interaction of the glycopeptidic antibiotic teicoplanin and its peptidic moiety with analogues of bacterial cell-wall peptides were studied by means of calorimetric and spectrophotometric techniques. The analysis of the thermodynamic data has allowed us to evaluate the contributions of the different peptide groups to the binding process. The nature of the primary binding forces is also discussed for each interacting group, on the basis of their enthalpic and entropic contribution and in connection with the detailed structural information available for these antibiotics from n.m.r. data. Similar analyses for the case of vancomycin and ristocetin are also reported.

INTRODUCTION

Teicoplanin is a glycopeptidic antibiotic of the vancomycin group with close structural resemblances to ristocetin (Borghi *et al.*, 1984). The major characteristic that differentiates teicoplanin from the other members of this family of antibiotics is the sugar composition, i.e. the presence of D-mannose and D-glucosamine, though the two chlorine atoms attached to aromatic rings make it similar to vancomycin (Bardone *et al.*, 1978*a,b*; Barna *et al.*, 1984).

Teicoplanin is of particular interest because it is of low toxicity (Parenti *et al.*, 1978) and also because studies performed *in vitro* have shown that it is more active than vancomycin against many Gram-positive bacteria (Varaldo *et al.*, 1983; Pallanza *et al.*, 1983). These properties might confer some clinical importance to it, as for vancomycin, in the treatment of serious infections that are resistant to penicillin and cephalosporine therapy. The teicoplanin aglycone, obtained by complete hydrolysis of the sugar components carried by the whole molecule, is more active *in vitro* than the parent molecule and shows a certain degree of activity against Gram-negative bacteria. However, it is less active *in vivo* than teicoplanin (Malabarba *et al.*, 1984). Like other glycopeptides (Marshall, 1965; Debono *et al.*, 1980, 1984; Nielsen *et al.*, 1982; McGahren *et al.*, 1985; Sitrin *et al.*, 1986) teicoplanin aglycone is also of importance for both structural and activity studies and as an intermediate for chemical modifications (Malabarba *et al.*, 1984).

Teicoplanin inhibits the biosynthesis of the bacterial cell wall, by interfering with the peptides terminating in -D-Ala-D-Ala (Somma *et al.*, 1984). Studies performed *in vitro* with vancomycin, ristocetin and several model peptides have shown that both hydrogen bonds and hydrophobic interactions are involved in the formation of the final antibiotic-peptide complex, and electrostatic interactions between the peptide carboxylate group and the terminal protonated amine group of the antibiotic may be responsible for the initial complex formed (Williamson & Williams, 1984; Williamson *et al.*, 1984; Barna *et al.*, 1985). N.m.r. studies of the binding of Ac-

D-Ala-D-Ala to teicoplanin and ristocetin showed that the chemical shifts of the methyl groups in the bound state are very similar for both antibiotics (Barna *et al.*, 1985), suggesting that at least the environments of the peptide methyl groups are similar. Analogously, results from n.m.r. have shown that the binding loci for the carboxylate group of this peptide are similar in vancomycin and ristocetin (Williams & Waltho, 1988). These conclusions were further supported from studies of the binding of Ac₂-Lys-D-Ala-D-Ala to these antibiotics (Williams *et al.*, 1983; Williamson & Williams, 1985; Fesik *et al.*, 1986; Kannan *et al.*, 1988). However, the contributions of the different interacting groups have not been quantified until now and there is only some qualitative information from n.m.r. studies, mainly from the nuclear Overhauser effect.

A first thermodynamic approach to this problem, by using calorimetric methods, was carried out by Rodriguez-Tebar *et al.* (1986), who studied the interaction of vancomycin and ristocetin with different models of cell-wall peptides, i.e. Ac-D-Ala, Ac-D-Ala-D-Ala, Ac-D-Ala-Gly and Ac-Gly-D-Ala. van't Hoff enthalpies have also been reported, from equilibrium studies for the binding of these peptides to vancomycin and ristocetin (Nieto & Perkins, 1971*a*; Williamson *et al.*, 1984). The present paper deals with the thermodynamic characterization of the interaction of teicoplanin and teicoplanin aglycone with the peptides mentioned above and also with Ac₂-Lys-D-Ala-D-Ala. In addition, the thermodynamic parameters for binding of the latter to vancomycin and ristocetin are also reported.

The experimental results were analysed in order to quantify the contributions of the different groups to the stabilization of the final complex.

MATERIALS AND METHODS

Chemicals

N-Acetyl-D-alanyl-D-alanine (Ac-D-Ala-D-Ala), N-acetyl-D-alanylglycine (Ac-D-Ala-Gly) and N-acetylglycyl-D-alanine (Ac-Gly-D-Ala) were prepared by acetyl-

* To whom correspondence should be addressed.

ation of their corresponding dipeptides (from Sigma Chemical Co., St. Louis, MO, U.S.A.) as described previously (Nieto & Perkins, 1971*a*). *N*-Acetyl-D-alanine (Ac-D-Ala) and *NN'*-diacetyl-lysyl-D-alanyl-D-alanine (Ac₂-Lys-D-Ala-D-Ala) were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Antibiotics

Vancomycin was obtained from Sigma Chemical Co. Ristocetin A (referred to in this paper simply as ristocetin) was a gift from Lundbeck, Copenhagen, Denmark. Teicoplanin and its aglycone were from Lepetit, Milan, Italy. All the antibiotics were used without further purification. Antibiotic concentrations were measured spectrophotometrically by employing the specific absorption coefficients reported elsewhere (Nieto & Perkins, 1971*a,b*; Borghi *et al.*, 1984).

Microcalorimetric measurements

Calorimetric titrations were carried out at different temperatures with an LKB batch 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 (Chen & Wadsö, 1982). The results were corrected for small differential effects due to compression, mixing and dilution, which were determined in separate control experiments. At least two independent sets of measurements were performed at each temperature for all the antibiotic–peptide complexes studied. Unless otherwise stated, titration experiments were performed in 0.1 M-sodium phosphate buffer at pH 7.0. In order to prevent self-aggregation of the antibiotics, their concentrations were kept as low as 0.5 mg/ml.

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, according to the Nieto & Perkins (1971*a*) method with slight modifications, with dilute solutions of the antibiotics (21 μ M in 0.1 M-sodium phosphate buffer, pH 7.0) and cells with 1 cm pathlength. The temperature was kept constant within 0.1 °C. The differences in absorbance were measured with an accuracy of ± 0.0005 A unit. The association constants were calculated by means of a non-linear least-squares program based on a 1:1 binding model.

RESULTS

The calorimetric titration measurements of teicoplanin and teicoplanin aglycone with Ac-D-Ala and the three dipeptides were performed at 25 °C, 30 °C and 37 °C. Figs. 1 and 2 show the experimental results obtained at 25 °C. Since the association constants for all these binding reactions were moderately large, it was possible to derive both the enthalpy change and the association constant from the calorimetric curves (Spink & Wadsö, 1976; Eftink & Biltonen, 1980). The experimental values were analysed according to the procedure described by Eftink & Biltonen (1980). A 1:1 binding model was assumed for all the complexes, on the basis of the values derived from the calorimetric titration curves for the complexes formed

Fig. 1. Calorimetric titration of teicoplanin (○ and △) and its aglycone (● and ▲) with Ac-Gly-D-Ala (○ and ●, lower scale) or Ac-D-Ala (△ and ▲, upper scale) in 0.1 M-phosphate buffer, pH 7, at 25 °C

The curves correspond to the theoretical fitting of the experimental data by the use of the thermodynamic parameters reported in Tables 1 and 2. The arrows indicate the peptide concentration at which a 1:1 peptide/antibiotic ratio is achieved.

Fig. 2. Calorimetric titration of teicoplanin (● and △) and its aglycone (○ and ▲) with Ac-D-Ala-D-Ala (● and ○, lower scale) or Ac-D-Ala-Gly (△ and ▲, upper scale) in 0.1 M-phosphate buffer, pH 7, at 25 °C

The curves were calculated by the use of the values of the thermodynamics parameters reported in Tables 1 and 2.

between teicoplanin and its aglycone with Ac-Gly-D-Ala or Ac-D-Ala-D-Ala (see Figs. 1 and 2). The curves plotted in these Figures correspond to the theoretical fits of the experimental data by using the thermodynamic parameters reported in Tables 1 and 2. The excellent agreement gives additional support to the assumed 1:1 binding model.

The high affinity of Ac₂-Lys-D-Ala-D-Ala towards teicoplanin and its aglycone makes spectrophotometric methods more suitable than calorimetry for determination of the binding constants. For this reason, the marked change in the absorbance around 243 nm of the

Table 1. Thermodynamic parameters of peptide binding to teicoplanin in 0.1 M-phosphate buffer, pH 7.0

Peptide	<i>T</i> (°C)	−Δ <i>G</i> (kJ/mol)	−Δ <i>S</i> (J/mol·K)	−Δ <i>H</i> (kJ/mol)	−Δ <i>C_p</i> (J/mol·K)
Ac-D-Ala	25	18.0±0.2	60±4	36±1	490±50
	30	17.5±0.2	71±7	39±1	
	37	16.8±0.2	81±4	42±1	
Ac-D-Ala-D-Ala	25	31.2±0.4	30±4	40±1	410±80
	28	35*	—	—	
	30	30.5±0.3	41±7	43±2	
	37	30.3±0.4	47±7	45±2	
Ac-Gly-D-Ala	25	28.4±0.4	66±7	48±1	
	30	27.6±0.3	71±6	49±1	
	37	27.3±0.2	72±2	49.5±0.5	
Ac-D-Ala-Gly	25	23.4±0.3	37±4	34±1	490±50
	30	23.4±0.4	45±5	37±1	
	37	23.4±0.2	53±4	40±1	
Ac ₂ -Lys-D-Ala-D-Ala	25	37.0±0.5	40±8	49±2	940±200
	25	36.4†	—	—	
	30	36.9±0.5	46±5	51±2	
	37	36.8±0.5	75±3	60±2	

* From Barna *et al.* (1985), pH 5.† From Somma *et al.* (1984), pH 7.**Table 2. Thermodynamic parameters of peptide binding to teicoplanin aglycone in 0.1 M-phosphate buffer, pH 7.0**

Peptide	<i>T</i> (°C)	−Δ <i>G</i> (kJ/mol)	−Δ <i>S</i> (J/mol·K)	−Δ <i>H</i> (kJ/mol)	−Δ <i>C_p</i> (J/mol·K)
Ac-D-Ala	25	17.1±0.2	87±4	43±1	88±70
	30	16.3±0.2	85±7	42±2	
	37	16.1±0.2	90±4	44±1	
Ac-D-Ala-D-Ala	25	23.9±0.1	61±4	42±1	330±30
	28	32.3*	—	—	
	30	23.0±0.1	69±4	44±1	
	37	21.8±0.1	78±4	46±1	
Ac-Gly-D-Ala	25	27.8±0.4	68±5	48±1	87±80
	30	26.5±0.3	71±5	48±1	
	37	26.8±0.2	72±4	49±1	
Ac-D-Ala-Gly	25	17.5±0.1	59±4	35±1	400±80
	30	17.5±0.3	68±8	38±2	
	37	16.5±0.2	76±4	40±1	
Ac ₂ -Lys-D-Ala-D-Ala	25	35.0±0.7	57±9	52±2	600±60
	30	34.6±0.7	71±5	56±1	
	37	33.2±0.4	86±4	60±1	

* From Barna *et al.* (1985), pH 5.0.

antibiotic generated by the peptide binding was employed in the spectrophotometric titrations of the four antibiotics with Ac₂-Lys-D-Ala-D-Ala. Fig. 3 shows the experimental data at 37 °C, as well as the theoretical fits calculated by use of the equilibrium constants collected, as Δ*G*⁰ values, in Tables 1–3.

The enthalpy changes for Ac₂-Lys-D-Ala-D-Ala binding to the four antibiotics were determined by using a saturating peptide concentration (see Tables 1–3).

The values for the Gibbs free energy, Δ*G*, found here for Ac-D-Ala-D-Ala binding to both teicoplanin and its aglycone are lower than those previously reported at pH 5 by 4 kJ/mol and 8 kJ/mol respectively (Barna *et al.*, 1985). In order to check the influence of pH on the

thermodynamic parameters, as well as the existence of changes in the protonation state of the antibiotics coupled to the peptide-binding process, calorimetric measurements of Ac-D-Ala-D-Ala binding to both antibiotics were performed at pH 7.0 and 5.0, with two buffers with different ionization enthalpies. Substitution of 0.085 M-NaCl/0.05 M-imidazole/HCl buffer at pH 7.0 for phosphate buffer decreased the enthalpy change of formation of Ac-D-Ala-D-Ala complexes with teicoplanin and its aglycone, with no significant effect on the binding constant (see Table 4). The number of proton equivalents absorbed per mol of complex formed, Δ*N*_{H⁺}, was determined by using eqn. (1):

$$\Delta H = \Delta H_{\text{app.}} + \Delta H_{\text{i,buffer}} \cdot \Delta N_{\text{H}^+} \quad (1)$$

Fig. 3. Difference absorption titration curves generated by Ac₂-Lys-D-Ala-D-Ala binding to teicoplanin (○), teicoplanin aglycone (●), ristocetin (△) and vancomycin (▲) at 37 °C in 0.1 M-phosphate buffer, pH 7

The curves correspond to the theoretical fitting of the experimental data by the use of thermodynamic parameters collected in Tables 1–3.

where $\Delta H_{i, \text{buffer}}$ is the ionization enthalpy of the buffer ($\Delta H_{i, \text{phosphate}} = 3.49$ kJ/mol, $\Delta H_{i, \text{imidazole}} = 36.9$ kJ/mol) (Christensen *et al.*, 1976). ΔN_{H^+} values of 0.27 and 0.37 were obtained for teicoplanin and its aglycone respect-

ively. The apparent enthalpy changes corrected for the buffering enthalpic contributions, ΔH_{app} , were found to be -40.9 kJ/mol for teicoplanin and -43.3 kJ/mol for the aglycone.

At pH 5.0, on the other hand, neither the antibiotics nor the peptide appreciably change their protonation states after formation of the complex, since the enthalpy changes measured in either phosphate buffer or imidazole buffer are approximately the same, within the experimental uncertainties. Table 4 summarizes the best-fitting thermodynamic parameters of the experimental curves at pH 5.0 and pH 7.0.

As for other glycopeptide antibiotics (Brown *et al.*, 1975; Popieniek & Pratt, 1988), the protonated form of the amino group of both antibiotics is stabilized as a result of formation of the complex, probably through the interaction with the peptide carboxylate anion. The dissociation constants of those groups in the bound state, K'_a , were calculated by using eqn. (2):

$$\Delta N_{H^+} = \frac{[H^+]}{[H^+] + K'_a} - \frac{[H^+]}{[H^+] + K_a} = f' - f \quad (2)$$

and the reported values for free teicoplanin ($pK_a = 7.1$) and its aglycone ($pK_a = 6.9$) (Malabarba *et al.*, 1984).

A pK'_a value of 7.65 was estimated in both complexes. The thermodynamic relationships for the peptide binding to the antibiotics at pH 7.0 are given by eqns. (3) and (4) (Eftink & Biltonen, 1980):

Table 3. Thermodynamic parameters of Ac₂-Lys-D-Ala-D-Ala binding to vancomycin and ristocetin in 0.1 M-phosphate buffer, pH 7.0

Antibiotic	T (°C)	−ΔG (kJ/mol)	−ΔS (J/mol·K)	−ΔH (kJ/mol)	−ΔCp (J/mol·K)
Vancomycin	25	33.1 ± 0.5	40 ± 8	45 ± 2	
	25	35.1*	—	—	
	25	34.1†	—	—	
	28	35.5‡	—	—	580 ± 10
	30	31.7 ± 0.5	54 ± 8	48 ± 2	
	37	30.8 ± 0.5	68 ± 5	52 ± 1	
Ristocetin	25	29.6 ± 0.4	24 ± 12	37 ± 1	
	25	32.7*	—	—	
	28	32.6‡	—	—	400 ± 200
	30	29.5 ± 0.3	26 ± 9	37 ± 1	
	37	29.6 ± 0.3	40 ± 8	42 ± 1	

* From Nieto & Perkins (1971a), pH 5.

† From Popieniek & Pratt (1987), pH 7.

‡ From Williamson *et al.* (1984), pH 5.

Table 4. Experimental thermodynamic parameters for Ac-D-Ala-D-Ala binding to teicoplanin and its aglycone at pH 7.0 and 5.0

Buffer	pH	Teicoplanin			Teicoplanin aglycone		
		−ΔH (kJ/mol)	−ΔG (kJ/mol)	−ΔS (J/mol·K)	−ΔH (kJ/mol)	−ΔG (kJ/mol)	−ΔS (J/mol·K)
Phosphate	7.0	40 ± 1	31.2 ± 0.4	29 ± 5	42 ± 1	23.9 ± 0.2	60 ± 4
	5.0	34 ± 1	31.8 ± 0.1	7 ± 3	32 ± 2	24.9 ± 0.1	24 ± 7
Imidazole	7.0	31 ± 1	31.5 ± 0.3	−2 ± 5	29 ± 1	—*	—
	5.0	35 ± 2	—*	—	37 ± 2	25.2 ± 0.2	40 ± 7

* The enthalpy change was determined at saturating concentrations of Ac-D-Ala-D-Ala.

$$\Delta G_{\text{app.}} = \Delta G^0 - RT \cdot \ln \left(\frac{1 + [\text{H}^+]/K_a'}{1 + [\text{H}^+]/K_a} \right) \quad (3)$$

$$\Delta H_{\text{app.}} = \Delta H^0 - f' \cdot \Delta H_i^{0'} + f \cdot \Delta H_i^0 \quad (4)$$

where $\Delta G_{\text{app.}}$ is the experimental Gibbs free energy change, ΔH^0 and ΔG^0 are the thermodynamic parameters of binding for an antibiotic in which the modified group was fully deprotonated, and ΔH_i^0 and $\Delta H_i^{0'}$ are the ionization enthalpies of the amino groups in the free and in the bound states respectively. From these equations and the $\text{p}K_a$ values given above, ΔG^0 values were calculated to be -29.1 kJ/mol for teicoplanin and -21.2 kJ/mol for the aglycone.

The thermodynamic parameters for the reaction of the peptide with the antibiotic when the modified residue is already protonated, ΔG_H^0 and ΔH_H^0 , are given by eqns. (5) and (6) (Eftink & Biltonen, 1980):

$$\Delta G_H^0 = \Delta G^0 - RT \cdot \ln(K_a/K_a') \quad (5)$$

$$\Delta H_H^0 = \Delta H^0 - \Delta H_i^0 - \Delta H_i^{0'} \quad (6)$$

The estimated Gibbs free energies, ΔG_H^0 , were -32.3 kJ/mol for teicoplanin and -25.5 kJ/mol for the aglycone.

At pH 5.0, where both the anionic and cationic residues involved in this interaction are fully charged, the experimental changes in Gibbs free energy agree fairly well with the calculated ΔG_H^0 values at pH 7.0. Thus the enthalpy change measured at pH 5.0 (-34 ± 2 kJ/mol for both complexes) may be considered as a good estimation of ΔH_H^0 at pH 7.0.

The experimental enthalpies collected in Tables 1–3 should be corrected by the effect of buffering, in order to obtain the apparent enthalpy changes of peptide binding to each antibiotic (Eftink & Biltonen, 1980). However, the low enthalpy of ionization of phosphate makes the magnitude of this correction very small, about the same order of magnitude as the experimental uncertainties (-0.9 and -1.3 kJ/mol for the enthalpy and -2.5 and -4 J/mol·K for the entropy of binding to teicoplanin and the aglycone respectively). Since the electrostatic interactions between the peptide carboxylate anion and the antibiotic amino residue may be assumed to be very similar for all the peptidic ligands studied here, these terms would be cancelled when the differences in the thermodynamic parameters of binding are used.

In order to verify the equilibrium constants derived from either the calorimetric or the spectrophotometric titration curves, the binding enthalpies were also calculated from van't Hoff plots (see Fig. 4). These values agreed, within the experimental uncertainties, with those derived from the calorimetric measurements. However, it is worth pointing out that the precision of the latter values is higher than those derived from the van't Hoff plots.

The plots of ΔH values versus temperature were linear for all the peptide–antibiotic interactions studied. The heat capacity changes, ΔC_p , calculated from the least-squares linear fits, taking into account the errors in the enthalpy values (Irving & Quickenden, 1983), are shown in Tables 1–3. The large standard errors in ΔC_p values arise from the inclusion of the enthalpy errors at each temperature, especially in those cases where a weak temperature-dependence of the enthalpy was observed.

Fig. 4. van't Hoff plots of the equilibrium constants of Ac₂Lys-D-Ala-D-Ala to teicoplanin (○), teicoplanin aglycone (●), vancomycin (□) and ristocetin (■)

The straight lines correspond to the fitting of the experimental values when the calorimetric enthalpy values are taken as the van't Hoff enthalpy.

DISCUSSION

The thermodynamic parameters collected in Table 4 show that the interaction between the peptide carboxylate anion and the charged amino group of the antibiotic increases the stability of teicoplanin and teicoplanin aglycone complexes by 3.1 and 4.3 kJ/mol respectively, values very similar to estimations for the charge–charge electrostatic attraction in vancomycin and ristocetin aglycone complexes (Kannan *et al.*, 1988; Herrin *et al.*, 1985). The influence of deprotonation on the stability is also comparable with the effect of *N*-acetylation on Ac-D-Ala-D-Ala binding to the 2-acetamido-2-deoxy- β -D-glucopyranosyl derivative of teicoplanin found by Barna *et al.* (1985). This result could be indicative that no direct interactions are established between the amino group and the carboxylate anion. An analogous model has also been suggested for the ristocetin and vancomycin complexes (Williams *et al.*, 1983; Williams & Waltho, 1988; Kannan *et al.*, 1988).

The association constants for Ac-D-Ala-D-Ala binding to either teicoplanin and teicoplanin aglycone at pH 7.0 and 5.0 (see Table 4) indicate that the pH change cannot account for the differences found between the present values and those given by Barna *et al.* (1985).

Let us now analyse the interaction of Ac-D-Ala with

the four antibiotics. The enthalpic character of the process indicates that van der Waals interactions and/or hydrogen-bonding are of primary significance for Ac-D-Ala binding to teicoplanin and its aglycone (Eftink & Biltonen, 1980) (see Tables 1 and 2). Nevertheless, hydrophobic interactions, as well as electrostatic forces (see above), may act as a stabilizing factor on formation of the complex, as already suggested for vancomycin and ristocetin (Williamson *et al.*, 1984; Williamson & Williams, 1984). The Gibbs free energy of Ac-D-Ala binding to vancomycin is about 3–4 kJ/mol less favourable than for the other three antibiotics, perhaps connected with the conformational change that vancomycin undergoes to form the binding locus for the peptide carboxylate group (Williams *et al.*, 1983; Williams & Waltho, 1988; Kannan *et al.*, 1988). The differences in the enthalpic and entropic contributions for the other three antibiotics clearly indicate that the nature and/or the intensity of the binding forces that are involved also depend on the structure of the antibiotic considered. The data in Table 5 show that, in spite of the additional conformational change that vancomycin undergoes before formation of the complex, the energetic balance between all the binding forces, except those concerning the methyl group, $\delta\Delta X$, is similar for this antibiotic and ristocetin. This result might indicate that the conformational change in aqueous solution does not contribute appreciably to the total values. It is worth noting the existence of a second conformation for vancomycin, as shown by nuclear-Overhauser-effect studies in $[^2\text{H}_6]$ dimethyl sulphoxide (Williams & Waltho, 1988) for which the carboxylate anion binding pocket is already formed to an appreciable extent. The thermodynamic parameters for the interaction of teicoplanin and its aglycone are almost identical within the estimated uncertainties in the calculated values. The more negative enthalpy and entropy changes suggest that the hydrogen bonds formed in the carboxylate pocket and/or dispersion forces would be more favourable for these than for the other two antibiotics.

From Table 6 it can be seen that the strong stabilization of the antibiotic-peptide complexes induced by addition of a glycine residue in position 2 is similar in the four cases (the C-terminal amino-acid is denoted residue 1). Whereas the interaction with this residue and teicoplanin is enthalpically driven, the positive entropy change, $\Delta\Delta S$, represents a substantial part of the $\Delta\Delta G$ values for the other antibiotics. Thus it can be concluded that for ristocetin and teicoplanin aglycone the transfer of glycine

from bulk water would account for most of the interacting forces, becoming the primary one in the vancomycin complex, as indicated by the positive $\Delta\Delta H$ and $\Delta\Delta S$ values (Eftink & Biltonen, 1980). Since both values for the teicoplanin complex are negative, van der Waals interactions and/or hydrogen-bonding should be the major forces involved in the interaction with the glycine residue. The formation of a hydrogen bond between the antibiotic and the carbonyl oxygen atom of the acetyl group (Sheldrick *et al.*, 1978; Williamson & Williams, 1984) would be compatible with the thermodynamic data of Ac-Gly-D-Ala binding in the latter case. However, neither the enthalpic nor the entropic terms for the vancomycin complex suggest the net formation of such a hydrogen bond. In the other two cases the negative enthalpy values and the less positive entropies might indicate that van der Waals interactions and/or a less favourable hydrogen-bond formation are of importance.

The presence of a methyl group in residue 2 of the dipeptide contributes to the Gibbs free energy by slightly variable amounts, ranging from –3 kJ/mol for teicoplanin to –0.6 kJ/mol for ristocetin. $\Delta\Delta H$ and $\Delta\Delta S$ values for these two antibiotics point to the importance of hydrophobic forces in the interaction with the methyl group at this locus, in accord with n.m.r. data for ristocetin, which located this group in the inside of a hydrophobic pocket (Williams *et al.*, 1983; Fesik *et al.*, 1986; Williams & Waltho, 1988).

The interaction with the methyl group in residue 2 accounts for most of the strong decrease in the stability of the Ac-D-Ala-D-Ala-teicoplanin complex associated with removal of sugar (4 kJ/mol), as it also occurs in ristocetin (Barna *et al.*, 1985), and it is comparable with the effect that removal of the α -D-mannopyranosyl residue has on the Gibbs free energy of Ac-D-Ala-D-Ala binding to teicoplanin (Barna *et al.*, 1984, 1985). Thus $\Delta\Delta H$ and $\Delta\Delta S$ values from Table 6 and also the structural analogies of teicoplanin with the region of the ristocetin molecule involved in this interaction suggest an analogous model of binding to this locus for both antibiotics.

In the vancomycin complex the negative enthalpy term determines the stabilization of the complex, showing clearly that van der Waals interactions or, more probably a slightly more favoured hydrogen bond (Eftink & Biltonen, 1980) are the factors that determine the bond energy. The second possibility seems to be the most plausible explanation according to previous results (Nieto & Perkins, 1971b; Williams *et al.*, 1983; Williamson & Williams, 1984; Kannan *et al.*, 1988).

Table 5. Thermodynamic parameters for Ac-D-Ala binding to teicoplanin, vancomycin, ristocetin and teicoplanin aglycone, corrected by the contributions of the alanyl side chain to peptide binding collected in Table 6, $\delta\Delta X$

$\delta\Delta X = \Delta X(\text{Ac-D-Ala}) - \Delta\Delta X(\text{CH}_3 \text{ group of residue 1})$, where X is G , H or S and $\Delta\Delta X$ refers to the differences between ΔX values for Ac-D-Ala-D-Ala and Ac-D-Ala-Gly binding to each antibiotic. Abbreviations: V, vancomycin; R, ristocetin; T, teicoplanin; TA, teicoplanin aglycone.

T (°C)	$-\delta\Delta H$ (kJ/mol)				$-\delta\Delta S$ (J/mol·K)				$-\delta\Delta G$ (kJ/mol)			
	V	R	T	TA	V	R	T	TA	V	R	T	TA
25	27±3	32±3	30±3	36±3	62±12	77±12	67±12	85±13	8.0±0.8	8.4±0.8	10.2±0.9	10.7±0.9
30	26±3	30±3	33±3	36±3	63±12	74±12	75±18	84±9	8.5±0.8	7.8±0.8	10.4±0.9	10.8±0.9
37	26±3	32±3	37±3	38±3	65±13	83±12	85±10	88±12	7.9±0.8	6.5±0.8	9.9±0.6	10.8±0.6

Table 6. Differences in the thermodynamics of antibiotic-cell-wall peptide analogue binding, generated when one amino acid is added or changed in the bonding peptide, $\Delta\Delta X$

Thermodynamic parameters for Ac-D-Ala and dipeptides binding to vancomycin and ristocetin are taken from Rodriguez-Tebar *et al.* (1986). Abbreviations: V, vancomycin; R, ristocetin; T, teicoplanin; TA, teicoplanin aglycone.

	T (°C)	$-\Delta\Delta G$ (kJ/mol)				$-\Delta\Delta H$ (kJ/mol)				$-\Delta\Delta S$ (J/mol·K)				Peptide change
		V	R	T	TA	V	R	T	TA	V	R	T	TA	
CH ₃ group of residue 2	25	9.8	8.7	10.4	10.7	-1	4	12	5	-38	-16	6	-19	Ac-D-Ala
	30	9.7	8.9	10.1	10.2	-1	3	10	6	-34	-18	1	-14	↓ Ac-Gly-D-Ala
	37	10.0	9.4	10.5	10.7	-1	3	9	5	-36	-21	11	-18	
	25	1.6	0.2	2.8	-4.0	1	-7	-8	-6	-1	-26	-36	-7	Ac-Gly-D-Ala
	30	1.6	0.4	2.9	-3.5	4	-5	-6	-4	8	-17	-30	-2	↓ Ac-D-Ala-D-Ala
CH ₃ group of residue 1	37	1.5	1.1	3.0	-5.0	6	-5	-4	-3	11	-21	-25	6	
	25	5.6	8.9	7.8	6.4	5	2	6	7	-2	-23	-7	2	Ac-D-Ala-Gly
	30	5.4	9.4	7.1	5.5	6	3	6	6	-3	-20	-4	1	↓ Ac-D-Ala-D-Ala
	37	5.5	9.8	6.9	5.3	7	5	5	6	-2	-16	-6	2	
	25	7.7	3.4	5.8	11.1	13	7	9	10	19	14	11	-4	Ac-D-Ala-D-Ala
	30	6.5	3.0	6.3	11.6	13	5	8	12	22	7	5	2	↓ Ac ₂ -Lys-D-Ala-D-Ala
	37	5.9	2.8	6.5	11.2	14	7	15	14	30	15	28	8	

The thermodynamic parameters for the interaction of a methyl group in position 1 of a dipeptide were derived from the corresponding values for Ac-D-Ala-Gly and Ac-D-Ala-D-Ala binding to the different antibiotics. The results in Table 6 indicate a moderately exothermic binding with also an entropic contribution to the change in Gibbs free energy that is particularly significant for the ristocetin complex. Again, the negative enthalpy changes associated with this interaction indicate the involvement of van der Waals forces and/or hydrogen-bonding in the binding of a methyl group in position 1 of a dipeptide to its pocket. Both vancomycin and teicoplanin, which bear a chlorine substituent near this locus, behave very similarly, suggesting that van der Waals interactions with this residue and with the sugar substituent in the side chain of the four amino acid residues (vancosamine in the vancomycin complex) may account for the differences found between them and ristocetin. It was postulated that the large change observed in ristocetin binding when a methyl group is added at residue 1 was mainly due, not to hydrophobic interactions, but to an increase in hydrogen-bond strength (Williamson & Williams, 1984). This hypothesis does not seem to hold for this locus, since both the entropic and enthalpic contributions are less negative than in the other two cases. This is contrary to what would be expected if the largest change in ΔG contributed by methyl group binding to this locus were due to the improvement of hydrogen-bond geometry. Furthermore, it should be taken into account that both enthalpic and entropic contributions by hydrophobic interactions and dispersion forces are opposite (Eftink & Biltonen, 1980), and that a more favourable geometry for the van der Waals contacts may result in a partial reversal of the stability of the complex. This effect may explain the differences in $\Delta\Delta G$ values found for this interaction between ristocetin and vancomycin, since the latter provides a better fit for this residue than the corresponding site in ristocetin (Williams *et al.*, 1983; Kannan *et al.*, 1988), mainly through vancosamine interaction. The differences found between teicoplanin and teicoplanin aglycone interaction with this group should denote the loss of a hydrophobic wall, for the binding of a methyl group in position 1 of a dipeptide to the pocket, which could be provided by the *N*-acyl-D-glucosamine moiety.

The value of the change in Gibbs free energy for tripeptide binding to teicoplanin agrees with that reported by Popieniek & Pratt (1987) at pH 7.0. The values for the Gibbs free energy for vancomycin and ristocetin binding to Ac₂-Lys-D-Ala-D-Ala are about 2–3 kJ/mol lower than those previously reported at pH 5.0 (Nieto & Perkins, 1971*a,b*; Williamson & Williams, 1984) and 1 kJ/mol lower than the change in Gibbs free energy found at pH 7.0 for vancomycin (Popieniek & Pratt, 1987). The most significant differences are those between the calorimetric enthalpies and the previous van't Hoff estimates reported for ristocetin (−41.8 kJ/mol) and vancomycin (−73 kJ/mol) (Williamson & Williams, 1984). Consequently, the entropy values are much less unfavourable than those calculated previously.

The interaction with the lysine residue is enthalpically driven in the four complexes studied, indicating that van der Waals interactions or hydrogen bonds are responsible for the increase in stability observed on passing from formation of dipeptide complexes to formation of tripeptide complexes with the antibiotic. N.m.r. studies

suggest stronger hydrogen bonding to the lysine carbonyl oxygen atom than to the acetyl carbonyl oxygen atom of Ac-D-Ala-D-Ala, through the NH proton of the amide bond between residues 6 and 7 of the antibiotic. On the contrary, few van der Waals contacts are established (Williams *et al.*, 1983; Williamson & Williams, 1985; Kannan *et al.*, 1988). Thus it appears that the large change in binding energy contributed by lysine is mainly due to the hydrogen-bonding, in agreement with a previous hypothesis (Williams *et al.*, 1983; Williamson & Williams, 1985; Kannan *et al.*, 1988). The enthalpic contributions listed in Table 6 suggest that this effect would be stronger in vancomycin complexes than in ristocetin complexes.

The binding constant of teicoplanin to the tripeptide is slightly changed upon removal of sugar, in contrast with the strong effect on Ac-D-Ala-D-Ala binding. Thus, although the mannose residue should be important in shielding the methyl group of the non-C-terminal alanine residue from water in the dipeptide, the lysine side chain should protect this residue from solvent, making the role of mannose less significant, as in ristocetin. The increment in the entropic contribution of the lysine residue associated with removal of sugar in teicoplanin gives further support for this conclusion.

Finally, it is possible to estimate the ΔG values of hydrogen-bond formation in the carboxylate pocket for the Ac-D-Ala-antibiotic complexes. If the changes in Gibbs free energies contributed by the electrostatic interactions (see above and Kannan *et al.*, 1988; Herrin *et al.*, 1985) and also by the methyl group of residue 1 (Table 6) are subtracted from the total ΔG values for Ac-D-Ala binding, the following quantities are obtained for hydrogen-bonding in the different complexes: teicoplanin, $\Delta G = -7.1$ kJ/mol; teicoplanin aglycone, $\Delta G = -7.5$ kJ/mol; vancomycin, $\Delta G = -5.2$ kJ/mol; ristocetin, $\Delta G = -5.3$ kJ/mol.

The present thermodynamic data suggest that the structural analogies of teicoplanin with either vancomycin or ristocetin result in analogous modes of interaction at the respective loci. Nevertheless, the particularities of teicoplanin confer on this antibiotic some of the structural requirements that improve the affinity of glycopeptidic antibiotics towards analogues of cell-wall peptides, as indicated by the following experimental results: (1) the affinities of teicoplanin towards any of the peptides here studied are higher than those of vancomycin and ristocetin; (2) the bonding forces established through a methyl group in position 1 or position 2 of a dipeptide and teicoplanin are stronger than those existing in vancomycin and ristocetin complexes respectively; (3) teicoplanin is the only antibiotic in this study for which the formation of a hydrogen bond with the carbonyl oxygen atom of the acetyl group of Ac-Gly-D-Ala is clearly compatible with thermodynamic data; (4) the energy of the hydrogen bonds formed in the carboxylate pocket is higher for teicoplanin complexes than for vancomycin and ristocetin ones.

We thank Dr. P. Sellers (Department of Physical Chemistry II, University of Lund, Lund, Sweden) for helpful discussions, M. V. Lopez Moyano for excellent technical assistance, and Gruppo Lepetit, Milan, Italy, for samples of teicoplanin and teicoplanin aglycone. P. A. acknowledges a fellowship from the Spanish 'Fondo de Investigaciones Sanitarias'. This work has been supported by Grants BT9/85 and PBO 352 from the Spanish Comisión Interministerial de Ciencia y Tecnología.

REFERENCES

- Bardone, M. R., Paternoster, M. & Coronelli, C. (1978a) *J. Antibiot.* **31**, 170-177.
- Bardone, M. R., Tuan, G. & Coronelli, C. (1978b) *Int. Symp. Chem. Nat. Prod.* 11th Abstr. Pap. no. 239.
- Barna, J. C. J., Williams, D. H., Stone, D. J. M., Leung, T. W. C. & Doddrell, D. M. (1984) *J. Am. Chem. Soc.* **106**, 4895-4902.
- Barna, J. C. J., Williams, D. H. & Williamson, M. P. (1985) *J. Chem. Soc. Chem. Commun.* 254-256.
- Borghi, A., Coronelli, C., Faniuolo, L., Allievi, G., Pallanza, R. & Gallo, G. G. (1984) *J. Antibiot.* **37**, 615-620.
- Brown, J. P., Terenius, L., Feeney, J. & Burgen, A. S. V. (1975) *Mol. Pharmacol.* **11**, 126-132.
- Chen, A. & Wadsö, I. (1982) *J. Biochem. Biophys. Methods* **6**, 307-316.
- Christensen, J. J., Hansen, L. D. & Izzat, R. M. (1976) *Handbook of Proton Ionization*, pp. 109 and 148, Wiley Interscience, New York.
- Debono, M., Molloy, R. M., Barnhart, M. & Dorman, D. E. (1980) *J. Antibiot.* **33**, 1407-1416.
- Debono, M., Merkel, K. E., Molloy, R. M., Barnhart, M., Presti, E., Hunt, A. H. & Hamill, R. L. (1984) *J. Antibiot.* **37**, 85-95.
- Eftink, M. & Biltonen, R. (1980) in *Biological Microcalorimetry* (Beezer, A. E., ed.), pp. 343-412, Academic Press, London.
- Fesik, S. W., O'Donnell, T. J., Gampe, R. T. & Olejniczak, E. T. (1986) *J. Am. Chem. Soc.* **108**, 3165-3170.
- Herrin, R., Thomas, A. H., Perun, T. J., Mao, J. C. & Fesik, S. W. (1985) *J. Med. Chem.* **28**, 1371-1375.
- Irving, J. A. & Quickenden, T. I. (1983) *J. Chem. Educ.* **60**, 711-712.
- Kannan, R., Harris, C. M., Harris, T. M., Waltho, J. P., Skelton, N. J. & Williams, D. H. (1988) *J. Am. Chem. Soc.* **110**, 2946-2953.
- Malabarba, A., Strazzolini, P., Depaoli, A., Landi, M., Berti, M. & Cavalleri, B. (1984) *J. Antibiot.* **37**, 988-999.
- Marshall, F. J. (1965) *J. Med. Chem.* **18**, 18-22.
- McGahren, W. J., Leese, R. A., Morton, G. O., Kuck, N. A. & Ellestad, G. A. (1985) *J. Antibiot.* **36**, 1671-1682.
- Nielsen, R. V., Hyldig-Nielsen, F. & Jacobsen, K. (1982) *J. Antibiot.* **35**, 1561-1564.
- Nieto, M. & Perkins, H. R. (1971a) *Biochem. J.* **123**, 773-787.
- Nieto, M. & Perkins, H. R. (1971b) *Biochem. J.* **124**, 845-852.
- Pallanza, R., Berti, M., Goldstein, B. P., Mapelli, E., Randisi, E., Scotti, E. & Arioli, V. (1983) *J. Antimicrob. Chemother.* **11**, 419-425.
- Parenti, F., Beretta, G., Berti, M. & Arioli, V. J. (1978) *J. Antibiot.* **31**, 276-283.
- Popieniek, P. H. & Pratt, R. F. (1987) *Anal. Biochem.* **156**, 108-113.
- Popieniek, P. H. & Pratt, R. F. (1988) *J. Am. Chem. Soc.* **110**, 1285-1286.
- Rodriguez-Tebar, A., Vazquez, D., Perez Velazquez, J. L., Laynez, J. & Wadsö, I. (1986) *J. Antibiot.* **39**, 1578-1583.
- Sheldrick, G. M., Jones, P. G., Kennard, O., Williams, D. H. & Smith, G. A. (1978) *Nature (London)* **271**, 223-225.
- Sitrin, R. D., Chan, G. W., Chapin, F., Giovenella, A. J., Grappel, S. F., Jeffs, P. W., Phillips, L., Snader, K. M. & Nisbet, L. J. (1986) *J. Antibiot.* **39**, 68-75.
- Somma, S., Gastaldo, L. & Corti, A. (1984) *Antimicrob. Agents Chemother.* **26**, 917-923.
- Spink, C. & Wadsö, I. (1976) *Methods Biochem. Anal.* **23**, 1-160.

- Varaldo, P. E., Debbia, E. & Schito, G. C. (1983) *Antimicrob. Agents Chemother.* **23**, 402–406
- Williams, D. H. & Waltho, J. P. (1988) *Biochem. Pharmacol.* **37**, 133–141
- Williams, D. H., Williamson, M. P., Butcher, D. N. & Hammond, S. J. (1983) *J. Am. Chem. Soc.* **105**, 1332–1339
- Williamson, M. P. & Williams, D. H. (1984) *Eur. J. Biochem.* **38**, 345–348
- Williamson, M. P. & Williams, D. H. (1985) *J. Chem. Soc. Perkin Trans. 1* 949–956
- Williamson, M. P., Williams, D. H. & Hammond, S. J. (1984) *Tetrahedron* **40**, 569–577

Received 22 March 1989/25 July 1989; accepted 4 August 1989