THERMOCHEMISTRY OF THE INTERACTION BETWEEN PEPTIDES AND VANCOMYCIN OR RISTOCETIN

ALFREDO RODRÍGUEZ-TEBAR, DAVID VÁZQUEZ, JOSÉ L. PÉREZ VELÁZQUEZ[a], JOSÉ LAYNEZ[a,b,†] and INGEMAR WADSÖ[b]

Centro de Biología Molecular CSIC,
UAM, Madrid 28034, Spain
[a]Instituto de Química Física CSIC,
Serrano, 119, Madrid 28006, Spain
[b]División of Thermochemistry, Chemical Center, University of Lund,
Box 124, S-221 00 Lund, Sweden

(Received for publication June 21, 1986)

The thermodynamics of the interaction between the glycopeptide antibiotics vancomycin and ristocetin and bacterial peptidoglycan peptide analogs have been studied by means of a microcalorimetric titration technique. From results of the calorimetric measurements, changes in Gibbs energies, enthalpies, entropies and heat capacities for the binding reactions have been calculated. The derived thermodynamic data have been discussed on the basis of stereochemical data available for the interaction of acetyl-D-alanyl-D-alanine with each of the two antibiotics. The significance of entropic factors connected with conformational changes of the antibiotics is stressed.

Both vancomycin and ristocetin are glycopeptide antibiotics active against Gram-positive bacteria (see reference 1 for a review on this group of antibiotics). The early work of PERKINS and his collaborators elucidated much of the mechanism of action of these two antibiotics. They first isolated 1:1 complexes between the peptidoglycan precursor UDP-*N*-acetylmuramylpeptide and the antibiotics from bacteria which had been treated with vancomycin or ristocetin[2]. Later, PERKINS demonstrated that the inhibitory effects of vancomycin were due to the specific binding of peptidoglycan precursors containing *C*-terminal D-alanyl-D-alanine residues[3]. A comprehensive study of the binding of both vancomycin and ristocetin with peptidoglycan peptide analogs was made by NIETO and PERKINS[4,5] who clarified the structural requirements in the peptides for productive interaction with the antibiotics. The studies were carried out at different temperatures by means of UV difference spectroscopy and some binding constants were obtained[4~6]. More recently, the NMR studies by WILLIAMS' and BURGEN's groups led to the proposal of a general topographical model for the interaction of vancomycin and ristocetin with peptidoglycan peptide analogs[1,6~11]. Equilibrium data and approximate values for changes in enthalpies and entropies were derived from spectroscopic investigations[10,11].

The recent developments in microcalorimetric titration methods have significantly improved the possibilities for accurate and detailed thermodynamic characterization of ligand binding reactions of the present type[12~16]. Rather surprisingly, microcalorimetric methods have earlier been used very little in connection with antibiotic binding reactions. In one study, the interaction between penicillin and albumin was measured[17] and in another the binding of ions to ion-carrier antibiotics[18]. We

† The correspondence should be addressed to: J. LAYNEZ, Instituto de Química y Física, Serrano, 119, Madrid 28006, Spain.

report here results from a microcalorimetric study of the interaction of vancomycin and ristocetin with several peptidoglycan peptide analogs. A preliminary report from the early part of this work has been given elsewhere[19].

Materials and Methods

Substrates (Peptides)

N-Acetyl-D-alanyl-D-alanine (Ac-D-Ala-D-Ala), *N*-acetyl-glycyl-D-alanine (Ac-Gly-D-Ala) and *N*-acetyl-D-alanyl-D-glycine (Ac-D-Ala-Gly) were prepared by acetylation of their corresponding dipeptides (Sigma Chem. Co., St. Louis, Mo.) as described previously[4]. *N*-Acetyl-D-alanine (Ac-D-Ala) was purchased from Sigma. The identity of all compounds was assesed by NMR measurements.

Antibiotics

Vancomycin was obtained from Sigma Chemical Company. Ristocetin A (referred to in this study simply as ristocetin) was a gift from Lundbeck, Copenhagen, Denmark. Both antibiotics were used without further purification.

Microcalorimetric Measurements

Calorimetric titrations were carried out at 25, 30 and 37°C with an LKB batch microcalorimeter equipped with a titration assembly (LKB 2107-350), using a method similar to that described by CHEN and WADSÖ[14]. 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[14]. In titration experiments, the reaction vessel was charged with 4 ml 0.10 M sodium phosphate buffer, pH 7.0, containing vancomycin or ristocetin. In order to prevent self-aggregation of the antibiotics, their concentrations were kept as low as 0.5 mg/ml.

The reference vessel was charged with 4 ml of the phosphate buffer. In the calorimetric titrations solutions of the peptides, in the same phosphate buffer, were injected stepwise into both vessels by use of motor driven Hamilton syringes. In each step 10 μl of solution was added. For each titration, about 10 additions were made. The results were corrected for small differential effects due to compression, mixing and dilution which were determined in separate control experiments.

A few binding experiments with vancomycin were carried out at 15 and 20°C by use of an LKB 2107 flow microcalorimeter. A flow rate of 0.13 ml/minute and a flow time of 15 minutes were used, leading to steady-state conditions. The baseline values for the binding experiments included the enthalpy of dilution of the peptide, whereas the dilution enthalpy for vancomycin was determined in separate experiments.

Calculation of Results

The association constants for the binding reactions studied in this work are moderately large, which makes it possible to derive both enthalpy values and the binding constants from the results of the calorimetric titrations, see *e.g.* references 12 and 13. The basic assumption made in such calculations is that the enthalpy change for each titration step (corrected for dilution effects, *etc.*) is directly proportional to the amount of ligand which interacts with the host molecules. In the present work, the calorimetric titration curves were analyzed either according to the procedure described by CHEN and WADSÖ[14] or by the method used by BJURULF *et al.*[20, *cf.* 12, 13].

From the derived value for the (apparent) equilibrium constant K'_c, the apparent standard Gibbs energy change was calculated, $\Delta G^{0\prime} = -RT \ln K'_c$.

Combination of $\Delta G^{0\prime}$ and corresponding enthalpy change, $\Delta H^{0\prime}$, will lead to the entropy change, $\Delta G^{0\prime} = \Delta H^{0\prime} - T\Delta S^{0\prime}$. From the enthalpy values determined at different temperatures, the change in heat capacity is derived, $\Delta C_p^{0\prime} = (d\Delta H^{0\prime}/dT)$.

In the flow calorimetric experiments with vancomycin, the concentrations of the peptide, were high enough to lead to saturation of the antibiotic. Therefore, results of these experiments gave directly values for the enthalpy changes, but no values could be derived for the equilibrium constants.

Fig. 1. Calorimetric record from the titration of vancomycin with Ac-Gly-D-Ala. 10 μl of Ac-Gly-D-Ala solution were injected in each step.

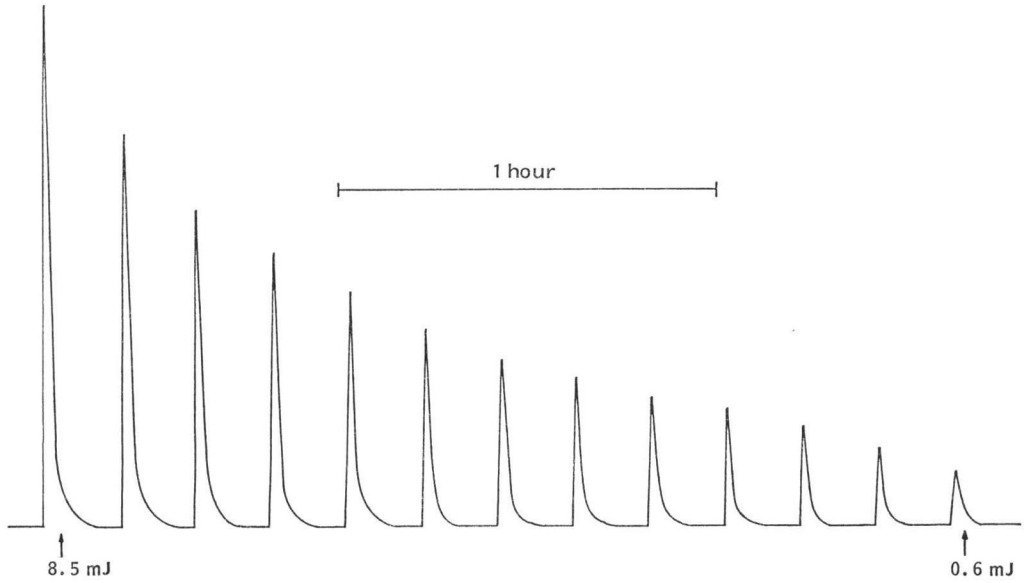


Table 1. Thermodynamic parameters of peptide binding to vancomycin.

	Temp (°C)	$-\Delta G^{0'}$ (kJmol^{-1})	$-\Delta H^{0'}$ (kJmol^{-1})	$-\Delta S^{0'}$ (Jmol^{-1}K^{-1})	$-\Delta C_p^{0'}$ (Jmol^{-1}K^{-1})
Ac-D-Ala	25	14.0±0.3	30.6±1.2	56±5	
	30	13.9±0.5	30.9±0.8	56±4	93±15
	37	13.4±0.4	31.7±1.3	59±5	
Ac-D-Ala-D-Ala	15	—	24.4±1.1[a]	—	
	20	—	26.0±1.3[a]	—	
	25	25.8±0.4	30.4±1.6	15±6	582±45
	30	23.4±0.6	33.4±1.8	33±8	
	37	20.7±0.8	36.5±1.7	51±8	
Ac-Gly-D-Ala	15	—	27.4±1.3[a]		
	20	—	28.2±1.1		
	25	23.8±0.3	29.2±1.1	18±5	
	30	23.8±0.4	30.6±1.5	22±6	160±28
	37	24.2±0.8	30.6±1.4	21±7	
Ac-D-Ala-Gly	25	19.7±0.4	25.6±1.3	20±6	
	30	19.8±0.4	26.8±1.2	23±5	320±36
	37	18.4±0.3	29.4±1.7	36±6	

[a] Flow calorimetric measurements under conditions where the antibiotic become saturated with the ligand.

Results and Discussion

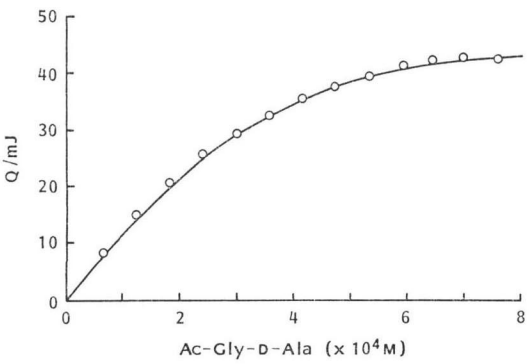
Fig. 1 shows a typical example of a record from a calorimetric titration experiment. The thermodynamic data derived from such titrations are summarized in Tables 1 and 2. The values for each ligand are based on at least three independent determinations. The same results were obtained by the two evaluation methods used, within expected limits of uncertainties.

In Fig. 2, the corrected values for the accumulated heat quantities are plotted against the total concentration (free and bound) of the ligand Ac-Gly-D-Ala. The curve was constructed by use of

Table 2. Thermodynamic parameters of peptide binding to ristocetin.

	Temp (°C)	$-\Delta G^{0\prime}$ (kJmol^{-1})	$-\Delta H^{0\prime}$ (kJmol^{-1})	$-\Delta S^{0\prime}$ (Jmol^{-1}K^{-1})	$-\Delta C_p^{0\prime}$ (Jmol^{-1}K^{-1})
Ac-D-Ala	25	17.4±0.5	28.3±1.4	37±6	
	30	17.2±0.3	29.2±1.7	39±7	332±59
	37	16.2±0.3	32.2±1.6	52±6	
Ac-D-Ala-D-Ala	25	28.8±0.4	25.4±1.6	−11±6	
	30	23.4±0.3	28.0±1.5	15±6	385±52
	37	20.7±0.3	30.1±1.8	30±7	
Ac-Gly-D-Ala	25	28.4±0.6	31.7±1.4	11±7	
	30	28.3±0.4	32.2±1.6	13±7	259±61
	37	25.5±0.3	34.7±1.3	30±5	
Ac-D-Ala-Gly	25	17.4±0.4	23.6±1.1	21±5	
	30	17.6±0.3	25.2±0.9	25±4	177±56
	37	16.8±0.3	25.8±0.8	29±4	

Fig. 2. Titration of vancomycin at 30°C.
The sum of experimental heat quantities, Q, plotted *versus* total ligand concentration. The curve was constructed from Q-values calculated, by use of derived values for K′ and $\Delta H^{0\prime}$.



the derived values for K′ and $\Delta H^{0\prime}$. The excellent agreement between the curve and the experimental points supports the assumed 1 : 1 binding model.

Association constants for the binding of peptides to vancomycin and ristocetin have been determined earlier. In the work by WILLIAMSON and WILLIAMS[11], the equilibrium between Ac-D-Ala-D-Ala and the two antibiotics was measured at four temperatures in the range 28~65°C. From those measurements, which were performed by UV difference spectroscopy at pH 5.0, values for the enthalpy and entropy changes were derived. The results are in general agreement with calorimetric values reported here but due to the differences in pH, no close comparison can be made.

Inspection of Tables 1 and 2 shows that the enthalpy values are significantly more negative than corresponding changes in Gibbs energies, *i.e.* the binding processes are accompanied by decreases in entropy. The interaction between Ac-D-Ala-D-Ala and ristocetin at 25°C forms an exception.

In all cases, the heat capacity changes are negative. The general picture of negative values for Gibbs energy, enthalpy, entropy and heat capacity are characteristic for many types of ligand binding processes in aqueous solution, see *e.g.* reference 21. In all cases the differences between comparable values for the two antibiotics are small, but for each ligand the binding processes are slightly more exothermic for vancomycin than for ristocetin.

A rather detailed description of the binding process of Ac-D-Ala-D-Ala and other peptides to the two antibiotics has emerged from the extensive NMR studies by WILLIAMS' group. These workers conclude that the process is initiated by the interaction between the peptide carboxylate group and a protonated amine exposed to the solvent on the antibiotic. This initial binding step will induce a conformational change in the antibiotic where the carboxylate group will fall into a hydrophobic

pocket. The pocket formed is similar for both antibiotics, but the conformation change is more pronounced for vancomycin. Outside the product, one can visualize H-bonding sites for the peptides. The picture developed by WILLIAMS and his collaborators is suitable as a starting point for a discussion of the thermodynamic data for the binding process. However, it is as yet not possible to conduct a detailed quantitative discussion in terms of contributions to the derived values from hydrogen bond formation, hydrophobic effects, electrostatic effects, *etc.*

The derived values for enthalpy and entropy changes are sufficiently precise to show significant differences between the different ligands and the different antibiotics. However, the differences are small compared to the different contributions from which they presumably are made up. Both the enthalpies and the entropies are increasingly negative with increasing temperatures. In particular for Ac-D-Ala-D-Ala, the temperature coefficient for $-\Delta S$ is large, leading to a significant weakening of the complex at higher temperatures.

ΔC_p-Values for ligand binding processes in aqueous solution are of particular interest. When a hydrophobic group is transferred from a dilute aqueous solution to a non-aqueous environment, the process is accompanied by a large negative ΔC_p. Typically the heat capacity decreases to about 1/3 of its value in water[22,23]. It is interesting to note that such ΔC_p-values vary only slightly with temperature and are highly additive[22], properties which for aqueous ligand binding processes make the ΔC_p-effect appear to be more suitable for analysis than the changes in enthalpies and entropies. The ΔC_p-values shown in Tables 1 and 2 are all negative and are of a magnitude which in some cases could imply a substantial hydrophobic interaction. However, the present peptides do not contain many hydrophobic groups and no correlation between them and the ΔC_p-values is apparent. It is therefore not likely that the pattern for the ΔC_p-values reflects decreased hydrophobic hydrations of the ligand molecules. However, the conformational changes of the antibiotics' molecules seem to involve a decreased exposure of hydrophobic groups to bulk water, in particular for vancomycin, which could give rise to a large decrease in C_p. But there is no consistent difference in the ΔC_p-values for the two antibiotics.

The conformational changes of the antibiotics lead to less flexible structures which, as was pointed out by WILLIAMSON and WILLIAMS[11], should give a negative contribution to ΔS. Negative ΔS-contributions are also expected from H-bond formation in low dielectric media, whereas a decreased exposure of hydrophobic groups to bulk water will give a positive contribution to ΔS[21]. The present results confirm the observation by WILLIAMSON and WILLIAMS[11] that ΔS for the binding of Ac-D-Ala-D-Ala to vancomycin is more negative than that for ristocetin. WILLIAMSON *et al.* took this as a sign of the antibiotic conformation change, which is more drastic for vancomycin than for ristocetin. We find the same tendency for Ac-D-Ala and for Ac-Gly-D-Ala, whereas the values are essentially the same for Ac-D-Ala-Gly. We believe that the negative ΔS-values are mainly due to effects connected with the conformational changes of the antibiotics. However, as for the ΔC_p-values, there are substantial differences in the values for the different processes which cannot easily be correlated with structural features for the reaction components.

Acknowledgments

J. LAYNEZ acknowledges a fellowship from "Fundation Juan March".

References

1) WILLIAMS, D. H.; V. RAJANANDA, M. P. WILLIAMSON & G. BOJASEN: The vancomycin and ristocetin group

of antibiotics. Top. Antibiotic Chem. 5: 119~158, 1980
2) CHATTERJEE, A. N. & H. R. PERKINS: Compounds formed between nucleotides related to the biosynthesis of bacterial cell wall and vancomycin. Biophys. Biochem. Res. Comm. 24: 489~494, 1966
3) PERKINS, H. R.: Specificity of combination between mucopeptide precursors and vancomycin or ristocetin. Biochem. J. 111: 195~205, 1969
4) NIETO, M. & H. R. PERKINS: Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. Biochem. J. 123: 789~803, 1971
5) NIETO, M. & H. R. PERKINS: The specificity of combination between ristocetin and peptides related to bacterial cell wall mucopeptide precursors. Biochem. J. 124: 845~852, 1971
6) BROWN, J. P.; J. FEENEY & A. S. V. BURGEN: A nuclear magnetic resonance study of the interaction between vancomycin and acetyl-D-alanyl-D-alanine in aqueous solution. Mol. Pharmacol. 11: 119~125, 1975
7) BROWN, J. P.; L. TERENIUS, J. FEENEY & A. S. V. BURGEN: A structure-activity study by nuclear magnetic resonance of peptide interactions with vancomycin. Mol. Pharmacol. 11: 126~132, 1975
8) KALMAN, J. R. & D. H. WILLIAMS: An NMR study of the interaction between the antibiotic ristocetin A and a cell wall peptide analogue. Negative nuclear Overhanser effects in the investigation of drug binding sites. J. Am. Chem. Soc. 102: 906~912, 1980
9) WILLIAMS, D. H.; M. P. WILLIAMSON, D. W. BUTCHER & S. J. 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
10) WILLIAMSON, M. P.; D. H. WILLIAMS & S. J. HAMMOND: Interactions of vancomycin and ristocetin with peptides as a model for protein binding. Tetrahedron 40: 569~577, 1984
11) WILLIAMSON, M. P. & D. H. WILLIAMS: Hydrophobic interactions affect hydrogen bond strengths in complexes between peptides and vancomycin or ristocetin. Eur. J. Biochem. 138: 345~348, 1984
12) SPINK, C. & I. WADSÖ: Calorimetry as an analytical tool in biochemistry and biology. *In* Methods of Biochemical Analysis. Vol. 23, *Ed.*, D. GICK, pp. 1~160, John Wiley and Sons, New York, 1976
13) EFTINK, M. & R. BILTONEN: Thermodynamics of interacting biological systems. *In* Biological Microcalorimetry. *Ed.*, A. E. BEEZER, pp. 343~412, Academic Press, London, 1980
14) 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
15) SPOKANE, R. B. & S. J. GILL: Titration microcalorimeter using nanomolar quantities of reactans. Rev. Sci. Instrum. 52: 1728~1733, 1981
16) NORDMARK, M. G.; J. LAYNEZ, A. SCHON, J. SUURKUUSK & I. WADSÖ: Design and testing of a new microcalorimetric vessel for use with living cellular systems and titration experiments. J. Biochem. Biophys. Methods 10: 187~202, 1984
17) LANDAU, M. A.; M. N. MARKOVICH & L. A. PIRUZYAN: Studies of the thermodynamics and nature of interaction between serum albumin and penicillins. Biochim. Biophys. Acta 493: 1~9, 1977
18) FRUH, P. U.; J. T. CLERC & W. SIMON: Determination of ΔH, ΔG and ΔS of the interaction of ions with carrier antibiotics by computerized microcalorimetry. Helv. Chim. Acta 54: 1445~1450, 1971
19) LAYNEZ, J.; A. RODRIGUEZ-TEBAR, V. ARAN & D. VAZQUEZ: Calorimetric studies on the binding of vancomycin-like antibiotics to peptidoglycan. *In* The Target of Penicillin. *Ed.*, R. HAKENBACK *et al.*, pp. 91~96, Walter de Gruyter & Co., Berlin, 1983
20) BJURULF, C.; J. LAYNEZ & I. WADSÖ: Thermochemistry of lysozyme-inhibitor binding. Eur. J. Biochem. 14: 47~52, 1970
21) ROSS, P. D. & S. SUBRAMANIAN: Thermodynamics of protein association reactions: Forces contributing to stability. Biochemistry 20: 3096~3102, 1981
22) NICHOLS, N.; R. SKÖLD, C. SPINK, J. SUURKUUSK & I. WADSÖ: Additivity relations for the heat capacities of non electrolytes in aqueous solution. J. Chem. Thermodyn. 8: 1081~1093, 1976
23) GILL, S. J. & I. WADSÖ: An equation of state describing hydrophobic interactions. Proc. Natl. Acad. Sci. U.S.A. 73: 2955~2958, 1976