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Thermodynamics and kinetics of dissociation of ligand-induced dimers of vancomycin antibiotics

Deborah McPhail and Alan Cooper*
Department of Chemistry, Glasgow University, Glasgow, UK G12 8QQ

The thermodynamics of dissociation of vancomycin and ristocetin dimers in the presence and absence of specific ligands has been studied by direct microcalorimetry over a range of temperature, pH and ionic strength conditions in H_2O and D_2O. Dimerization of these antibiotics is exothermic with large temperature dependence (ΔC_p) and consequent entropy–enthalpy compensation effects that may be consistent with solvation changes associated with burial of non-polar surfaces during macromolecular association. For vancomycin, no significant ionic strength effects are observed, so non-specific electrostatic contributions are probably discounted, but pH and buffer effects on the thermodynamic parameters are consistent with hydrogen ion uptake and pK shift in the dimerization process. Vancomycin dimerization is significantly enhanced in the presence of specifically binding ligands: acetate, *N*-acetyl-D-Ala, and N_α,N_ε-diacetyl-Lys-D-Ala-D-Ala, in increasing order of effectiveness. The dipeptide ligand *N*-acetyl-D-Ala-D-Ala promotes higher oligomerization and crystallization of the complex. Ristocetin, in contrast, displays no such ligand effects; it shows a slight reduction in dimerization in the presence of strongly binding N_α, N_ε-diacetyl-Lys-D-Ala-D-Ala. This difference may reflect the need for flexibility in the antibiotic structure to allow ligand-induced aggregation. Eremomycin dimerizes strongly even in the absence of ligand. Dissociation of the vancomycin–N_α, N_ε-diacetyl-Lys-D-Ala-D-Ala dimer complex is slow (k_{diss} *ca.* 0.005 s^{-1}) and kinetics can be measured by conventional UV difference techniques.

Vancomycin is one of a family of glycopeptide antibiotics, first isolated from a strain of *Streptomyces orientalis*, effective against Gram-positive bacterial infections, especially methicillin resistant 'superbug' *Staphylococcus aureus*.[1] Due to increasing bacterial resistance to other antibiotics, vancomycin is now commonly the last line of defence with staphylococcal infections,[2,3] though vancomycin-resistant strains are now being reported. Vancomycin acts by specific complexation with peptides containing terminal -D-alanyl-D-alanine units in the peptidoglycan structural components of the surface of bacterial cell walls, where interaction with these dipeptide units blocks their incorporation into peptidoglycan by preventing the transpeptidase from completing the cross-linking process and therefore preventing the cell wall from forming properly.[4–7] Once cell wall biosynthesis is interrupted, death of the cell by lysis can occur.

Experimental studies of the interaction of vancomycin antibiotics with peptide analogues are complicated by formation of vancomycin dimers, or possibly higher oligomers, at the higher concentrations frequently required for biophysical techniques.[4] Previous microcalorimetric binding studies using vancomycin and the dipeptide *N*-acetyl-D-Ala-D-Ala, and related peptide analogues, showed that the apparent binding constants and enthalpies depend on antibiotic concentration.[8] Thermal titration curves of the association between vancomycin and peptides show anomalous behaviour at high antibiotic concentrations which are inconsistent with the simple 1:1 complex formation observed at lower concentrations, suggesting ligand-induced aggregation of the antibiotic. In contrast, calorimetric binding studies using the related antibiotic, ristocetin, indicated rather that ligand binding induces dissociation of antibiotic dimers.[8] Similar proposals have been made earlier from detailed NMR studies of vancomycin and its analogues in aqueous and non-aqueous mixtures,[9,10] and it has also been suggested that dimerization may play an important functional role *in vivo* during interactions at the cell wall surface.[11] Vancomycin also crystallizes as a dimer, as shown in recent X-ray diffraction studies.[12]

In view of the central importance of vancomycin antibiotics in the fight against infections, and the need to understand the basis of their action when designing potential variants, we have now extended our calorimetric studies to explore more directly the energetics of the dimerization process through heats of dilution measurements. Here we compare the thermodynamics of dissociation of vancomycin and ristocetin dimers in the presence and absence of weakly binding (acetate, *N*-acetyl-D-Ala) and strongly binding (N_α, N_ε-diacetyl-Lys-D-Ala-D-Ala) ligands over a range of conditions. In the course of these experiments we also observed that under certain conditions the rate of dissociation of vancomycin–ligand dimer complexes is sufficiently slow to follow by conventional UV difference spectroscopy, and this allowed us to determine some kinetic parameters for the dissociation process at room temperature.

Experimental

Materials

Vancomycin, ristocetin, *N*-acetyl-D-Ala, *N*-acetyl-D-Ala-D-Ala, N_α, N_ε-diacetyl-Lys-D-Ala-D-Ala (LAA), and D_2O (99.9%) were obtained from Sigma and used without further purification. Eremomycin was provided by Dr. D.H. Williams (Cambridge University). Buffers used (usually at 0.1 M) were: citrate (pH 3); acetate (pH 5); phosphate, 3-(*N*-morpholino) propanesulfonic acid (MOPS), piperazine-*N*, *N*′-bis(2-ethanesulfonic acid (PIPES), imidazole (pH 7); tris(hydroxymethyl)aminomethane (TRIS) (pH 8); and phosphate (pH 11). Non-specific electrostatic or ionic strength effects were investigated in MOPS buffer (pH 7) by addition of KCl or $CaCl_2$. For experiments in D_2O (0.1 M buffer, pD 7.0), an empirical correction of 0.45 was added to the observed meter reading to convert the operational pH to an equivalent value on the pD scale.[13] Deuteriated salts were not used; consequently there will have been an additional trace of H_2O (*ca.* 0.3 mol%) in these mixtures. Peptide and antibiotic concentrations were determined by weight, confirmed in the case of vancomycin by UV spectroscopy of diluted samples using a molar absorption coefficient $a_{280\,nm} = 6690\ dm^3\ mol^{-1}\ cm^{-1}$ at pH 7.0.[4]

Microcalorimetry

Calorimetric data for the dilution of vancomycin and ristocetin solutions, both in the presence and absence of appropriate

ligands, were obtained over a range of temperature, pH (vancomycin only), and other conditions using a Microcal Omega Isothermal Titration Calorimeter (ITC).[14,15] Dilution experiments involved sequential injections of concentrated antibiotic solution (2–3 mM) into the stirred calorimeter cell (1.4 ml) initially containing buffer alone, with a typical injection sequence of 12 × 20 μl at 3–5 min intervals. This gave rise to a series of endothermic heat pulses which, after correction for appropriate buffer mixing control experiments under identical conditions, were analysed using Microcal Origin and purpose-made software in terms of a simple dimer dissociation model (see Appendix A) to give K_{dim} and ΔH_{dim}.

In experiments involving ligands, the free peptide concentration was kept the same in both cell and injection syringe solutions, typically 90–100 mM for *N*-acetyl-D-Ala and 1–5 mM for LAA. These ligand concentrations are sufficient to ensure that antibiotic molecules are predominantly in their ligand-complexed form in such experiments.[8] Standard thermodynamic quantities were calculated in the usual manner using $\Delta G^\circ = -RT \ln K = \Delta H^\circ - T\Delta S^\circ$.

Kinetics

The rates of dilution-induced changes in vancomycin in the presence of LAA were determined from changes in UV absorbance at 283 nm using a Shimadzu UV-160A UV–VIS spectrophotometer with thermostatted cell holder. For a typical experiment, small aliquots (10, 20 or 30 μl) of concentrated vancomycin/ligand solution (3–6 mM each) were mixed rapidly into 1 ml of buffer in a 1 cm pathlength quartz cuvette. Absorbance changes (A) at 283 nm were recorded over several minutes and subsequently analysed in terms of the conventional first-order expression: $\ln\{(A - A_\infty)/(A_0 - A_\infty)\} = -kt$, (or the equivalent single exponential decay expression) where A_0 and A_∞ are estimated absorbance readings at $t = 0$ and $t = \infty$, respectively, and k is the apparent first-order rate constant for the process. Kinetic experiments were carried out over the pH range 3–8 and over the temperature range 25–40 °C.

Results and Discussion

Dilution of vancomycin solutions by the injection of small aliquots into the calorimeter cell containing a larger volume of buffer is an endothermic process consistent with dissociation of vancomycin dimers or higher oligomers. Examples of typical calorimetric dilution data are shown in Fig. 1. A sequence of dilution injections gives a series of endothermic heat pulses which, after integration and correction for control mixing experiments, gives the absolute heat uptake per injection. In such a dilution series, successive injections become progressively less endothermic as the antibiotic concentration builds up in the calorimeter cell. The resulting thermal dilution profile is consistent with a simple monomer–dimer dissociation model and can be analysed by non-linear regression techniques to give estimates of the dimerization constant (K_{dim}) and enthalpy of dimerization (ΔH°_{dim}). [Although calorimetric dilution experiments alone are insufficient to discriminate between simple dimerization and infinite polymerization models (see Appendix A),[16] previous NMR, structural and other evidence is consistent with dimer formation,[4,10,12] and we shall assume this in subsequent analysis of data presented here. Qualitative trends will be unaffected by this assumption.]

Thermodynamic data for antibiotic dimerization over a range of pH and temperature conditions are given in Tables 1 and 2, respectively. In the absence of ligands the aggregation of vancomycin molecules in solution is relatively weak, but is significantly enhanced in the presence of ligand. For example, at 25 °C, pH 7, vancomycin alone has a $K_{dim,0}$ of 475 dm^3 mol^{-1} and a $\Delta H^\circ_{dim,0}$ of −29.2 kJ mol^{-1}. However, in the presence of specific ligand this dimerization constant is increased up to 10-fold (*e.g.* $K_{dim,L}$ is about 5000 dm^3 mol^{-1} with LAA) and aggregation is significantly more exothermic ($\Delta H^\circ_{dim,L} = -39.3$ kJ mol^{-1} with LAA). Similar trends are observed over a range of temperature and pH with both LAA or with the more weakly binding ligands, acetate and *N*-acetyl-D-Ala (Tables 1 and 2). Trial experiments using the dipeptide, *N*-acetyl-D-Ala-D-Ala, with vancomycin at concen-

Table 1 Effects of pH and ligand binding on thermodynamic data for the formation of antibiotic dimers in aqueous buffers, determined from calorimetric dilution data at 25 °C

antibiotic	ligand	pH {pD}	K_{dim}[a] /dm^3 mol^{-1}	ΔH_{dim}[a] /kJ mol^{-1}	ΔG°_{dim} / kJ mol^{-1}	ΔS°_{dim} / J K^{-1} mol^{-1}
vancomycin	none	3	470(200)	−28.6(5.8)	−15.2	−45
		5	380(40)	−24.0(4.5)	−14.7	−31
		7	475(80)	−29.2(1.7)	−15.3	−47
{in D_2O}		{7}	745(15)	−26.2(0.5)	−16.3	−33
		8	610(70)	−27.1(4.2)	−15.9	−38
		11	170(5)	−49.5(7.6)	−12.7	−123
	N-acetyl-D-ala	3	1085(185)	−35.7(0.2)	−17.3	−61
		5	1250(75)	−36.2(1.2)	−17.7	−62
		7	2300(230)	−36.5(1.8)	−19.2	−58
		8	900(20)	−33.5(1.7)	−16.8	−56
		11	395(10)	−27.6(2.0)	−14.8	−43
	LAA	3	2655(540)	−30.1(2.0)	−19.5	−36
		5	3775(—)	−30.9 (—)	−20.4	−35
		7	5050(1080)	−39.3(2.8)	−21.1	−61
{in D_2O}		{7}	6700(1300)	−26.2(1.5)	−21.8	−15
		8	2285(—)	−25.3(—)	−19.2	−21
	acetate[b]	7	860(80)	−36.2(1.5)	−16.7	−65
ristocetin	none	7	280(95)	−21.5(2.0)	−14.0	−25
{in D_2O}		{7}	595(65)	−20.7(1.4)	−15.8	−16
	N-acetyl-D-ala	7	390(85)	−15.6(2.7)	−14.8	−3
	LAA	7	185(25)	−32.1(0.3)	−12.9	−64
{in D_2O}		{7}	335(—)	−30.6(—)	−14.4	−54
eremomycin	none	7	46000(1000)	−4.6(0.5)	−26.6	+74

[a] From non-linear regression of calorimetric dilution data, assuming a monomer–dimer model. Figures in parentheses are standard deviations of multiple experiments. [b] 0.5 M Na acetate in phosphate buffer. (—) Single determination only.

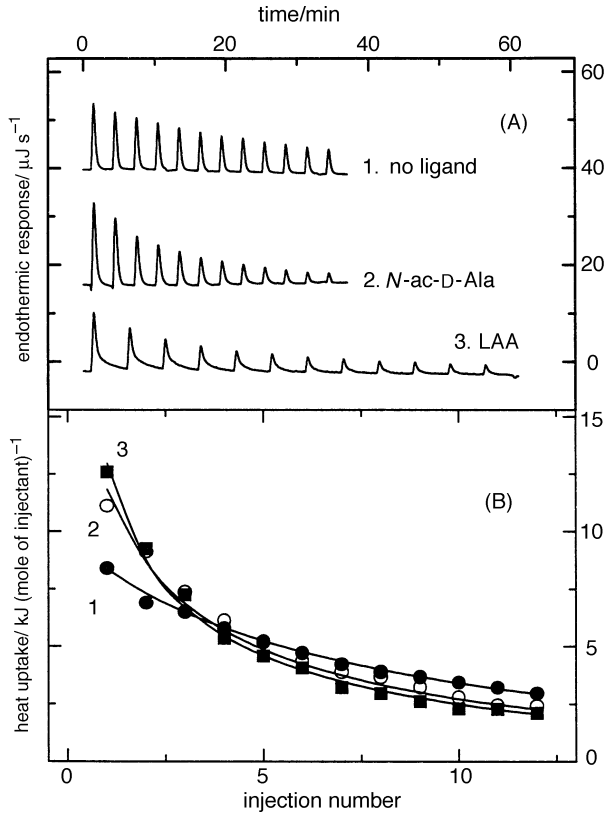


Fig. 1 Typical calorimetric dilution data (pH 7, phosphate buffer, 25 °C) for the dissociation of vancomycin dimers in the presence or absence of ligands. (A) Endothermic responses for sequential 20 μl injections of vancomycin (2.65 mM) into buffer alone, or ligand solution (98 mM *N*-acetyl-D-Ala or 1.5 mM LAA) under the same conditions. (B) integrated dilution heat effects, with theoretical fits to a dimer dissociation model using parameters given in Table 1.

trations required for these calorimetric dilution measurements led to precipitation of the dipeptide–antibiotic complex which precluded any further study with this peptide in this context. Interestingly, however, this did give us conditions suitable for growth of single crystals of the complex, and X-ray crystallographic determination of the structure is currently in progress.

At the concentrations used here in antibiotic–ligand mixtures, the vancomycin (either monomer or dimer) will be present predominantly as the ligand complex, so any complications arising from change in liganded state during the dilution/dissociation process may be discounted. However, ligand binding and dimerization are clearly linked, and it must follow that any enhancement in vancomycin dimerization brought about by ligand-binding must be mirrored by a similar enhancement in ligand-binding affinities to the dimeric antibiotic. The overall ligand-binding–dimerization equilibrium scheme will involve several steps, including the binding of a ligand to a monomeric antibiotic:

$$A + L \rightleftharpoons AL;\ K_L = [AL]/[A][L];\ \Delta H_L$$

together with dimerization of liganded or un-liganded species:

$$A + A \rightleftharpoons A_2;\ K_{dim,\,0} = [A_2]/[A]^2;\ \Delta H_{dim,\,0}$$

$$AL + AL \rightleftharpoons A_2L_2;\ K_{dim,\,L} = [A_2L_2]/[AL]^2;\ \Delta H_{dim,\,L}$$

where A = antibiotic (*e.g.* vancomycin), L = ligand, and the square brackets indicate molar concentrations (strictly activities).

Binding of ligand to dimeric species will be described by:

$$A_2 + L \rightleftharpoons A_2L;\ K_{L1} = [A_2L]/[A_2][L];\ \Delta H_{L1}$$

and

$$A_2L + L \rightleftharpoons A_2L_2;\ K_{L2} = [A_2L_2]/[A_2L][L];\ \Delta H_{L2}$$

So, for sequential binding of ligand to dimer:

$$K_{L1}K_{L2} = K_L^2(K_{dim,\,L}/K_{dim,\,0})$$

showing that, though we cannot necessarily resolve the individual constants K_{L1} and K_{L2}, the overall binding affinity to the dimer is enhanced in cases where $K_{dim,\,L} > K_{dim,\,0}$. With LAA as ligand, and assuming $K_{L1} = K_{L2}$, the ligand binding affinity would therefore be enhanced roughly three-fold in the dimer compared to the monomer, corresponding to a change in standard Gibbs free energy of ligand binding, $\Delta\Delta G^\circ$, of *ca.* -3 kJ mol^{-1}. (For LAA binding to monomeric vancomycin under similar conditions, $K_L \approx 5.2 \times 10^5$ M^{-1}, $\Delta G^\circ = -32.6$ kJ mol^{-1}, $\Delta H_L = -53.3$ kJ mol^{-1}, $\Delta S^\circ = -69$ J K^{-1} mol^{-1}; see ref. 8.)

The enthalpies and entropies of ligand binding are similarly affected by dimerization. From the complete thermodynamic cycle, the change in the overall ligand binding enthalpy is given by the difference in dimerization enthalpies thus:

$$\Delta H_{dim,\,L} - \Delta H_{dim,\,0} = \Delta H_{L1} + \Delta H_{L2} - 2\Delta H_L$$

with a similar expression for entropies. Again taking the data for LAA at 25 °C (Table 1), this suggests that binding of LAA to the dimer is more exothermic than to the monomer by about -5 kJ mol^{-1}, expressed as an average per mole of ligand bound. This is offset by a positive change in ligand binding entropy, $\Delta\Delta S^\circ$, of the order $+7$ J K^{-1} mol^{-1}. None

Table 2 Effect of temperature on the thermodynamic parameters for vancomycin dimerization at pH 7.0 (phosphate buffer, H_2O)

ligand	T/°C	K_{dim}[a] /dm^3 mol^{-1}	ΔH^a_{dim} /kJ mol^{-1}	ΔG°_{dim} /kJ mol^{-1}	ΔS°_{dim} /J K^{-1} mol^{-1}	ΔC_p[b] /J K^{-1} mol^{-1}
none	15	480(70)	−27.5(0.5)	−14.8	−44	
	25	475(80)	−29.2(1.7)	−15.3	−47	−550(90)
	35	265(25)	−36.9(0.4)	−14.3	−73	
	45	225(65)	−43.3(0.1)	−14.3	−91	
N-acetyl-D-ala	15	5100(—)	−35.0(—)	−20.4	−51	
	25	2300(230)	−36.5(1.8)	−19.2	−58	−445(90)
	35	980(135)	−41.2(0.1)	−17.6	−76	
	45	570(40)	−48.3(0.1)	−16.8	−99	
LAA	15	9600(4000)	−21.1(0.9)	−21.9	3	
	25	5050(1080)	−39.3(2.8)	−21.1	−61	−1745(155)
	35	3150(985)	−61.1(6.1)	−20.6	−131	
	45	1800(50)	−72.0(15.0)	−19.8	−164	

[a] From non-linear regression of calorimetric dilution data, assuming a monomer–dimer model. [b] From linear regression of ΔH_{dim} *vs.* T data. (—) Single determination only.

of these changes is particularly large compared to the overall ligand-binding parameters, and for the weaker binding ligand, the changes will be correspondingly less.

Temperature effects

The vancomycin dimerization enthalpies show significant variation with temperature both in the presence and absence of ligands, generally becoming more exothermic (ΔH_{dim} more negative) with increasing temperature (Table 2, Fig. 2), and the negative ΔC_p values implied are typical of those found in many macromolecular associations in water.[17–19] As a consequence of ΔC_p (see Appendix B), these data show apparent entropy–enthalpy compensation and linear correlation between ΔH_{dim} and $T\Delta S_{dim}$ (Fig. 2), with relatively small temperature dependence in the free energy of dimer formation.

Though contributions from other interactions and changes in macromolecular dynamics cannot be ruled out,[20–22] negative ΔC_p values are frequently taken as evidence of significant hydrophobic interaction in the binding process and may be correlated with changes in exposed non-polar surface area (ΔA_{np}) during complexation.[19] Using the empirical procedure of Spolar and Record,[19] together with the estimated heat capacity changes in Table 2, we estimate burial of non-polar surface areas ranging from about 400 Å^2 for vancomycin alone, up to 1300 Å^2 for the vancomycin-LAA dimer complex. This compares to an estimate from model building of about 300 Å^2, assuming no conformational change in the monomers during dimerization.[23] This discrepancy, and particularly the significantly larger apparent ΔA_{np} in the presence of LAA, might suggest that vancomycin dimerization, especially in the ligand-bound form, involves extensive conformational rearrangement. However, such interpretations, though intriguing, should be treated with some caution at this stage since the empirical basis for the $\Delta A_{np} - \Delta C_p$ correlation has yet to be tested for such relatively small molecular complexes, and major empirical discrepancies have recently been found in much larger systems.[24]

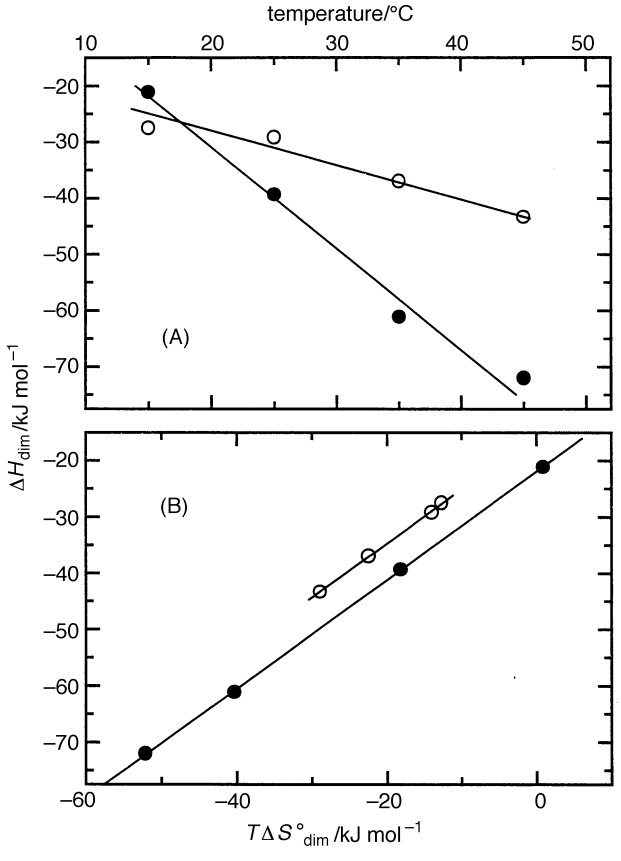


Fig. 2 (A) Variation with temperature of the enthalpy of dimerization of vancomycin alone (open circles) or in the presence of LAA (filled circles). (B) Apparent enthalpy–entropy compensation plot under the same conditions.

pH and ionic strength effects

Vancomycin dimers show relatively little variation in stability with pH in the acid to neutral region, but dimerization falls off significantly above pH 8.0. Various ionizable groups have been identified in vancomycin, including groups having pK_a values of about 2.9 (—COOH), 7.2 (terminal α-NH_3^+), with a further four at pK_a 8.6, 9.6, 10.5 and 11.7 assigned to three phenolic groups and a vancosamine—NH_3^+.[4] Other researchers have similarly assigned pK_a values of 8.7 and 9.6 to groups involving phenolic dissociation.[25] It seems possible, therefore, that ionization of these phenolic groups may be responsible for the reduction in dimerization at high pH. High pH also reduces the stability of the vancomycin–peptide complex since peptide binding requires antibiotic carboxyl groups to be ionized and phenolic groups to be unionized,[4] and this will also contribute to a reduction in dimer stability at higher pH in the ligand-bound situation.

It is feasible, at least in principle, that the ligand-induced dimerization seen here is simply an electrostatic effect arising from a reduction in the overall electrostatic repulsion between monomers in the complex. At neutral pH the vancomycin monomers will carry a net positive charge, while the ligands will be negatively charged. Consequently, ligand binding to vancomycin monomers might reduce repulsion between the molecules. However, addition of salts (0.5 M KCl or 0.1 M $CaCl_2$) to the buffer mixture had no significant effect on vancomycin dimerization in the absence of ligands (data not shown), suggesting that the energetics of association of the unliganded monomers has little non-specific electrostatic contribution. High concentrations of acetate ions, however, do show a small but significant enhancement in K_{dim} (Table 1), consistent with its action as a very weak ligand,[8] mimicking the binding of the terminal carboxylate of larger peptide ligands in the flexible anion-binding pocket of vancomycin.[26]

Buffer ionization effects

More specific charge effects can be detected in calorimetric experiments by use of different buffers. Specific ligand-binding is known to involve stabilization of the terminal —NH_3^+ cation of vancomycin through interaction with ligand carboxylate groups,[5] and it is possible that protonation of this group is also required for association between vancomycin molecules. The reduction in dimerization above pH 7–8 is consistent with this. If this is the case, then dimer formation will involve the uptake of H^+ ions from solution under pH conditions where the relevant groups are only partly protonated, equivalent to an increased effective pK_a in the vancomycin dimer. In calorimetric experiments this will give rise to an additional heat effect that can be used to quantify the protonation changes.[27,28] Recall that calorimetric measurements record the totality of heat effects associated with any process and, if proton ionization effects are involved, this will include a component from buffer ionization heats.[28] Consequently, the same process observed at the same pH but in different buffers may give rise to different apparent enthalpies (ΔH_{obs}), though with the same K and $\Delta G°$, due to the different proton ionization enthalpies (ΔH_I) of the different buffers.[27] Specifically, $\Delta H_{obs} = \Delta H_0 + \nu_{H+}\Delta H_I$, where ν_{H+} is the number of protons taken up in the process.

Calorimetric dilution experiments with vancomycin at pH 7.0 carried out using a series of buffers with different heats of proton ionization show that the apparent observed dimerization enthalpy does vary significantly with buffer (Table 3),

Table 3 Effect of different buffers on the thermodynamic parameters for vancomycin dimerization at pH 7.0 and 25 °C

buffer	ΔH_I[a] /kJ mol^{-1}	K_{dim}[b] /dm^3 mol^{-1}	ΔH_{dim}[b,c] /kJ mol^{-1}
phosphate	3.3	475(80)	−29.2(1.7)
PIPES	11.3	360(250)	−24.4(5.4)
MOPS	20.5	470(180)	−19.6(1.9)
imidazole	36.6	410(240)	−18.2(1.2)

[a] Buffer proton ionization enthalpy (literature values[35,36]). [b] From calorimetric dilution experiments. [c] Apparent observed dimerization enthalpy, uncorrected for buffer effects.

but without any significant variation in K_{dim}. This is consistent with an uptake (ν_{H+}) of approximately 0.3 (±0.06) H^+ ions per dimer under these conditions. For a vancomycin group with a (monomer) pK_a of 7.2, this would correspond to a pK shift of about +0.3 units to pK_a 7.5. Alternatively, this might arise from a summation of smaller pK_a shifts of a number of groups.

(Note: the very low heat of proton ionization of phosphate buffers in the neutral pH range means that enthalpies reported in Tables 1 and 2 are relatively unaffected by buffer protonation heat effects.)

D_2O effects

A limited number of calorimetric dilution experiments with both vancomycin and ristocetin carried out in D_2O rather than H_2O, summarized in Table 1, indicate that the same overall trends are found in deuteriated solvent, with only minor changes in thermodynamic parameters. Dimerization is slightly more favoured in D_2O and for vancomycin, significantly enhanced by LAA binding, though in this case the effect appears entirely entropic (dimerization enthalpies are the same). This is consistent with a significant thermodynamic contribution from solvation effects to the dimerization process, though it is not possible to conclude much more from this at this stage in our understanding of macromolecular interactions in aqueous systems.

Ristocetin and eremomycin

Discussion so far has concentrated on data for vancomycin, where it is evident that ligand-induced dimerization is observed over a wide range of temperature and pH conditions, and is clearly an important feature of vancomycin–peptide interaction at high antibiotic concentrations. In contrast, dilution experiments with the related antibiotic ristocetin show only small or even opposite effects of added ligands (Table 1). In the absence of ligands the dimerization parameters of ristocetin are similar to vancomycin ($K_{dim,0} = 280$ dm^3 mol^{-1}, $\Delta H_{dim,0} = -21.5$ kJ mol^{-1} at 25 °C, pH 7), but in agreement with NMR studies;[29] addition of LAA here gives rise to a slight reduction in dimerization constant ($K_{dim,L} = 185$ dm^3 mol^{-1}), albeit with an increase in exothermicity $\Delta H_{dim,L} = -32.1$ kJ mol^{-1}) comparable to vancomycin under similar conditions. Addition of *N*-acetyl-D-Ala has little significant effect on ristocetin dimerization constant, though it possibly reduces the dimerization enthalpy slightly. No significant buffer effects are observed (data not shown). Dimerization of ristocetin appears, therefore, relatively insignificant below millimolar concentrations even in the presence of ligand. Similar effects are seen in D_2O (Table 1). Consequently, if ligand-induced dimerization is a significant feature of the vancomycin antibiotic mechanism,[11,30] it seems unlikely to be common to other members of this family. Interestingly, ristocetin lacks the flexibility in the carboxylate binding pocket shown by vancomycin,[31] suggesting that some ligand-induced conformational change in this region may be involved in enhancing dimerization in the latter.

Preliminary calorimetric dilution experiments with other members of the vancomycin group show a wide range of K_{dim} values, which show that dimerization under physiological conditions may be relevant in some circumstances, though we have not yet been able to explore the effects of ligand binding in such cases. Eremomycin for example (Table 1) dimerizes more strongly than vancomycin or ristocetin in the absence of ligands under comparable conditions, and the process is entropy driven with a much lower dimerization enthalpy. Clearly one must exercise caution in extrapolating thermodynamic observations even between closely related molecules such as these.

Kinetics

In addition to establishing the energetics of the process, calorimetric dilution experiments give some indication of the kinetics of dimer dissociation. Although the process is normally too fast to be resolved on the relatively slow timescale of the Omega ITC ($t_{1/2} \approx 20$ s), dissociation of vancomycin in the presence of LAA gave endothermic heat pulses that were significantly broader and took longer to return to baseline than normal for fast reactions (see Fig. 1 and 3) suggesting that dissociation is slow under such conditions. This was confirmed by observing the slow decrease in UV absorbance upon dilution of vancomycin and LAA solutions into buffer. Typical data are shown in Fig. 3. The process obeyed simple first-order kinetics from which apparent rate constants could be determined over a range of temperature and pH conditions (Fig. 4). (Dissociation in the absence of ligand or with the weakly binding ligand *N*-acetyl-D-Ala was too fast to measure with this technique.) Apparent dissociation rate constants showed no significant variation with LAA concentration over a three-fold concentration range, provided that the ligands were in excess. Dimer dissociation is slowest around pH 5–6 [Fig. 4(B)], in which range the vancomycin–LAA complex will carry zero net charge. The Arrhenius activation energy of dissociation is estimated at 73 (±6) kJ mol^{-1} from the temperature dependence at pH 7 [Fig. 4(A)].

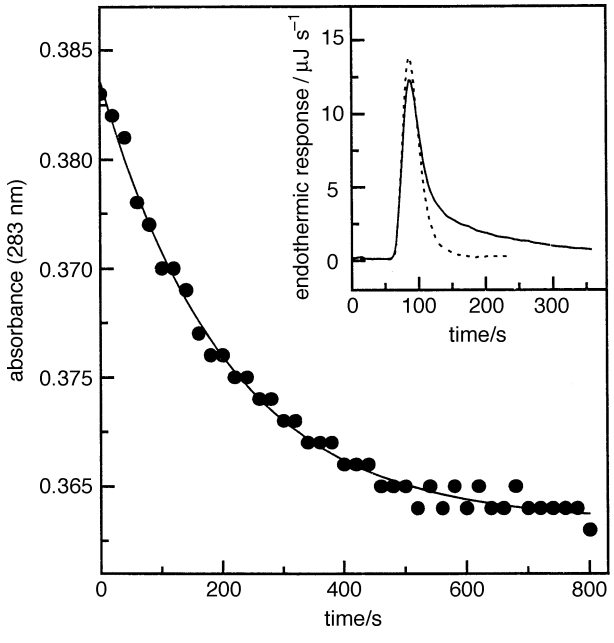


Fig. 3 Example of apparent first order kinetic data for the dissociation of vancomycin dimers in the presence of excess ligand (LAA), determined from absorbance changes at 283 nm. Experimental data points are fitted to a single exponential decay expression with parameters given in the text. Inset: comparison of ITC dilution heat pulses for vancomycin alone (dotted line) or in the presence of LAA (solid line).

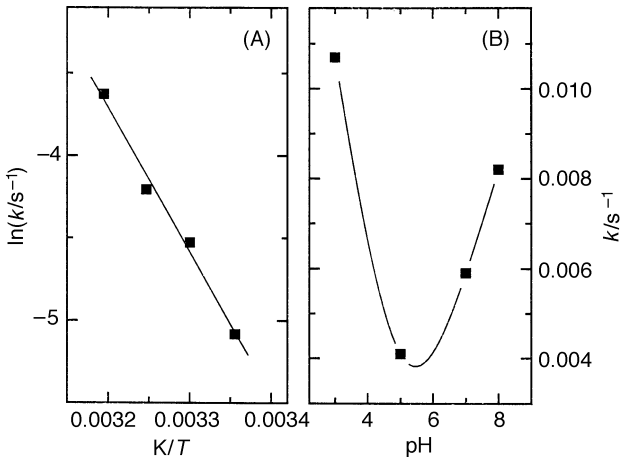


Fig. 4 Variation of vancomycin–LAA dimer dissociation rate as a function of (A) temperature (as an Arrhenius plot) and (B) pH

The rate constant for dissociation of the vancomycin dimer in the presence of LAA (0.006 s^{-1} at 25 °C, pH 7.0) is considerably slower than the rate for ligand dissociation from vancomycin under similar conditions. For LAA and similar ligands a dissociation rate of the order of 50 s^{-1} has been reported at low vancomycin concentrations ($\leqslant$100 μM) where dimerization is not significant.[25] This leads to questions regarding the mechanism of dimer dissociation in the presence of ligands. Under the conditions used here in dilution experiments to follow dimer dissociation, the ligand LAA concentration remained significantly high to guarantee that the vancomycin is predominately in the ligand-bound form either as monomer or dimer. Consequently, we may ask whether dissociation of the ligand-bound dimer occurs directly (Scheme 1, step 1) or requires prior release of the ligand, for example following steps 2 → 3 → 4.

For direct dissociation (step 1) we would expect a rate law of the form:

$$\text{rate} = k_1[A_2L_2]$$

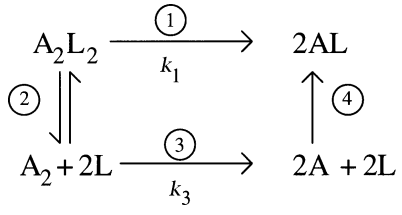
with no dependence on free ligand concentration. However, an indirect dissociation mechanism involving ligand dissociation might involve ligand concentration dependent steps. For example, for steps 2 → 3 → 4 with step 3 rate determining we might expect:

$$\text{rate} = k_3[A_2] = k_3^*[A_2L_2]$$

where

$$k_3^* = k_3 K_{\text{dim, 0}}/K_{\text{dim, L}} K_L{}^2[L]^2$$

is the apparent first-order rate constant for dissociation of the liganded dimer. In this case, under the conditions used here where the free ligand [L] is in excess, a strong inverse-square dependence on ligand concentration for the apparent first-order rate constant would be anticipated. Dilution kinetic studies over a three-fold range of ligand concentration show no significant variation in apparent rate constant, so the second may be discounted. Direct dissociation, in any case, would appear to be the more likely since the free energy for



Scheme 1

vancomycin–LAA complex formation is much greater than that for dimerization.

Combination of the measured dissociation rate and equilibrium constants gives an estimate of around 30 s^{-1} dm^3 mol^{-1} for the apparent second-order rate constant for formation of dimers from vancomycin–LAA monomers. This is much slower than ligand binding rates under similar conditions and very much slower than would be expected for diffusion-controlled association in this system.[25]

Taken together with ligand binding information[8] and the detailed structural models that are now emerging,[12] these thermodynamic and partial kinetic data on dimerization effects in the vancomycin system add to the now quite comprehensive body of knowledge relating to the behaviour of these antibiotics in solution and their interaction with small cell wall analogue ligands, forming a solid physico-chemical basis for understanding this kind of antibiotic action.

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Appendix A: dimer dissociation heats (analysis)

Heats of dilution data for a simple monomer–dimer system are analysed as follows. If only monomer or dimer states of (macro)molecule P are possible:

$$P + P \rightleftharpoons P_2\ ;\ \Delta H_{\text{dim}}\ ;\ K_{\text{dim}} = [P_2]/[P]^2$$

the equilibrium concentration of monomers is given by:

$$[P] = \{(1 + 8K_{\text{dim}}C)^{1/2} - 1\}/4(K_{\text{dim}}) \quad \text{(A1)}$$

where C is the total concentration of P, expressed as monomer:

$$C = [P] + 2[P_2] \quad \text{(A2)}$$

In an ITC dilution experiment we measure the heat change (δq) when a small volume (δV) of concentrated solution (concentration C_0) is injected into the calorimeter cell (volume V_0) containing initially buffer but, for later injections, more dilute solution. The heat arises from dimers present in the higher concentration solution that dissociate upon entering the lower concentration environment.

For the ith injection of a series the observed heat is given by:

$$\delta q_i = \Delta H_{\text{dim}}\{V_0([P_2]_i - [P_2]_{i-1}) - V([P_2]_0 - [P_2]_{i\text{-}1})\} \quad \text{(A3)}$$

where $[P_2]_0$, $[P_2]_i$ and $[P_2]_{i-1}$ are the dimer concentrations in the original (syringe) solution and in the calorimeter cell after the ith and $(i-1)$th injections: total concentrations C_0, C_i and C_{i-1}, respectively. [The last term in this expression is a small correction factor to allow for the quantity of solution displaced from the constant-volume calorimeter cell during each δV addition.]

Eqn. (A1) to (A3) are used in standard non-linear regression (least-squares) procedures to fit experimental dilution data and obtain estimates of K_{dim} and ΔH_{dim}. Similar, though more algebraically complex expressions may be derived for dissociation processes involving higher oligomers or other mechanisms. Such mechanisms frequently give sigmoidal dilution thermograms, in contrast to the hyperbolic shapes for the dimer dissociation shown here, and this might give empirical indications that the process under investigation is more complex than simple dimers can model. (No such effects were seen for the experiments reported above.)

Interestingly, Eqn. (A1) is algebraically identical (apart from a factor 2) to that giving free monomer concentrations in a simple infinite-polymerization model.[16] Consequently, calorimetric dilution data alone might be insufficient to discriminate between dimer or polymer interaction models, and other experimental approaches might be needed to resolve possible ambiguities.

Appendix B: ΔC_p and entropy–enthalpy compensation

A finite heat capacity (ΔC_p) gives rise to temperature dependence in enthalpy and entropy changes that cancel, at least partially, to give relatively much smaller changes in the Gibbs free energy for the process. With respect to some arbitrary reference temperature, T_{ref}, the temperature variation of enthalpy and entropy changes, respectively, are given by the classic relations:

$$\Delta H(T) = \Delta H(T_{\mathrm{ref}}) + \int_{T_{\mathrm{ref}}}^{T} \Delta C_p \, \mathrm{d}T$$

and

$$\Delta S(T) = \Delta S(T_{\mathrm{ref}}) + \int_{T_{\mathrm{ref}}}^{T} (\Delta C_p/T) \, \mathrm{d}T$$

If ΔC_p is constant, independent of temperature (not necessarily true, but usually a reasonable approximation over a limited temperature range), then integration gives:

$$\Delta H(T) = \Delta H(T_{\mathrm{ref}}) + \Delta C_p(T - T_{\mathrm{ref}})$$

$$\Delta S(T) = \Delta S(T_{\mathrm{ref}}) + \Delta C_p \ln(T/T_{\mathrm{ref}})$$

For small changes in temperature with respect to absolute T_{ref}, $\delta T = T - T_{\mathrm{ref}}$, these become:

$$\Delta H(T) = \Delta H(T_{\mathrm{ref}}) + \Delta C_p \delta T$$

$$\Delta S(T) = \Delta S(T_{\mathrm{ref}}) + \Delta C_p \ln(1 + \delta T/T_{\mathrm{ref}})$$

$$\approx \Delta S(T_{\mathrm{ref}}) + \Delta C_p \delta T/T_{\mathrm{ref}}$$

using the approximation $\ln(1 + x) \approx x$, for $x \ll 1$. Consequently, to the extent that this approximation is valid:

$$\Delta G(T) = \Delta H - T\Delta S$$

$$\approx \Delta H(T_{\mathrm{ref}}) - T\Delta S(T_{\mathrm{ref}}) - \Delta C_p \delta T^2/T_{\mathrm{ref}}$$

$$= \Delta G(T_{\mathrm{ref}})$$

to first order in δT. Moreover, over the limited temperature range for which this approximation is valid:

$$\Delta H(T) \approx \Delta H(T_{\mathrm{ref}}) + T_{\mathrm{ref}}[\Delta S - \Delta S(T_{\mathrm{ref}})]$$

so that a plot of ΔH *vs.* ΔS would appear linear with slope T_{ref}. Though much could be made of the significance of such a linear correlation, and the nature of T_{ref} as some sort of 'characteristic temperature', it is simply a mathematical consequence arising from experimental data covering a limited temperature range. The T_{ref} arising from such a correlation would simply be that temperature for which the approximation (δT small) is most appropriate, *i.e.* somewhere in the experimentally observable range. These effects are one example of the much broader phenomenon of 'enthalpy–entropy compensation' seen in many systems.[17,18,32–34]

References

1 M. H. McCormick, W. M. Stark, G. E. Pittenger, R. C. Pittenger, and J. M. McGuire, *Antibiotics Annual*, 1955–1956, p. 606.
2 C. T. Walsh, *Science*, 1993, **261**, 308.
3 P. Groves, M. S. Searle, J. P. Mackay and D. H. Williams, *Structure*, 1994, **2**, 747.
4 M. Nieto and H. R. Perkins, *Biochem. J*, 1971, **123**, 773.
5 M. Nieto and H. R. Perkins, *Biochem. J*, 1971, **123**, 789.
6 J. C. J. Barna and D. H. Williams, *Ann. Rev. Microbiol.*, 1984, **38**, 339.
7 G. D. Wright and C. T. Walsh, *Acc. Chem. Res.*, 1992, **25**, 468.
8 A. Cooper and K. McAuley-Hecht, *Philos. Trans. R. Soc. London A*, 1993, **345**, 23.
9 J. P. Waltho and D. H. Williams, *J. Am. Chem. Soc.*, 1989, **111**, 2475.
10 U. Gerhard, J. P. Mackay, R. A. Maplestone and D. H. Williams, *J. Am. Chem. Soc.*, 1993, **115**, 232.
11 J. P. Mackay, U. Gerhard, D. A. Beauregard, M. S. Westwell, M. S. Searle and D. H. Williams, *J. Am. Chem. Soc.*, 1994, **116**, 4581.
12 M. Schäfer, T. R. Schneider and G. M. Sheldrick, *Structure*, 1996, **4**, 1509.
13 A. K. Covington, M. Paabo, R. A. Robinson and R. G. Bates, *Anal. Chem.*, 1968, **40**, 700.
14 T. Wiseman, S. Williston, J. F. Brandts and L. N. Lin, *Anal. Biochem.*, 1989, **179**, 131.
15 A. Cooper and C. M. Johnson, in *Methods in Molecular Biology*, ed. C. Jones, B. Mulloy and A. H. Thomas, Humana Press, Totowa, NJ, 1994, **22**, 137.
16 P. R. Stoesser and S. J. Gill, *J. Phys. Chem.*, 1967, **71**, 564.
17 G. Weber, *J. Phys. Chem.*, 1993, **97**, 7108.
18 G. Weber, *J. Phys. Chem.*, 1995, **99**, 1052.
19 R. S. Spolar and M. T. Record, *Science*, 1994, **263**, 777.
20 A. Cooper, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 2740.
21 A. Cooper, *Prog. Biophys. Mol. Biol.*, 1984, **44**, 181.
22 J. M. Sturtevant, *Proc. Natl. Acad. Sci. USA*, 1977, **74**, 2236.
23 D. H. Williams, M. S. Searle, J. P. Mackay, U. Gerhard and R. A. Maplestone, *Proc. Natl. Acad. Sci. USA*, 1993, **99**, 1172.
24 D. Xu, S. L. Lin and R. Nussinov, *J. Mol. Biol.*, 1997, **265**, 68.
25 P. H. Popieniek and R. F. Pratt, *J. Am. Chem. Soc.*, 1991, **113**, 2264.
26. D. H. Williams, M. J. Stone, R. J. Mortishire-Smith and P. R. Hauck, *Biochem. Pharmacol.*, 1990, **40**, 27.
27 J. M. Sturtevant, in *Experimental Thermochemistry*, ed. H. A. Skinner, Interscience, New York, 1962, vol. II, p. 427.
28 A. Cooper and C. A. Converse, *Biochemistry*, 1976, **15**, 2970.
29 P. Groves, M. S. Searle, I. Chicarelli-Robinson and D. H. Williams, *J. Chem. Soc., Perkin Trans., 1*, 1994, 659.
30 D. H. Williams, M. S. Searle, P. Groves, J. P. Mackay, M. S. Westwell, D. A. Beauregard and M. F. Cristofaro, *Pure Appl. Chem.*, 1994, **66**, 1975.
31 M. P. Williamson, D. H. Williams and S. J. Hammond, *Tetrahedron*, 1984, **40**, 569.
32 R. Lumry and S. Rajender, *Biopolymers*, 1970, **9**, 1125.
33 E. Grunwald and C. Steel, *J. Am. Chem. Soc.*, 1995, **117**, 5687.
34 J. D. Dunitz, *Chem. Biol.*, 1995, **2**, 709.
35 J. J. Christensen, L. D. Hansen and R. M. Izatt, *Handbook of Proton Ionization Heats*, Wiley, New York, 1976.
36 A. Cooper and C. M. Johnson, in *Methods in Molecular Biology*, ed. C. Jones, B. Mulloy and A. H. Thomas, Humana Press, Totowa, NJ, 1994, **22**, 109.

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