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Kinetic barriers and ordering of non-covalently bound states

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Binding of a dimer of a glycopeptide antibiotic to two molecules of a ligand that are bound to a membrane surface (by a hydrocarbon anchor) has been investigated. This binding on a surface is cooperatively enhanced (surface enhancement) relative to the binding in solution, because the former occurs intramolecularly on a template. Previously a correlation between surface enhancement and thermodynamic stability of the dimer in free solution (K_{dim}^{sol}) was hypothesised. However, we found that two weakly dimerising antibiotics (vancomycin and ristocetin A) with similar K_{dim}^{sol} give very different surface enhancements. We propose a model to explain the data correlating surface enhancement to the kinetic barrier to dissociation of the dimer. The surface enhancement of binding can be expected to increase with increasing tightness of the non-covalent interactions formed at the dimer interface. The effect should be found in general where cooperativity is exercised within an organised template (*e.g.*, DNA duplexes and proteins).

Introduction

Antibiotics of the vancomycin group bind strongly to bacterial mucopeptide cell wall precursors terminating in -Lys-D-Ala-D-Ala (-KDADA).[1,2] Additionally, they typically form dimers (Fig. 1).[3] Antibiotic dimers have the capability of exercising

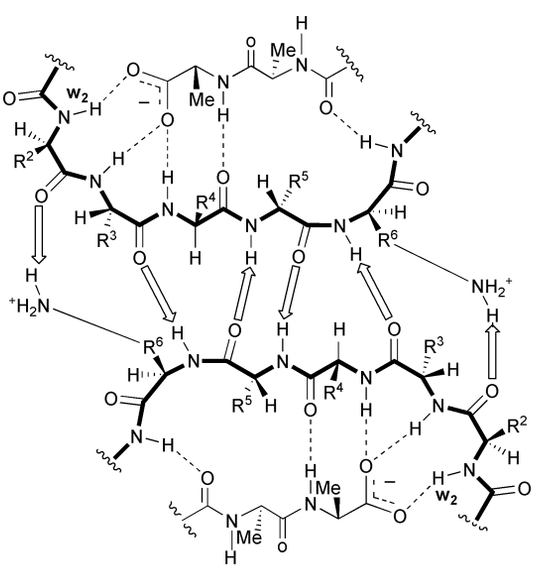

Fig. 1 Structure of the ligand-bound dimer formed by the glycopeptides (for example, ristocetin A). Hydrogen bonds to a cell wall analogue Ac-D-Ala-D-Ala are indicated by broken lines, and hydrogen bonds between the two halves of the dimer by broad arrows. The peptide backbones of the antibiotic molecules are indicated in bold and the monitored proton w_2 is indicated.

cooperativity by binding simultaneously (effectively intramolecularly, Fig. 2a) to two cell wall precursors terminating in -KDADA at a cell surface.[4–6] The intramolecular (templated) association is favoured because of a chelate type enhancement, *i.e.*, the loss in entropy associated with the second binding event is considerably less than the first. There is also the possibility of an enthalpic benefit associated with this templated complex.[7]

The first evidence of surface binding enhancements due to dimerisation was provided by experiments in which antibiotic

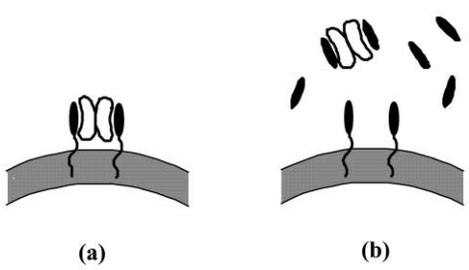

Fig. 2 Schematic diagram illustrating the basis of the competition experiments at a surface (curved shaded area). Antibiotic dimers are indicated by the paired open structures, and cell wall analogues by filled ovals (with hydrocarbon membrane anchors illustrated by tails). (a) Surface-enhanced bidentate binding of a dimer. (b) Antibiotic displaced into free solution by a competing ligand.

action against bacteria on agar plates was antagonised by the addition of external Ac_2-KDADA.[4,8] It was found that the larger the dimerisation constant of the antibiotic in solution, the greater the concentration of external Ac_2-KDADA required to inhibit the activity of the antibiotic. The effect was very large; the concentration of external Ac_2-KDADA required to half-antagonise antibiotic action was a 1000-fold larger for the most strongly dimerising antibiotics relative to the most weakly dimerising. These data indicate that the extent to which binding is enhanced at the bacterial surface (relative to free solution) increases with the increasing ability of an antibiotic to dimerise in solution.

Subsequent experiments have attempted to determine the extent to which binding is enhanced when it occurs intramolecularly at a model cell surface relative to the equivalent event when in free solution (intermolecular binding). Phosphatidylcholine (PC) vesicles were used to mimic the bacterial membrane. A binding enhancement of ~100-fold was determined for the binding of chloroeremomycin (also referred to as LY264826 or A82846B, Fig. 3c, which dimerises strongly) to vesicle anchored -D-Ala terminating peptides over analogous binding events in free solution.[6] Additionally, the enhancement associated with the surface binding to -D-lactate (-D-Lac) terminating peptides has been investigated.[9] This was of interest since resistant bacteria utilise -D-Lac terminating cell wall precursors.[10–12] These precursors have much weaker affinity to the

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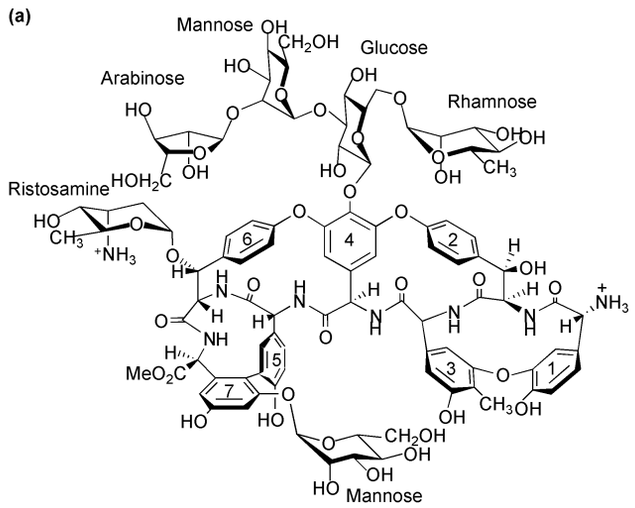

(b) R_1 = Cl, R_2 = H, R_3 = vancosaminylglucose
(c) R_1 = Cl, R_2 = 4-epivancosamine, R_3 = 4-epivancosaminylglucose
(d) R_1 = H, R_2 = 4-epivancosamine, R_3 = 4-epivancosaminylglucose

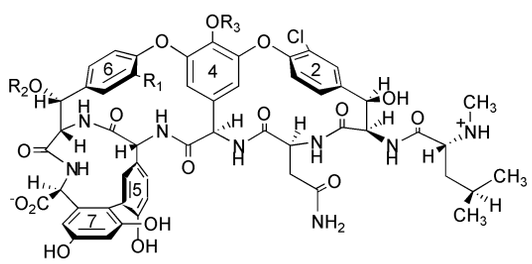

Fig. 3 Structures of the antibiotics: (a) ristocetin A; (b) vancomycin; (c) chloroeremomycin; and (d) eremomycin.

antibiotics than the -D-Ala terminating precursors.[13] It was again found that the surface enhancement of binding is very large for the strongly dimerising antibiotic chloroeremomycin, but small for the weakly dimerising antibiotic vancomycin (Fig. 3b).[9]

The surface enhancement of ligand binding was previously correlated with the thermodynamic stability of the dimer in solutions.[4,9] We have now investigated further the enhancement exercised by various glycopeptide antibiotics at the surface of a lipid bilayer by proton NMR spectroscopy and found here that this correlation is not universally valid. Here, we hypothesise that the surface binding enhancements are causally related to the kinetic barriers for the dimer dissociations, and thereby to the tightness of the dimer interfaces. This hypothesis is well supported by the data described below.

Results and discussion

Ligand binding constants on a membrane surface

In the cell wall biosynthesis of resistant bacteria, a pentapeptide is attached to a C_{55} membrane anchor *via* a sugar–phosphate link. In our work, a glycine residue was inserted between the pentapeptide and the membrane anchor in order to mimic the width of this link.[9] The anchored depsipeptide used in the present work was *N*-docosanoyl-Gly-Ala-γ-D-Glu-Lys-(*N*-ε-Ac)-D-Ala-D-Lac (doc-hex-Lac).

Phosphatidylcholine vesicles were used to provide a membrane surface. Direct NMR observation of the complexes bound to the vesicles was not possible due to the large size and slow correlation time of the vesicular aggregates which results in severe line broadening of the NMR signals. However, this line-broadening effect allowed surface binding affinities to be measured using competition experiments. In these experiments, antibiotic was displaced from the vesicle surface into free solution by the addition of a non-membrane anchored cell wall precursor analogue (Fig. 2b). Removal of the antibiotic from its vesicle-attached state resulted in its ^{1}H NMR signals no longer being severely line broadened, *i.e.*, resonances arising from the antibiotic–ligand complex in free solution could be observed. The ease, or difficulty, with which the antibiotic was displaced from the vesicle surface was then used to estimate the strength of the surface binding.

Fig. 4 shows the results of a competition experiment used to

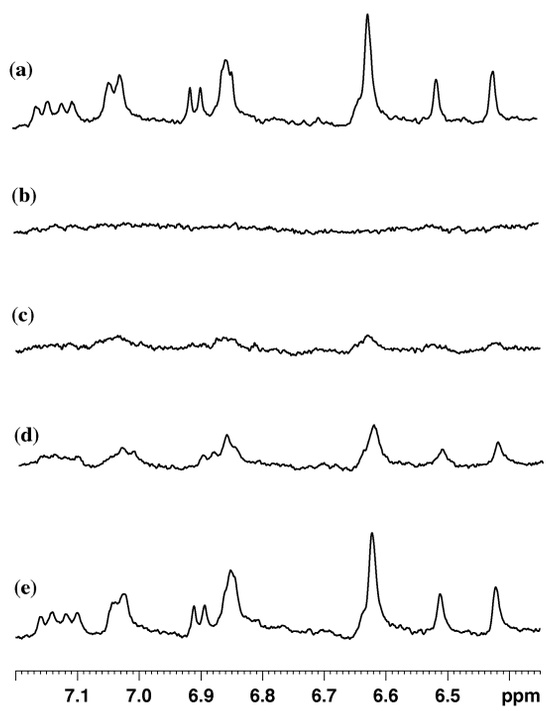

Fig. 4 Aromatic region of the proton NMR spectra (500 MHz, 300 K, D_2O, pD 7) of ristocetin A (0.2 mM) with Ac_2-KDADA (varying concentration) and/or doc-hex-Lac (1 mM) in the presence of vesicles (10 mM). (a) Control spectrum of the ristocetin A–Ac_2-KDADA complex in free solution (Ac_2-KDADA 1 mM); (b) spectrum of ristocetin A when surface bound to the membrane-anchored -KDADLac terminating peptide; (c), (d) and (e) spectra of the ristocetin A–Ac_2-KDADA complex when the antibiotic is displaced from its surface binding by adding Ac_2-KDADA to the solution. The concentration in free solution for Ac_2-KDADA was (c) 1 mM; (d) 10.7 mM and (e) 28.7 mM.

determine the binding affinity of ristocetin A to vesicle bound doc-hex-Lac. The binding of the antibiotic to the anchored cell wall precursor was sufficiently strong that Ac_2-KDADA was necessary to displace the antibiotic into free solution. As an example, the aromatic region of the ^{1}H NMR spectrum of the complex of ristocetin A and Ac_2-KDADA in the presence of vesicles is shown in Fig. 4a. The resonances are sharp, as the antibiotic is not associated with the vesicle. Fig. 4b shows the spectrum of ristocetin A in the presence of doc-hex-Lac and vesicles. The complete line broadening indicates the antibiotic is fully bound to the ligand at the vesicle surface. Fig. 4c shows the spectrum when Ac_2-KDADA and doc-hex-Lac are present in approximately equal concentrations. Clearly, the antibiotic is still predominantly associated with the vesicle, and therefore the binding constant to doc-hex-Lac at the surface of the vesicle must be larger than that to Ac_2-KDADA in free solution (*i.e.*, K_{lig}^{surf} for doc-hex-Lac is greater than 5.9×10^5 M^{-1} which corresponds to K_{lig}^{sol} for Ac_2-KDADA [1]). Fig. 4d and 4e indicate that a large excess of Ac_2-KDADA over the membrane-bound doc-hex-Lac is required to remove by competition most of the antibiotic from the vesicle.

A more rigorous analysis of the NMR spectra obtained from the competition experiment allowed the calculation of the binding constant between the antibiotic and the surface bound ligand. Integration of a specific antibiotic peak, relative to a 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (TSP) standard, at any point in the titration, enabled the concentration of antibiotic displaced from the vesicle surface by Ac_2-KDADA to be determined. The concentration of the antibiotic bound to the surface-anchored doc-hex-Lac was then calculated from the difference of this value and the known total concentration of the antibiotic. The binding constant between the antibiotic and the surface bound doc-hex-Lac could then be determined by an analysis of the equilibrium present within the system. The quantitative arguments have been presented previously.[14] The binding constant for ristocetin A binding to lipid-bound doc-hex-Lac was found to be $(3 \pm 2) \times 10^6$ M^{-1}. An analogous titration was performed for eremomycin (also referred to as MM45289 or A82846A, Fig. 3d) and again a large surface binding constant [$(4 \pm 2) \times 10^5$ M^{-1}] was obtained. Thus, the surface binding constants for both antibiotics are very large even though they have very different dimerisation constants in free solution (see Table 1). However their kinetic barriers to dimer dissociation are the same within experimental error (*ca.* 62 kJ mol^{-1}) supporting the hypothesis that the kinetic barriers determine the surface enhancement rather than thermodynamic stabilities. We estimated the kinetic barrier for ristocetin A from the coalescence temperature of proton resonances at the dimer interface, while the coalescence temperature of eremomycin was taken from Gerhard *et al.*[15]

We can compare the surface binding constants of ristocetin A and eremomycin with those of the other two antibiotics, chloroeremomycin[14] and vancomycin[9] (see Table 1). The contrast between the data obtained for ristocetin A (Fig. 4) and vancomycin (Fig. 5) is striking. These two antibiotics have similar dimerisation constants in free solution (see Table 1), but very different kinetic barriers to dimer dissociation. In fact the vancomycin dimer is in fast exchange on the NMR time-scale with its momomeric form even at 280 K. Its kinetic barrier is calculated to be less than 53 kJ mol^{-1}, which corresponds to a dissociation rate >3700 s^{-1} (ristocetin A has a dissociation rate of *ca.* 100 s^{-1}) at 300 K. In our competition experiments, 28.7 mM of Ac_2-KDADA was required to displace the surface binding of ristocetin A, whereas only 0.4 mM of Ac_2-KDADA was sufficient in the case of vancomycin (Fig. 5).

Surface plasmon resonance (SPR) analysis has also been previously used to investigate the binding of vancomycin group antibiotics to *N*-α-docosanoyl-Lys(*N*-ε-Ac)-D-Ala-D-lactate (doc-tri-Lac) anchored in a supported lipid mono-layer. These experiments showed that ristocetin A had surface binding constants in excess of 50–150 times larger than vancomycin,[16] well in agreement with our data. However, quantification of the extent of surface enhanced binding ($K_{lig}^{surf}/K_{lig}^{sol}$) for both antibiotics was not available from that study[16] as the ligand binding constant in solution for ristocetin A could not be determined by UV analysis and capillary electrophoresis.

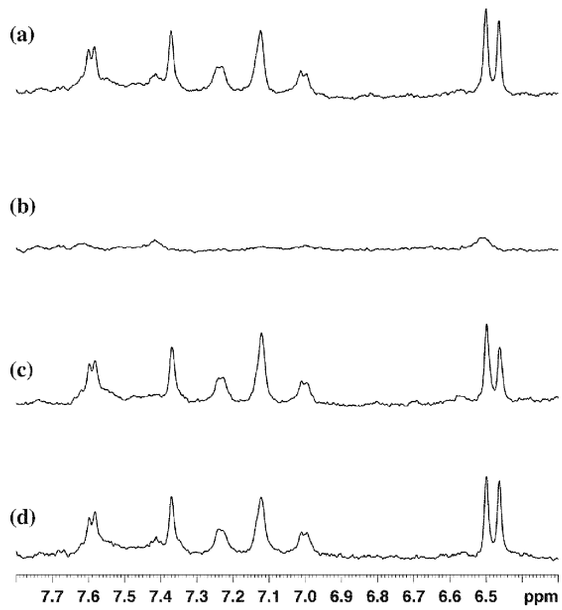

Fig. 5 Aromatic region of the proton NMR spectra (500 MHz, 300 K, D_2O, pD 7) of vancomycin (0.2 mM) with Ac_2-KDADA (varying concentration) and/or doc-hex-Lac (1 mM) in the presence of vesicles (10 mM). (a) Control spectrum of the vancomycin–Ac_2-KDADA complex in free solution; (b) spectrum of vancomycin when surface bound to the membrane-anchored -KDADLac terminating peptide; (c) and (d) spectra of the vancomycin–Ac_2-KDADA complex when the antibiotic is displaced from its surface binding by relatively low concentrations of Ac_2-KDADA. The concentration in free solution for Ac_2-KDADA was (c) 0.4 mM and (d) 1.29 mM.

Ligand binding constants in free solutions

The binding constants in free solution of all antibiotics to -D-Lac terminating ligands are listed in Table 1. The binding constants of chloroeremomycin and eremomycin to Ac_2-KDADLac in solution were obtained by measuring the change in chemical shift of the proton w_2 at the ligand interface (see Fig. 1) with increasing Ac_2-KDADLac concentration. Since w_2 undergoes a large downfield shift on ligand binding, it is an excellent probe for quantification of the ligand affinities. The ligand binding constants to chloroeremomycin and eremomycin were found to be $(9.2 \pm 0.5) \times 10^2$ M^{-1} and $(5.5 \pm 0.5) \times 10^2$ M^{-1}, respectively. These values are within a factor of two (in the case of chloroeremomycin[9]) and three (in the case of eremomycin, present work) of the corresponding binding constants to *N*-Ac-Gly-Ala-γ-D-Glu-Lys(*N*-ε-Ac)-D-Ala-D-Lac (Ac-hex-Lac). Thus it appears the *N*-terminal residues (-Gly-Ala-γ-D-Glu) have a relatively small effect on ligand binding (in agreement with earlier work on the binding of extended -DADA analogues to vancomycin group antibiotics[1,2]). The binding constant of Ac_2-KDADLac to ristocetin A was also obtained by NMR in a similar way as described above and found to be $(2.6 \pm 1.0) \times 10^2$ M^{-1}. In the case of vancomycin the binding constant to Ac-hex-Lac or Ac_2-KDADLac could not be determined using

Table 1 Comparison of solution and surface binding constants to -KDADLac terminating ligands, for antibiotics of differing dimerization constants

Antibiotic	$K_{dim}^{sol\,a}/M^{-1}$	$K_{lig}^{sol\,b}M^{-1}$	$K_{lig}^{surf\,c}M^{-1}$	$\rho^{surf/sol\,d}$
Ristocetin A	500	$(2.6 \pm 1.0) \times 10^{2\,e}$	$(3 \pm 2) \times 10^{6\,f}$	$(1.2 \pm 0.9) \times 10^4$
Vancomycin	700	$4.1 \times 10^{2\,g}$	$<10^{4\,h}$	<24
Chloroeremomycin	2×10^5	$(9.2 \pm 0.5) \times 10^{2\,e}$	$(6.6 \pm 2.1) \times 10^{6\,i}$	$(7.2 \pm 2.3) \times 10^3$
Eremomycin	3×10^6	$(5.5 \pm 0.5) \times 10^{2\,e}$	$(4 \pm 2) \times 10^{5\,f}$	$(7.3 \pm 3.7) \times 10^2$

[a] Dimerization constants in free solution.[27,28] [b] Binding constants to Ac_2-KDADLac in free solution. [c] Binding constants to vesicle bound doc-hex-Lac. [d] $\rho^{surf/sol}$ is defined as $K_{lig}^{surf}/K_{lig}^{sol}$. [e] Determined by NMR in the present work. [f] Present work. [g] Determined by affinity capillary electrophoresis.[13] [h] Data taken from O'Brien *et al.*[9] [i] Data taken from Sharman *et al.*[14]

the w_2 proton probe due to the resulting complex being in intermediate exchange on the NMR time-scale. However, we can use the value determined by affinity capillary electrophoresis for vancomycin bound to Ac_2-KDADLac (410 M^{-1}).[13] Binding constants obtained by the two different methods (NMR or capillary electrophoresis) have been shown to give comparable results (this work and O'Brien *et al.*[9]). For example, we found by NMR the binding constants of chloroeremomycin to Ac_2-KDADLac to be $(9.2 \pm 0.5) \times 10^2$ M^{-1}, while 1.6×10^3 M^{-1} was the value obtained by capillary electrophoresis.[13]

Surface enhancement of the ligand binding constants

Comparison of the surface binding constants with the equivalent binding constants in free solution provides a measure of the enhancement gained by binding a ligand at the lipid surface ($\rho^{surf/sol}$) (Table 1). In particular, for ristocetin A, the surface enhancement of binding to -D-Lac terminating peptides is remarkable. Although in free solution the -D-Lac terminating peptide (Ac_2-KDADLac) has much weaker affinity (2.6×10^2 M^{-1}) for ristocetin A than does the -D-Ala terminating peptide (Ac_2-KDADA) (5.9×10^5 M^{-1}),[1] a large excess of Ac_2-KDADA is required to antagonise the surface binding to the -D-Lac terminating peptide. Importantly, from Table 1, it can also be seen that eremomycin, chloroeremomycin and ristocetin A have larger binding enhancements for templated binding at the membrane surface than vancomycin. The striking property of the vancomycin dimer is that it is in fast exchange with its monomeric form, whereas the dimers of the other three antibiotics are in slow exchange on the NMR time-scale with their monomers at room temperature. We conclude here that it is likely to be their relatively large kinetic barriers to dimer dissociation that allows them to promote relatively large cooperative effects. The much greater enhancement of surface binding by ristocetin A in comparison to vancomycin is particularly noteworthy, since they have very similar dimerisation constants (Table 1). Usually antibiotics that have a large kinetic barrier to dimer dissociation have also a large thermodynamic stability of the dimer.[17] Thus, not surprisingly in previous work, it was the correlation of surface enhancement of ligand binding and thermodynamic stability of the dimers that was noted.

Tight binding

Tight binding is sometimes used to mean high affinity binding.[18] However, we use the term to define binding that occurs with a large restriction of the relative translational motion of the associating molecules (eqn. (1), contrast eqn. (2) where a large relative translational motion is depicted as remaining).

$$A + B \rightleftarrows A \leftrightarrow B \quad (1)$$

$$A + B \rightleftarrows A \longleftrightarrow B \quad (2)$$

Several variables can affect the tightness of binding. First, a large restriction of translational motion requires relatively large restraining forces to be associated with the non-covalent bonds that hold the molecules together. Thus, among non-covalent bonds those with the larger force constants will be the more effective in promoting tight binding. For example, amide–amide hydrogen bonds are associated with larger force constants than are hydrocarbon/hydrocarbon (van der Waals C–H/C–H) interactions.

Second, for a given type of non-covalent bond, its elasticity is dependent on how deeply it lies inside its characteristic potential energy (PE) well (Fig. 6). For a given available thermal energy (temperature), a specified type of non-covalent interaction will permit less relative translational motion of **A** and **B** in **A·B** where lower vibrational energies are occupied. The average bond length is thus shorter for tigher binding [Fig. 6(a)] than for looser binding [Fig. 6(b)]. It is not only the temperature that determines the vibrational levels that are occupied (as would be the case for a non-covalent bond acting without aid from others). Rather, the addition of an adjacent non-covalent interaction can also reduce the dynamic behaviour of the non-covalent interaction under consideration.[19] The mechanical analogy of this behaviour occurs where a displacement associated with a given kinetic energy is more effectively constrained by two springs (*cf.* bonds) acting simultaneously in comparison to each spring acting in isolation. Thus, the addition of the second interaction results in less dynamic and "tighter" binding [*i.e.*, results in the conversion from (b) to (a) in Fig. 6 without a change in temperature]. In this way, using one non-covalent interaction to tighten another results in thermodynamically stronger binding, with a benefit in enthalpy (ΔH) that is larger than the cost in entropy (in terms of $T\Delta S$).[19]

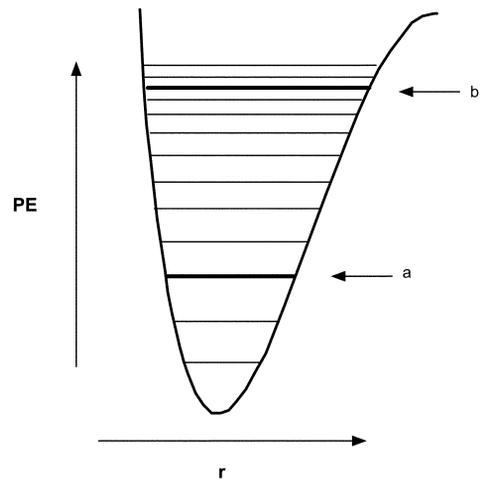

Fig. 6 Schematic illustration of a potential energy well for a non-covalent interaction. For a given temperature, when a non-covalent bond lies more deeply in the potential energy well its translational motion is more restricted [Fig. 6(a) *vs.* Fig. 6(b)]. The average bond lengths are shorter for tight binding [Fig. 6(a)] than for loose binding [Fig. 6(b)].

A third variable that can affect the dynamic behaviour of a non-covalently bonded interface is the degree of solvent accessibility. Where some of the functional groups that are "bared" as the non-covalent bonds are broken can be solvated as their stretching proceeds, then a more dynamic behaviour of the interface is promoted.

In summary, tight binding should be promoted by non-covalent bonds that (i) have relatively large force constants (ii) are strengthened by adjacent interactions (Fig. 6) and (iii) have poor solvent accessibility.

Tighter complexes will typically lie in deeper free energy wells.[17] Consider a dimer X·X which must either surmount a high barrier (path A), or a low barrier (path B) in order to reach its dissociated state X + X (Fig. 7). Suppose the dimer has a mean thermal energy indicated by the horizontal line (Fig. 7). In the case of a low barrier to dissociation (path B), this thermal energy will bring the dimer close to the transition state for dissociation, but in the case of a high barrier (path A) the non-covalent bonds at the X·X interface will be shorter. Thus, in the case of two antibiotic dimers with the same thermodynamic stabilities, that with a high barrier to dissociation should have the tighter structure. We have provided NMR evidence to support this conclusion.[17] Specifically, the dimers of ristocetin A and vancomycin have very similar thermodynamic stabilities, but the former has a higher barrier to dissociation of the dimer and a more tightly bonded dimer interface. Tightness at the dimer interface was demonstrated by the downfield changes upon dimerisation in the chemical shift of the proton

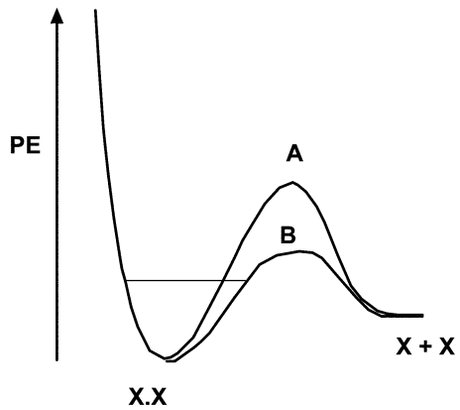

Fig. 7 Free energy profiles for the dissociation of a dimer (X·X ⟶ X + X) over either a high barrier (path A), or a low barrier (path B). At a given mean thermal energy (*e.g.*, represented by the horizontal line in the X·X. well), the expectation is that the high barrier results in shorter bonds, and less motion, at the X·X interface.

x_4 ($\Delta\delta_{x_4}$) which lies at the dimer interface. While a remarkably large $\Delta\delta_{x_4}$ was measured for ristocetin A, a much smaller value was detected for vancomycin.[17] We note here two effects that may be the causes of the relatively large barrier for dissociation of the ristocetin A dimer, relative to that for the vancomycin dimer. First, the large tetrasaccharide of ristocetin A shields the hydrogen bonds at the dimer interface (Fig. 1) from solvent. Second, the interactions between the tetrasaccharides in the two halves of the dimer may cooperatively increase the hydrogen bonding efficiency. Consistent with this possibility, removal of the tetrasaccharide causes the formation of a looser dimer with increased non-covalent bond lengths near the centre of the dimer interface.[17] The resulting antibiotic (ristocetin Ψ) is now in fast exchange on the NMR time-scale.

Tight binding has relevance to the function of biochemical systems. Helical structures are frayed near their termini, establishing in terms of the definition used here that their structures are less tight at these termini. The tighter binding near the centre of DNA duplexes (known from measuring distances across the duplex) is associated with slower NH exchange rates observed nearer to the centre of these structures.[20,21] In a similar manner, where the ends of an alanine-based peptide helix are frayed, amide proton exchange rates are slower near to the centre of the helix.[22]

Models which can account for the data

Ristocetin A, eremomycin, and chloroeremomycin give surface enhancements of binding approaching 10^3–10^4 in binding to bacterial cell wall precursor analogues at a membrane. The corresponding value for vancomycin is <24 (Table 1). These findings provide persuasive evidence that a large kinetic barrier to dimer dissociation is a cause of larger surface cooperativity. In free solution, all four antibiotics have rather similar (small or negligible) cooperative enhancements of binding Ac_2-KdAdA to dimer over monomer (0.8 for ristocetin A,[23] 1.4 for vancomycin,[5] 8–10 for eremomycin and chloroeremomycin[5]). We elaborate below on the model that indicates that high kinetic barriers to dimer dissociation are much more important in promoting cooperative binding at a surface than in promoting cooperative binding in free solution.

(a) Factors promoting positively cooperative binding in free solution. Consider the case where, in free solution, a second molecule of ligand binds to a dimer already carrying one molecule of ligand (Fig. 8a, where the latter entity is boxed). The boxed entity that associates with the second molecule of ligand (to bind it at the interface indicated by the heavy double-headed arrow) rotates and translates independently of the incoming ligand prior to the association. In making this interface therefore, an adverse entropy of translation and overall rotation has to be overcome. This adverse entropy is due to the restriction of the relative motions of the second molecule of ligand and the boxed entity. It is essentially independent of whether the dimer interface is loose (see the two extended double-headed arrows at the dimer interface in Fig. 8c), or tight (see the two contracted double-headed arrows at the dimer interface in Fig. 8a).

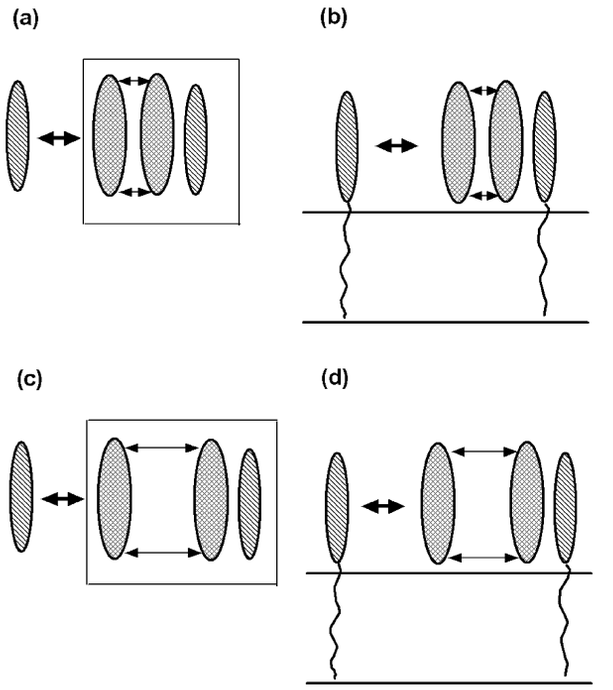

Fig. 8 Ligand molecules represented by hatching, and antibiotic molecules by cross-hatching. Binding of a tight antibiotic dimer (a) in free solution and (b) at the membrane surface; binding of a loose antibiotic dimer (c) in free solution and (d) at the membrane surface. See text for details.

(b) Factors promoting positively cooperative binding at a surface template. On a surface, we can consider cases (Fig. 8b and 8d) analogous to those above. Binding at the surface is enhanced relative to binding in free solution since the adverse entropy to be overcome in binding the boxed entity to a second molecule of ligand is reduced by attaching both of them to the same membrane. Additionally, the orientating effect of the membrane depends on how efficiently it is transmitted across the dimer interface. This transmission is efficient if the dimer interface is tight (Fig. 8b) as a more organised template is provided for the binding of the second molecule, but less so if the dimer interface is loose (Fig. 8d). Thus, a tight dimer with a large barrier to its dissociation (*e.g.*, ristocetin A) will give a larger surface cooperativity than a loose dimer with a smaller barrier to its dissociation (*e.g.*, vancomycin).

Conclusions

We have considered systems in which bacterial cell wall analogue precursors (ligands) and dimers of the vancomycin group antibiotics bind one to the other. Larger surface cooperativity of ligand binding to dimer is correlated with larger barriers to dissociation of the dimer and tightness, rather than with their thermodynamic stability. The importance of kinetic barriers and tightness in the determination of cooperative phenomena is therefore suggested. A simple model can account for the enhancement of the surface binding.

Experimental

Materials

Ristocetin A sulfate was purchased from abp Co. Chloroeremomycin acetate was donated by Eli Lilly, and eremomycin hydrochloride by SmithKline Beecham. Vancomycin hydrochloride was purchased from Aldrich. Ac_2-KDADA and Ac_2-KDADLac were purchased from Sigma. Doc-hex-Lac[24] and Ac-hex-Lac[9] were synthesised according to the procedures previously published. PC vesicles were prepared in a concentration of 10 mM according to the published method.[9]

NMR Spectroscopy

All NMR experiments were performed on a Bruker DRX-500 spectrometer at 300 K. Spectra were referenced to TSP. Samples were prepared in either D_2O (100%) or H_2O–D_2O (9 : 1). No corrections were made for isotope effects. The 3–9–19 WATERGATE pulse sequence[25,26] (for samples in H_2O–D_2O) or presaturation (samples in D_2O) was used to suppress the water signal.

Determination of surface binding constants on PC vesicles

Surface binding constants were obtained using titration experiments as described in the previously published paper.[9] The antibiotic concentration was 0.2 mM for all titrations. Antibiotics were dissolved in vesicle solution (10 mM in D_2O) and the lipopeptide (1 mM) was added. The competing ligand solutions were prepared by dissolving the appropriate amount in the antibiotic–vesicle solutions and the pD was adjusted to 7. TSP (6 or 10 mM in D_2O) was used as an external reference. The procedure for calculating the surface binding constants has previously been published.[14]

Determination of binding constants in free solution

The binding constants in free solution were obtained using titration experiments as described previously.[9] Antibiotic solutions were prepared in H_2O–D_2O (9 : 1) at a concentration of 5 mM and pH 4.5 with the exception of ristocetin A (10 mM and pH 3.5). The different conditions used for ristocetin A were required to follow the broader amide resonances of this antibiotic during the titration.

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