High Affinity Surface Binding of a Strongly Dimerizing Vancomycin-Group Antibiotic to a Model of Resistant Bacteria

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Received November 17, 1998. Revised Manuscript Received March 29, 1999

Abstract: The factors that give rise to binding enhancements when a strongly dimerizing vancomycin-group antibiotic (chloroeremomycin) binds to a model cell surface of vancomycin-resistant enterococci (VRE) have been semiquantitated. The model cell surface is comprised of vesicles to which have been anchored cell wall precursor analogues of vancomycin-resistant bacteria (which terminate in -D-lactate) via a hydrophobic docosanoyl (C_{22}) chain. Using ^1H and ^{19}F NMR spectroscopy, a large binding enhancement at the model cell surface (compared to the binding of an analogous ligand in free solution) has been observed. This enhancement can be partitioned into two distinct factors: a simple concentrating factor arising from an increase in local concentration of ligand when it is located at the vesicle surface and a factor arising from the cooperative interaction of species mutually bound to the membrane surface. The overall enhancement to binding at a surface compared to binding in free solution was found to be a factor of 10^2-10^3. In contrast, no significant surface binding enhancement was observed for the weakly dimerizing antibiotic vancomycin.

Introduction

The widespread occurrence of bacterial resistance to most common antibiotics[1] including the antibiotics of "last resort", the vancomycin-group (glycopeptide) antibiotics, means that there is now an urgent need to develop further our understanding of their mode of action. The vancomycin-group antibiotics act by binding to bacterial cell wall precursor peptides which terminate in the sequence -L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala.[2] Vancomycin-resistant bacteria, however, synthesize mucopeptides which terminate with the sequence -L-Lys-D-Ala-D-lactate,[3−7] and analogues of these precursors bind to the antibiotics with a much lower affinity in free solution than -D-Ala-terminating precursors.[8,9]

In previous work, we have modeled the binding of vancomycin-group antibiotics binding to cell wall precursors growing at the surfaces of bacteria.[10−14] Specifically, we quantitated the binding of vancomycin-group antibiotics to membrane-anchored cell wall precursor analogues (terminating in -Lys-D-Ala-D-Ala, or -Lys-D-Ala-D-Lac). The membrane surfaces used were sodium dodecyl sulfate micelles,[14] small unilamellar (phosphatidylcholine) vesicles,[10,13] or actual bacterial surfaces.[11,12] The derived binding constants were expressed in terms of M^{-1} even though we did not take into account the potential complications in defining the appropriate standard state for the binding of a templated aggregate at a surface. In this paper, we illustrate the validity of expressing these binding constants in terms of M^{-1} but also demonstrate that the derived values are dependent on the concentration of the membrane-anchored cell wall peptide (R-Lys-D-Ala-D-Lac, where R includes a hydrophobic group that can act as a membrane anchor) per unit of volume swept out by an anchored peptide at the vesicle surface (which defines the "surface" concentration of the anchored peptide). The results and conclusions presented here have implications for studies of the activity of glycopeptide antibiotics and more generally for the interaction of dimeric species with receptors at surfaces.

Results and Discussion

Two analogues of the mucopeptide precursors found in vancomycin-resistant bacteria were synthesized by solid-phase peptide synthesis: *N*-acetyl-Gly-Ala-D-γ-Glu-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate (**1**) and *N*-docosanoyl-Gly-Ala-D-γ-Glu-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate (**2**). The natural peptide is a pentapep-

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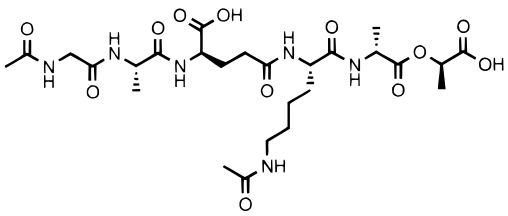
10.1021/ja9839769 CCC: $18.00
Published on Web 05/20/1999



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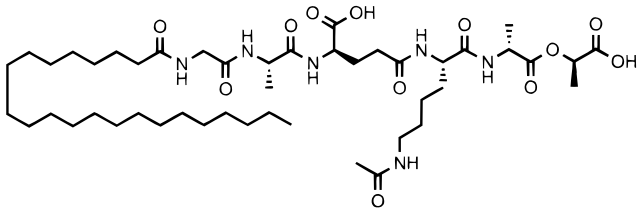

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tide attached to a C_{55} membrane anchor via a sugar−phosphate linkage, and therefore a glycine residue was inserted between the pentapeptide and the membrane anchor in order to mimic the width of this linkage. The former of these ligands was used to measure binding constants to antibiotic in free solution (since it cannot anchor to vesicles). The latter ligand can insert into small unilamellar vesicles via its N-terminal lipophilic anchor. Vesicles are a much better model of bacterial membranes than SDS micelles, since they are much larger and thus have a radius of curvature which better approximates that of a bacterial cell. Unfortunately, direct observation of an antibiotic−ligand complex on the surface of a vesicle by NMR is not possible due to the large size and slow correlation time of the vesicular aggregate which results in severe line broadening of the NMR signals.[13] Competition experiments, in which the antibiotic is displaced from an anchored complex on the vesicle surface into a complex in free solution by the addition of nonmembrane-anchored cell wall precursor analogues circumvent this problem and allow the measurement of surface-binding affinities (Scheme 1).[10,13]

In the present work, antibiotic bound to anchored ligand at the surface of a vesicle was displaced into free solution by two methods. First, by the addition of the fluorinated peptide *N*-α-acetyl-Lys(*N*-ϵ-trifluoroacetyl)-D-Ala-D-Ala (TFAc-KAA).[15] In this method, TFAc-KAA was titrated into a solution containing the antibiotic/anchored-ligand/vesicle aggregate. Two signals were observed in the ^{19}F NMR spectra; free (unbound) TFAc-KAA and TFAc-KAA bound to antibiotic in solution (not attached to the vesicle). The amount of antibiotic bound to TFAc-KAA in solution was calculated by integration of the bound TFAc-KAA signal in the ^{19}F NMR spectra. This was then used to calculate the amount of antibiotic bound to the membrane-anchored ligand. The affinity of TFAc-KAA for the glycopeptide antibiotic chloroeremomycin in free solution was measured by UV difference spectrophotometry.[15] By using an equation derived from a consideration of the equilibria present, the binding constant between the antibiotic and the surface bound ligand could be calculated as described by Entress et al.[15] The second method relied on a similar principle, but used *N*-α-acetyl-Lys(*N*-ϵ-acetyl)-D-Ala-D-Ala (Ac-KAA) as the competing ligand and ^{1}H NMR spectroscopy to observe the results.[10] Antibiotic which is bound to the surface-anchored ligand is not observed in the ^{1}H NMR spectrum due to the severe line broadening that occurs when it is associated with the vesicle. Integration of antibiotic ^{1}H NMR signals (arising from antibiotic complexed with Ac-KAA in free solution) enabled the determination of the concentration of antibiotic displaced from the vesicle surface by Ac-KAA, and hence, the concentration of antibiotic bound to the surface-anchored ligand could be calculated from the difference between this value and the known total concentration of antibiotic. The binding constant between the antibiotic and the surface bound ligand was then calculated as described by Sharman et al. by using equations analogous to those used in the ^{19}F NMR method.[10] Representative NMR data for both ^{1}H and ^{19}F methods can be found in the references[10,15] cited above.

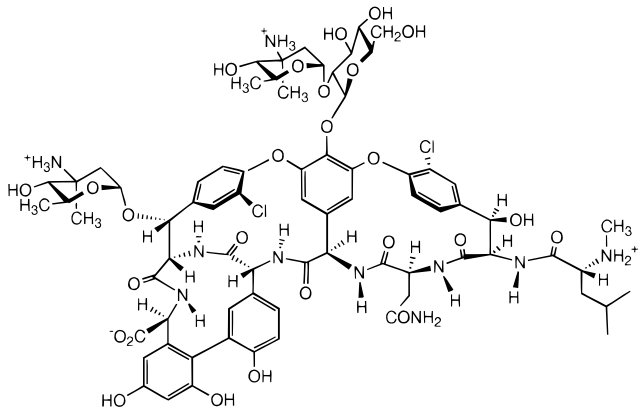
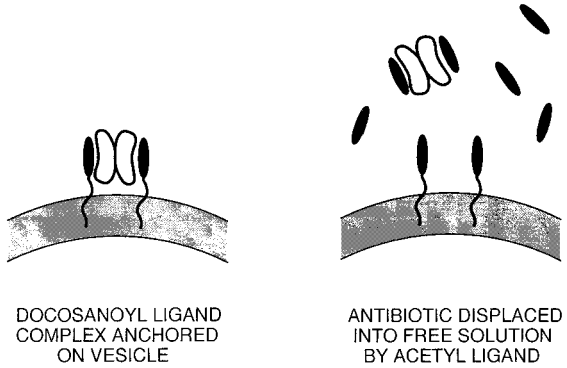


Figure 1. Structure of chloroeremomycin.

Scheme 1



Analysis of Binding in Free Solution. We give an analysis for the binding of a strongly dimerizing antibiotic chloroeremomycin (also known as LY264826B, Figure 1); such strongly dimerizing antibiotics are important because of the potential for their adaptation to give compounds active against vancomycin-resistant enterococci.[9] The dimerization constant (K_{dim}) of chloroeremomycin is 2×10^6 M^{-1} in the presence of ligand.[16] Therefore, at the concentrations used in the NMR experiments, the dimeric form of the antibiotic will be approximately completely populated, and only the equilibria shown in Scheme 2 need be considered.

In principle the two values of K_1 and K_2 (Scheme 2) could be different, not only because the second binding event oc-

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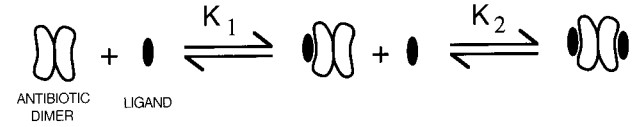
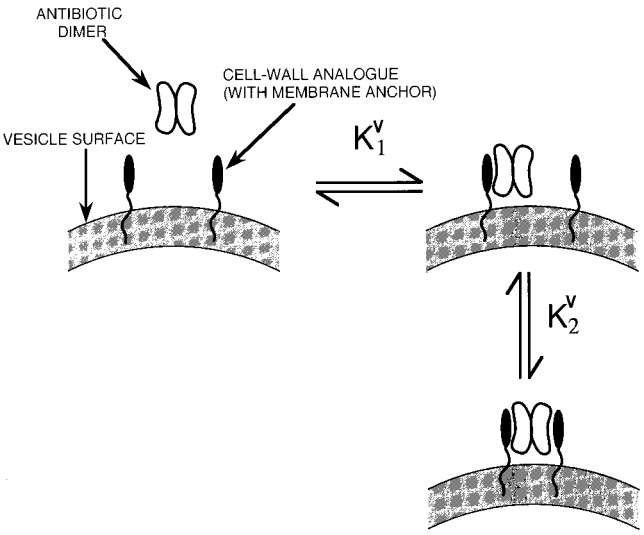
Scheme 2



Scheme 3



curs when the first ligand is already bound but also because the dimer of chloroeremomycin is asymmetric.[17,18] However, we have previously shown that only in the case of the glycopeptide ristocetin A are the two K_1 affinities significantly different.[19] We can therefore assume that the binding sites of chloroeremomycin do not differ significantly and thus assume $K_1 = K_2$.

Solution binding constants (K_{sol}) were determined by plotting the change in ^{1}H NMR chemical shift of an antibiotic amide proton as a function of ligand concentration.[20] K_{sol} is calculated such that it is the *equilibrium constant for the binding of ligand per mole of antibiotic binding site*. Thus, assuming K_1 is equal to K_2, we can approximate

$$K_{sol} = K_1$$

For the binding of chloroeremomycin to the bacterial mucopeptide analogue **1**, K_1 was determined to be 2000 ± 200 M^{-1}.

Analysis of Binding at the Vesicle Surface. For binding at the vesicle surface, we can consider a dimer binding to two vesicle-anchored ligands in a simple two-step process, with equilibrium constants, K^{v_1} and K^{v_2} (Scheme 3)

$$A + 2L \overset{K^{v_1}}{\rightleftharpoons} A_1 + L \overset{K^{v_2}}{\rightleftharpoons} A_2$$

where A is antibiotic dimer, A_1 is singly bound dimer, A_2 is doubly bound dimer, and L is anchored ligand.

The first binding event involves an antibiotic dimer in free solution associating with one of the membrane-anchored ligands. For this binding event, the relevant concentrations of L and A are their molar concentration in the aqueous solution.

$$K^{v_1} = \frac{[A_1]}{2[A][L]}\,M^{-1} \quad (1)$$

The factor of 2 in this equation is due to the fact that ligand can bind dimer in either of two sites but can dissociate from only one (the binding site which has ligand bound).

The second binding event can also be treated as a bimolecular association. If L is again taken as the concentration of free ligand in the total volume of the aqueous solution, then

$$K^{v_2} = \frac{2[A_2]}{[A_1][L]}\,M^{-1} \quad (2)$$

The factor of 2 in this equation reflects the fact that the ligand can now bind dimer in only one site but can dissociate from either of two sites. However, since the associating entities are anchored to the same vesicle, the ligand is much more likely to encounter anchored A_1 in the second step than was A to encounter anchored L in the first step. As a consequence, K^{v_2} will be greater than K^{v_1}. In the second step, the anchored ligand, in relation to its probability of finding the membrane-attached A_1, would have to be regarded as existing at a concentration greater than that available for the first step. This concentrating factor is the ratio of the volume available to the ligand in the aqueous solution ($V_{aq\ soln}$) to the volume swept out by the ligand on the lipid surface (V_{surf}). Assuming that the hydrophobic docosanoyl chain is inserted into the vesicle, the volume swept out by the peptide headgroup is approximately equal to (the length of the peptide headgroup) × (area of vesicle surface), integrated over all vesicles. In estimating this value, we take the length of the peptide headgroup to correspond to that of the extended form, i.e., when it is approximately linear. This value defines the total volume available to the peptide at the surface of the vesicle, within which any other conformation (where the peptide headgroup is not in its extended form) must exist. Thus, for a given concentration of phosphatidylcholine (p), the volume swept out by a ligand (V_{surf}) in one liter of solution ($V_{aq\ soln}$) is

$$V_{surf} = y4\pi r^2 \frac{pN_A}{z} \quad (3)$$

where y is the length of the peptide -Gly-L-Ala-D-γ-Glu-Lys-(N-ϵ-acetyl)-D-Ala-D-lactate, r is the radius of the vesicle, N_A is Avogadro's constant, and z is the average number of phosphatidylcholine molecules per vesicle.

Estimating y to be 27 Å (estimated from a CPK model of the peptide), r to be 50 nm (ca. 100-nm diameter vesicles were used in all experiments), and z to be 1.5×10^5 for 100-nm vesicles [calculated by dividing the surface area of a 100-nm vesicle by the area occupied by a phosphatidylcholine molecule headgroup in the vesicle (42 Å^2),[21] and multiplying by two as the vesicle is a bilayer], then

$$V_{surf} = 340p\ (cm^3)$$

Thus, the concentrating factor $V_{aq\ soln}/V_{surf}$ is

$$\frac{V_{aq\ soln}}{V_{surf}} = \frac{1000}{340p} = \frac{2.94}{p} \quad (4)$$

The position of the equilibrium will also be affected by at least four other factors. First, the peptide headgroup of membrane-

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bound L which binds to A_1 is not simply randomly orientated at the surface of the vesicles but rather orientated in a geometry which is probably favorable for association. Second, we have previously shown that in strain-free associations where (for two otherwise analogous binding events) there is an entropic advantage of one over the other, there is also an associated enthalpic benefit (since the restriction of motion favors bonding).[22,23] Third, the binding of a second ligand may provide a cooperative enhancement to the binding of the ligand already bound. Fourth, these benefits may be offset by a geometry of the membrane-templated complex which is less than ideal. The benefit to K^{v_2} over K^{v_1} from the combined operation of these four effects is not predictable, and thus we therefore allow for them by the introduction of a factor *m*. Thus, the total factor by which binding will be enhanced as a result of binding at the membrane surface rather than in solution is represented by $2.94m/p$.

In summary, if the input parameter used to determine K^{v_2} is the molar concentration of membrane-bound ligand in the aqueous volume of one liter, then K^{v_2} will be derived (using eq 2) as being much larger than K^{v_1} and is predicted to vary with the vesicle concentration. However, if the ligand-concentrating and ligand-orientating effects of the vesicles can be allowed for, then K^{v_2} would be expected to have a value close to K^{v_1}. This is because, apart from the effect of an increase in the effective concentration and binding affinity of ligand due to anchoring in the membrane, the two processes are essentially physically the same event. Therefore, as a good approximation, we can write

$$K^{v_2} = \frac{2.94mK^{v_1}}{p}\ (\mathrm{M}^{-1}) \tag{5}$$

We note that the dimensions of eq 5 are appropriate since *m* is a dimensionless factor, and $2.94/p$ is also dimensionless [a ratio of two volumes (eq 4)]. If the overall measured equilibrium constant for the binding of antibiotic dimer in free solution to two vesicle-bound ligands is expressed as K^v *per mole of antibiotic binding site*, then

$$K^v = \sqrt{K^{v_1}K^{v_2}} = K^{v_1}\sqrt{\frac{2.94m}{p}} \tag{6}$$

In deriving eq 6, we note that taking K^v as the geometric mean of K^{v_1} and K^{v_2} simply assumes that the average free energy of binding a ligand to antibiotic is given by the arithmetic mean of the free energies of binding in the two associative steps depicted in Scheme 3. Equation 6 predicts that K^v is dependent on the vesicle concentration; the smaller this value, the larger K^v.

To test this theory, K^v was determined for the binding of chloroeremomycin to vesicle-anchored **2** at a range of lipid concentrations at constant ligand concentration, using both ^{1}H NMR and ^{19}F NMR techniques (Table 1 and Figure 2). The overall trends observed by the two quite different NMR methods are remarkably consistent, although there is a systematic difference between the two data sets insofar as the proton experiments give K^v values which are a factor of 10−30 greater than the ^{19}F experiments. As expected from theory, K^v increases with decreasing lipid concentration but reaches a maximum at $p = 10$ mM, after which it decreases. A possible explanation for the lower K^v at a lipid concentration of 5 mM is steric crowding at the vesicle surface, where the close proximity of the ligands relative to each other makes antibiotic binding less favorable. At this low lipid concentration, there are 25 lipid molecules available at the external surface of the vesicle for each antibiotic dimer (taking into account the fact that the vesicle is a bilayer). If the antibiotic sits at the vesicle surface by contacts through the face bearing the sugars (for which there is supportive evidence[13]), then the appropriate cross section of the antibiotic dimer has an area of ∼500 Å^2, whereas the cross-sectional area of 25 lipid molecules at a vesicle surface is ∼1000 Å^2. Thus, since the cross-sectional area of the antibiotic dimer is approaching that of the available vesicle surface (per dimer), the possibility of steric crowding appears plausible. In addition, there will be a significant accumulation of positively charged antibiotic on the surface at low *p*. Therefore, another possible explanation for the decrease in *m* when $p < 0.01$ M is electrostatic repulsion.

Table 1. Variation in Binding Constant (K^v) for Chloroermomycin to **2** with Phospatidylcholine Concentration *p*[a]

p, mM	K^v, M^{-1} (^{19}F)	K^v, M^{-1} (^{1}H)
5	$(7.2 \pm 0.3) \times 10^4$	$(1.2 \pm 0.7) \times 10^6$
10	$(2.5 \pm 0.3) \times 10^5$	$(5.0 \pm 1.8) \times 10^6$
15	$(1.4 \pm 0.3) \times 10^5$	
20	$(7.0 \pm 0.2) \times 10^4$	$(2.4 \pm 0.9) \times 10^6$
30	$(2.1 \pm 0.2) \times 10^4$	$(2.1 \pm 0.8) \times 10^5$
40	$(1.0 \pm 0.2) \times 10^4$	$(1.8 \pm 0.8) \times 10^5$

[a] Values determined from both ^{1}H and ^{19}F NMR methods are given. Chloroeremomycin and **2** concentrations are fixed at 0.2 mM and 1 mM, respectively.

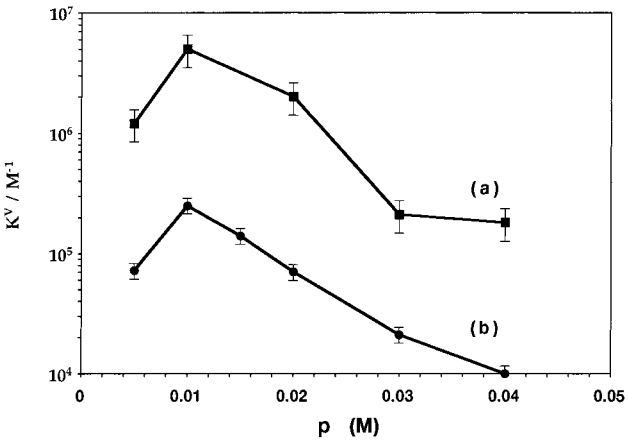

Figure 2. Variation of K^v with phosphatidylcholine concentration (*p*). (a) Values determined from ^{1}H NMR spectroscopy and (b) those determined by ^{19}F NMR spectroscopy.

Partitioning Cooperativity: The "*m*" factor. Up to this point, we have not considered whether "*m*" is constant or whether it varies with *p*. From eq 6 it is possible to calculate *m* for each lipid concentration (Table 2 and Figure 3), since K^{v_1} should be effectively the same as K_1 (solution binding constant which was measured to be 2000 M^{-1} -see above). Thus, from eq 6

$$m = \frac{p}{2.94}\left(\frac{K^v}{K^{v_1}}\right)^2 \tag{7}$$

Values of K^{v_2} and *m* determined at different lipid concentrations from both ^{1}H and ^{19}F NMR methods are given in Table 2. Although the ^{1}H NMR method gives values of *m* that are

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Table 2. Variation in *m* with Phosphatidylcholine Concentration (*p*) as Determined by Each NMR Method

	^{19}F		^{1}H	
p, mM	K^{v_2}	*m*	K^{v_2}	*m*
5	2.6×10^6	2	7.2×10^8	610
10	3.1×10^7	53	1.3×10^{10}	2.1×10^4
15	9.8×10^6	25		
20	2.5×10^6	8	2.9×10^9	9.8×10^3
30	2.2×10^5	1	2.2×10^7	110
40	5.0×10^4	0.3	1.6×10^7	110

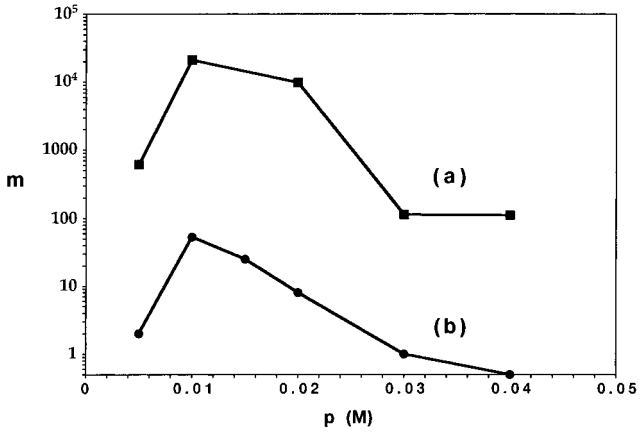

Figure 3. Variation in *m* with phosphatidylcholine concentration (*p*). (a) Values determined from ^{1}H NMR spectroscopy and (b) those determined by ^{19}F NMR spectroscopy.

systematically higher than those calculated from the ^{19}F NMR method (Table 2 and Figure 3), both data sets show a general trend in that *m* increases with decreasing vesicle concentration (until the surface of the vesicle cannot bind antibiotic without steric crowding—see above).

At a lipid concentration of $p = 0.01$ M (where the maximum cooperativity is expressed), K^{v_2} is in the range 3×10^7 M^{-1} (^{19}F data) to 10^{10} M^{-1} (^{1}H data). Thus, the surface cooperativity expressed in terms of the extent to which the second binding event is promoted over the first (i.e. as K^{v_2}/K^{v_1}) is in the range 1.5×10^4 to 5×10^6. At this lipid concentration, the simple concentrating effect is predicted to enhance K^{v_2} (relative to K^{v_1}) by a factor of only 300, and therefore effects due to other aspects of surface cooperativity (the net effects in *m*) are also large (Table 2). It must be noted that in our theory, the surface cooperativity *m* is exercised in the second binding step (K^{v_2}) only. The surface cooperativity *per mole of antibiotic binding site* is $\sqrt{m}$ since in the model we have used there is no cooperativity exercised in the first binding step. At a lipid concentration of 10 mM, this corresponds to a binding enhancement (in excess of the simple concentrating effect) of approximately 10 and 150 per mole of antibiotic binding site for the ^{19}F and ^{1}H NMR methods, respectively. Under the same conditions, the surface binding constant, K^{v}, for the weakly dimerizing antibiotic vancomycin was measured (using both the ^{19}F and ^{1}H NMR methods) to be $<10^4$ M^{-1}, emphazing the importance of dimerization in the expression of enhanced surface binding. Thus, when $p = 10$ mM, the surface binding constant to the D-Lac terminating peptide is 25 (^{19}F) to 500 (^{2}H) times less to vancomycin than to chloroeremomycin.

Our results raise the question of why *m* should vary with *p*. At this point we must note that excluded volume effects (the surface that is inaccessible due to being already occupied by other ligands) are not significant and cannot explain the *p*-dependence of *m*.[24] We suggest that as the concentration of lipopeptide in the vesicles increases (as *p* is reduced), the lipopeptide−antibiotic dimer complexes have an increasing tendency to associate together on the vesicle surface. This phenomenon is known in cellular systems where divalent antibodies induce "patching" and "capping" of immunoglobulin receptors on the cell membrane of lymphocytes.[25−29] The possible occurrence of associations between complexes in the present work implies that as the entropic cost of such associations is reduced (upon increasing lipopeptide-to-vesicle ratio), the favorable interactions between adjacent complexes can overcome the adverse entropy component. Relevant to this suggestion is the recent determination by Loll et al. of the crystal structure of vancomycin bound to the truncated ligand *N*-acetyl-D-alanine in which there exists an extensive array of interactions between two vancomycin molecules in the face-to-face configuration.[30] Although this interaction could be an artifact of crystal packing, it is consistent with the cooperativity observed in the present work. This work suggests that bacteria which express more of the "resistant" peptide on their surface will then become more susceptible to vancomycin antibiotics via enhanced binding.

Conclusion

Lipopeptides which terminate in -Lys-D-Ala-D-Lac have been synthesized and allowed to insert into vesicles, thereby generating assemblies which simulate VanA and VanB resistant bacteria. It has been shown that a strongly dimerizing antibiotic (chloroeremomycin) shows strongly enhanced binding to these assemblies relative to the corresponding binding in free solution. Using two independent NMR probes (^{19}F and ^{1}H), we have shown that surface binding of chloroeremomycin to the resistant bacteria cell wall precursor analogues varies as a function of vesicle concentration. At the lowest vesicle concentration examined (5 mM phosphatidylcholine) there is a decrease in binding ascribed to crowding at the vesicle surface. However, as the vesicle concentration is lowered in the range 40 → 10 mM phosphatidylcholine, the binding increases as a function of decreasing vesicle concentration, as predicted by theory. It has been possible to separate the enhancement of binding due to a simple surface concentration effect from other effects (expected to include surface cooperativity and orientation effects involving the lipopeptides). These latter influences are concluded to give a net enhancement of 1 to 2 orders of magnitude (depending on the NMR method employed) per mole of lipopeptide bound to the surface. Perhaps the most striking result from our work is that the second binding event of Scheme 3 corresponds to a value of K^{v_2} in the range 3×10^7 M^{-1} (^{19}F data) to 10^{10} M^{-1} (^{1}H data) at 10 mM phosphatidylcholine and 1 mM lipopeptide. Bearing in mind that this binding is to a -D-lactate-terminating peptide, the experiments and the analysis

(24) In the range $p = 0.01$ M (where the maximum cooperativity is expressed) to $p = 0.04$ M, the percentage volume occupied by lipopeptide is in the range 20−2.5%. If the excluded volume effect due to ligand is a favorable one, the maximum error in *m* caused by ignoring this effect will lie in the range of 20% (at $p = 0.01$ M) to 2.5% (at $p = 0.04$ M), which is not enough to significantly affect the values in Table 2.

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indicate the remarkable enhancement of binding that is achieved due to concentrating and cooperativity effects.

Experimental Section

Materials. Chloroeremomycin was provided by Eli Lilly and Co; XV1-E L-α-phosphatidylcholine and acetic anhydride were purchased from Sigma; 1,3-diisopropylcarbodiimide (DIC), diisopropylethyamine (DIEA), 4-(dimethylamino)pyridine (DMAP), and triisoproplsilane (TIPS) were purchased from Aldrich; 2-chlorotrityl chloride resin, *N*-α-Fmoc-D-alanine, *N*-α-Fmoc-alanine, *N*-α-Fmoc-*N*-ϵ-acetyl-L-lysine, *N*-α-Fmoc-D-glutamic acid-γ-*tert*-butyl ester, *N*-α-Fmoc-glycine, 1-hydroxybenzotriazole (HOBt), and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) were purchased from Novabiochem; lithium-D-lactate, dimethylformamide, dimethyl sulfoxide, and tetrakis(triphenylphosphine) palladium (0) were purchased from Fluka; *N*-docosanoyl-Gly-Ala-D-γ-Glu-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate was synthesized according to the previously published procedure.[15]

Preparation of Phosphatidylcholine Vesicles. Typical Preparation of Vesicle Suspension, Phosphatidylcholine Concentration 10 mM. Type XV1-E L-α-phosphatidylcholine from fresh egg yolk (Sigma, 64 mg) was dissolved in chloroform (10 mL) which had been rendered ethanol-free by passage through a column of activated alumina. The solution was then evaporated under reduced pressure to yield a thin film on the wall of the flask which was dried under high vacuum for 2 h. The flask was flushed with argon, and D_2O or pH 7.4 NaH_2PO_4 buffer (50 mM, 8 mL) was added. The mixture was shaken for 30 min and sonicated for 30 min to yield a slightly turbid suspension of vesicles. This suspension was then passed 17 times through a 100-nm pore size polycarbonate filter in an Avestin Lipofast Basic extrusion apparatus, to yield a clear suspension of vesicles, phosphatidylcholine concentration 10 mM. The samples in D_2O were adjusted to pH 7.0 with NaOD/D_2O and DCl/D_2O solutions, and these deuterated samples were used for the ^1H experiments. Control experiments showed that the competition experiments were pH insensitive in the range pH 7.0−7.4. All quoted pD values were measured using a Corning pH meter fitted with a combination glass electrode, and no attempt was made to correct for isotope effects.

NMR Spectroscopy. All ^1H NMR experiments were performed on a Bruker DRX-500 spectrometer at 300 K. Suppression of solvent was achieved using presaturation for samples dissolved in D_2O or H_2O. One-dimensional spectra were recorded using 32k complex data points. All ^1H NMR spectra were referenced to 3-trimethylsilyl-2,2,3,3-d_4-propionic acid, sodium salt (TSP, $\delta = 0.00$ ppm).

All ^{19}F NMR experiments were performed on a Bruker AM-400 spectrometer equipped with a $^{19}F/^1H$ probe at 300 K. One-dimensional spectra were typically recorded at 376.47 MHz using 8k data points over a spectral width of 6.9 ppm. TfOH was used as an external reference.

General Procedure for NMR Titrations. Titrations with Vesicles. All titrations were performed at 300 K. The antibiotic concentration used in all the titrations was 0.2 mM, and the lipopeptide concentration 1.0 mM. The lipopeptide was added to the vesicle solution and the resulting solution was mixed using agitation only; no sonication was used. The competing ligand (Ac-KAA for ^1H NMR and TFAc-KAA for ^{19}F NMR) was prepared from a solution identical to that in the NMR tube, so as not to change the pH or concentration of the other components in the tube (vesicles, antibiotic, lipopeptide). During the titrations, a total of up to 100 μL of the competing solution was added in 10 μL aliquots to 500 μL of the antibiotic lipopeptide solution. The ^1H NMR method used TSP in water (10 mM) as an external reference (in a glass insert in the NMR tube) for both chemical shift and relative peak integrals. The ^{19}F NMR method used TfOH in water as an external reference for chemical shift and relative peak integrals. The procedures for calculating K^v using the ^1H NMR and ^{19}F NMR methods have previously been published.[10,15]

Determination of K_{sol}. A solution of antibiotic (5 mM, 1.5 mL) in 9:1 H_2O:D_2O was prepared and adjusted to pH 4.5 with HCl and NaOH. This solution (600 μL) was added to an NMR tube and a 1-D ^1H NMR spectrum was recorded. A part of the remaining antibiotic solution (600 μL) was used to dissolve an amount of ligand [*N*-acetyl-Gly-Ala-D-γ-Glu-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate (analogue **1**)] such that when 400 μL of the ligand solution was added to the NMR tube, the antibiotic would be >90% bound by ligand based on an estimated binding constant for the association. The pH of the ligand solution was readjusted to 4.5 with NaOH and HCl. Aliquots of the ligand solution were added to the NMR tube (rising from 5 μL initially, to 100 μL at the end of the titration) and the change in chemical shift of the antibiotic proton ω_2[20] was recorded after each addition. The association constant was determined by performing a least-squares curve fit on a plot of change in chemical shift of ω_2 vs ligand concentration using the commercial software package *Kaleidagraph* version 3.1.2 (Abelbeck Software). The value obtained (2000 ± 200 M^{-1}) lies within a factor of 2 of the values obtained for other -D-Lac terminating ligands, both at ph 4.5 and pH 7.[9,15]

Synthesis of Precursor for Solid-Phase Synthesis. *N*-α-Fmoc-D-glutamic acid-γ-*tert*-butyl ester-α-allyl ester. *N*-α-Fmoc-D-glutamic acid-γ-*tert*-butyl ester (0.5 g, 1.16 mmol), HOBt (0.2 g, 1.47 mmol), and PyBOP (0.77 g, 1.47 mmol) were dissolved in allyl alcohol (10 mL). After stirring for 15 min, DIEA (0.2 g, 1.47 mmol) was added and the solution stirred for 15 h. Excess allyl alcohol was removed under reduced pressure and the crude mixture chromatographed over silica (chloroform) to afford pure *N*-α-Fmoc-D-glutamic acid-γ-*tert*-butyl ester-α-allyl ester (520 mg, 96%) as a white solid. The product gave the expected spectra by FT-ICR electrospray mass spectrometry ($C_{27}H_{32}O_6N$, $[M + H]^+$ found: 466.19792, calculated: 466.2224). ^1H NMR (500 MHz; $CDCl_3$, 300K) δ 1.44 (9H, s, 3 × CH_3), 1.95−1.99 (1H, m, Glu-β), 2.12−2.20 (1H, m, Glu-β), 2.41−2.50 (2H, m, 2 × Glu-γ), 4.20 (1H, t, *J* 7.2 Hz, Fmoc CH), 4.35−4.42 (1H, m, Glu-α), 4.48 (2H, d, *J* 7.2 Hz, Fmoc CH_2), 4.64 (2H, d, *J* 7.0 Hz, CH_2), 5.25 (1H, d, *J* 9.0 Hz, CH_{cis}), 5.38 (1H, d, *J* 14.0 Hz, CH_{trans}), 5.40 (1H, d, *J* 7.2 Hz, Glu NH), 5.93−5.99 (1H, m, CH), 7.35 (2H, t, *J* 6.1 Hz Fmoc), 7.40 (2H, t, *J* 6.1 Hz, Fmoc), 7.61 (2H, d, *J* 6.1 Hz, Fmoc), 7.79 (2H, d, *J* 6.1 Hz, Fmoc).

***N*-α-Fmoc-D-glutamic acid-α-allyl Ester.** *N*-α-Fmoc-D-glutamic acid-γ-*tert*-butyl ester-α-allyl ester (520 mg, 1.12 mmol) was dissolved in dichloromethane (10 mL), and triflouroacetic acid (10 mL) added. The mixture was stirred for 4 h, and the solvent was evaporated under reduced pressure. The crude mixture was chromatographed over silica (9:1 chloroform:methanol) to afford pure *N*-α-Fmoc-D-glutamic acid-α-allyl ester as a white solid (410 mg, 89%). The final product gave the expected spectra by FT-ICR electrospray mass spectrometry ($C_{23}H_{23}O_6NNa$, $[M + Na]^+$ found: 432.1395, calculated: 432.1418). ^1H NMR (500 MHz; $CDCl_3$, 300K) δ 1.95−1.99 (1H, m, Glu-β), 2.12−2.20 (1H, m, Glu-β), 2.41−2.50 (2H, m, 2 × Glu-γ), 4.20 (1H, t, *J* 7.2 Hz, Fmoc), 4.35−4.42 (1H, m, Glu-α), 4.48 (2H, d, 7.2 Hz, Fmoc), 4.64 (2H, d, *J* 7.0 Hz, CH_2), 5.25 (1H, d, *J* 9.0 Hz, CH_{cis}), 5.38 (1H, d, *J* 14.0 Hz, CH_{trans}), 5.40 (1H, d, *J* 7.2 Hz, Glu NH), 5.93−5.99 (1H, m, CH), 7.35 (2H, t, *J* 6.1 Hz, Fmoc), 7.40 (2H, t, *J* 6.1 Hz, Fmoc), 7.61 (2H, d, *J* 6.1 Hz, Fmoc), 7.79 (2H, d, *J* 6.1 Hz, Fmoc).

Solid-Phase Synthesis of *N*-acetyl-Gly-Ala-D-γ-Glu-Lys(*N*-ϵ-acetyl)-D-Ala-D-lactate. Solid-phase synthesis was carried out on 2-chlorotrityl chloride resin (Nova-Biochem).

D-Lactate-2-chlorotrityl Chloride Resin. Lithium D-Lactate (0.38 g, 3.9 mmol) was dissolved in a mixture of dimethyl sulfoxide/dichloromethane (1:1 v/v, 10 mL) and added to the 2-chlorotrityl chloride resin (1.3 mmol, 1 g resin), and the resulting mixture was agitated overnight (15 h, under N_2) and then washed successively with dimethysulfoxide (15 mL × 6), dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6) to afford D-Lactate-P.

D-Alanyl-D-lactate-P. *N*-α-Fmoc-D-alanine (1.214 g, 3.9 mmol) was dissolved in dimethylformamide (6 mL) and then added to the resin, and the resulting mixture was cooled to 0 °C. DIC (492 mg, 3.9 mmol) and 4-(dimethylamino)pyridine (24 mg, 0.20 mmol) were dissolved in dimethylformamide (4 mL) and added dropwise to the resin mixture over 30 min (0 °C, under N_2), and the mixture was agitated (under N_2) at room temperature for 6 h. The resin was washed successfully with dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6), and dried under vacuum for 2 h. Two samples of the resin complex (1 mg, 1 mmol of original resin loading) were placed in solutions of 20% piperidine in dimethylformamide (3 mL) at 290 nm. The average absorbance of 0.9 gave an estimated loading of 0.9 mmol of D-lactate/

gram resin. To remove the terminal Fmoc group, a solution of 20% piperidine in dimethylformamide (7 mL) was added to the resin, and N_2 was bubbled for 15 min. This procedure was repeated twice. The resin was washed with dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6). A Kaiser test confirmed complete deprotection to afford D-alanyl-D-lactate-P.

Lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. *N*-α-Fmoc-*N*-ϵ-acetyl-lysine (1.60 g, 3.90 mmol), PyBOP (2.03 g, 3.90 mmol), and HOBt (0.60 g, 3.90 mmol) were dissolved in dimethylformamide (6 mL), added to the resin mixture, and agitated for 10 min before DIEA (1.00 g, 7.8 mmol) was added. The resulting mixture was agitated for 6 h (under N_2) and then washed with dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6). A negative Kaiser test confirmed the reaction had gone to completion to afford Fmoc-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. The Fmoc group was removed as above to afford lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.

D-γ-Glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. *N*-α-Fmoc-D-glutamic acid-α-allyl ester (1.59 g, 3.90 mmol), PyBOP (2.03 g, 3.90 mmol), and HOBt (0.60 g, 3.90 mmol) were dissolved in dimethylformamide (6 mL), added to the resin mixture, and agitated for 10 min before DIEA (1.00 g, 7.80 mmol) was added. The resulting mixture was agitated for 6 h (under N_2) and then washed with dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6). A negative Kaiser test confirmed the reaction had gone to completion to afford Fmoc-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. The Fmoc group was removed as above to afford D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.

Alanyl-D-γ-Glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. *N*-α-Fmoc-alanine (1.21 g, 3.90 mmol), PyBOP (2.03 g, 3.90 mmol), and HOBt (0.60 g, 3.90 mmol) were dissolved in dimethylformamide (6 mL), added to the resin mixture, and agitated for 10 min before adding DIEA (1.00 g, 7.80 mmol). The resulting mixture was agitated for 6 h (under N_2) and then washed with dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6). A negative Kaiser test confirmed the reaction had gone to completion to afford Fmoc-alanyl-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. The Fmoc group was removed as above to afford alanyl-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.

Glycyl-alanyl-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. *N*-α-Fmoc-glycine (1.16 g, 3.60 mmol), PyBOP (2.03 g, 3.90 mmol), and HOBt (0.60 g, 3.90 mmol) were dissolved in dimethylformamide (6 mL), added to the resin mixture, and agitated for 10 min before DIEA (1.00 g, 7.80 mmol) was added. The resulting mixture was agitated for 6 h (under N_2) and then washed with dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6). A negative Kaiser test confirmed the reaction had gone to completion to afford Fmoc-glycyl-alanyl-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P. The Fmoc group was removed as above to afford glycyl-alanyl-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.

***N*-Acetyl-glycyl-alanyl-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.** Acetic anhydride (4.328 g, 42.39 mmol) and dichloromethane (6 mL) were added to the resin mixture and agitated for 10 min before DIEA (2.96 g, 22.96 mmol) was added. The resulting mixture was agitated for 6 h (under N_2) and then washed with dimethylformamide (15 mL × 6) and dichloromethane (15 mL × 6). A negative Kaiser test confirmed the reaction had gone to completion to afford *N*-acetyl-glycyl-alanyl-D-γ-glutamyl(α-allyl)-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.

***N*-Acetyl-glycyl-alanyl-D-γ-glutamyl-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.** The resin was swollen in dimethyl sulfoxide (6 mL) and tetrakis(triphenylphosphine) palladium (0) (0.35 g, 0.30 mmol) dissolved in 1:1 tetrahydrofuran:dimethyl sulfoxide (5 mL) was added. The mixture was agitated under N_2 for 30 min, HOBt (0.08 g, 0.50 mmol) was added, and the resulting mixture was agitated for 6 h. The resin was washed thorougly with tetrahydrofuran (15 mL × 10) and dichloromethane (15 mL × 6) or until the washings were no longer colored. The procedure was repeated to afford *N*-acetyl-glycyl-alanyl-D-γ-glutamyl-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.

***N*-Acetyl-glycyl-alanyl-D-γ-glutamyl-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P.** A solution of trifluoroacetic acid:tri-isopropyl silane:H_2O (38:1:1, 10 mL) was added to the resin complex and the mixture agitated for 1 h, under N_2. The resulting solution was collected and the resin washed with trifluoroacetic acid (2 mL × 3) and dichloromethane (30 mL × 5), all collected. The combined solvents were removed under pressure to give a yellow oil. The residue was dissolved in water and lyophilized to give an off-white powder. The crude product was purified by reverse phase HPLC and then lyophilized to afford *N*-acetyl-glycyl-alanyl-D-γ-glutamyl-lysyl(*N*-ϵ-acetyl)-D-alanyl-D-lactate-P as a white solid (227 mg, 40% yield based on the initial loading of D-lactate on resin). The final product gave the expected spectra by FT-ICR electrospray mass spectrometry ($C_{26}H_{42}O_{12}N_6$, $[M + H]^+$ found: 631.2925, calculated: 631.2933). ^{1}H NMR (500 MHz; DMSO-d_6, 300 K) δ 1.24 (3H, *J* 7.1 Hz, Ala CH_3), 1.27 (2H, m, 2 × Lys-γ), 1.33 (3H, d, *J* 7.1 Hz, Lac CH_3), 1.37 (2H, m, 2 × Lys-δ), 1.42 (3H, d, *J* 7.1 Hz, Ala CH_3), 1.50 (1H, m, Lys-β), 1.61 (1H, m, Glu-β), 1.79 (3H, s, acetyl CH_3), 1.87 (3H, s, acetyl CH_3), 1.99 (1H, m, Glu-β), 2.19 (2H, m, 2 × Glu-γ), 2.99 (2H, app q, 2 × Lys-ϵ), 3.72 (2H, d, *J* 5.5 Hz, 2 × Gly-α), 4.17 (1H, m, Glu-α), 4.25 (1H, m, Lys-α), 4.32 (2H, m, 2 × Ala-α), 4.95 (1H, q, *J* 7.3 Hz, Lac-α), 7.77 (1H, m, Lys-ϵ NH), 7.88 (1H, d, *J* 7.7 Hz, Lys-α NH), 8.04 (1H, d, *J* 7.3 Hz, Ala NH), 8.10−8.15 (2H, m, gly NH, glu NH), 8.31 (1H, d, Ala NH), 12.51 (1H, s, Lac OH).

Acknowledgment. Eli Lilly and Co. are thanked for generously providing a sample of chloroeremomycin. The EPSRC and Unilever (D.P.O.), the BBSRC and Roussel (R.M.H.E.), Eli Lilly and Co. (S.W.O.), and the BBSRC and the George Murray Foundation (M.A.C.) are thanked for financial support. We thank Mike Gradwell and Tom Frenkiel of the NMR Laboratory, NIMR, Mill Hill, London, for helpful discussions and access to NMR equipment.

JA9839769