Glycopeptide Antibiotic Activity and the Possible Role of Dimerization: A Model for Biological Signaling

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Abstract: It is demonstrated that the presence of bacterial cell wall analogues may either enhance or, in the case of ristocetin A, oppose dimerization of glycopeptide antibiotics. These observations may imply that dimerization plays a role in the mode of action of these antibiotics, and a mechanism is proposed to take account of this possibility. The glycopeptide dimers are also found to be formed more exothermically in the presence of cell wall analogues, and the nature of biological signaling events is discussed in this context. It is pointed out that binding enthalpy (rather than simply binding free energy, ΔG) may be an important quantity in signaling events. If this is so, then oligomers may be abundant in signaling processes partly because the extended aggregates they form are able to cooperatively amplify the conformational changes which are incurred on ligand binding, which occur through relatively small changes in free energy but larger opposing changes in enthalpy and entropy.

Introduction

Research into the molecular basis for the mode of action of the vancomycin glycopeptide antibiotics[1-3] has been stimulated by the growing clinical importance of vancomycin and teicoplanin for the treatment of Gram-positive bacterial infections, particularly those which are multiply drug resistant.[4] More than 100 variants of the antibiotics are known to be produced by actinomycetes, all sharing a very similar extended heptapeptide backbone. The side chains are extensively cross-linked, and the resulting rigid framework forms a concave pocket along the peptide backbone (Figure 1). The immature cell wall of Gram-positive bacteria carries a terminal -L-Lys-D-Ala-D-Ala sequence which binds noncovalently into this pocket. Cell wall mimics such as di-*N*-acetyl-L-Lys-D-Ala-D-Ala (DALAA, which has a binding affinity for vancomycin of 10^6 M^{-1}) have been used extensively to characterize the molecular nature of the interaction with the antibiotics, most notably vancomycin and ristocetin A (Figure 2).[1-3] Recently, this system has been used to develop an approach toward the factorization of free energy contributions to binding in terms of defined "costs" and "benefits".[5,6] The basis of such an approach has depended on the relative rigidity of the antibiotics, which ensures a well-defined receptor conformation.[7]

In 1971, during the course of early ligand binding studies, Nieto and Perkins[3] observed aggregation of vancomycin in aqueous solution. Although the structure of vancomycin was not then known, a dimerization constant of 800 M^{-1} was calculated from circular dichroism measurements, on the assumption that the aggregate was a dimer. The structure of the aggregated glycopeptides remained uncharacterized for 18 years. NMR experiments then showed that the DALAA complexes of ristocetin A and ristocetin pseudoaglycon (ristocetin-Ψ, Figure 2) form hydrogen bonded dimers.[8a] A similar structure was proposed for the dimer of eremomycin.[8b] The hydrogen bond interactions are formed between the "back" faces of two antibiotic molecules,

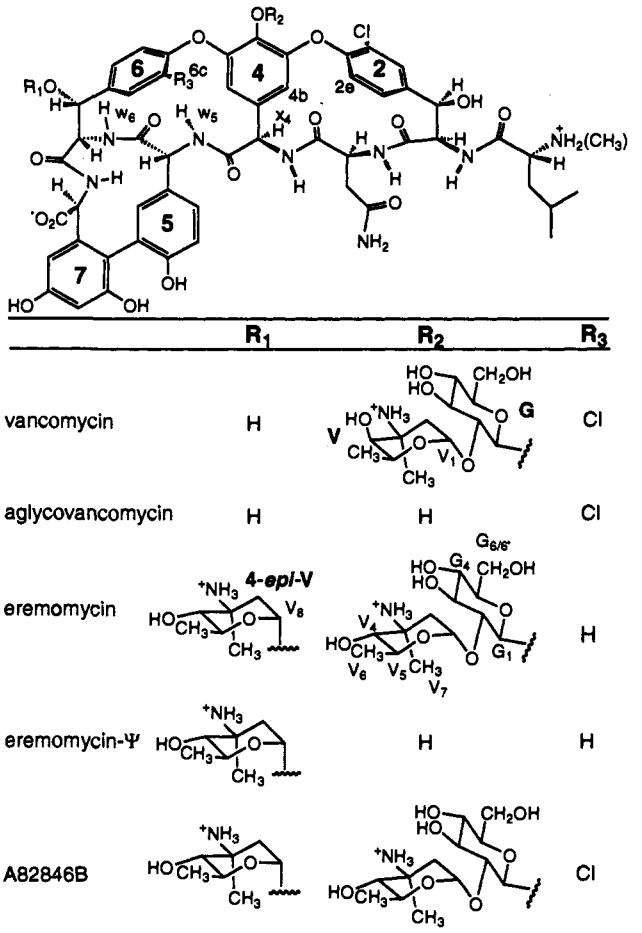

Figure 1. The structures of vancomycin, aglycovancomycin, eremomycin, eremomycin-Ψ, and A82846B (V = vancosamine; 4-*epi*-V = 4-*epi*-vancosamine; G = glucose). Protons which are referred to in the text are labeled.

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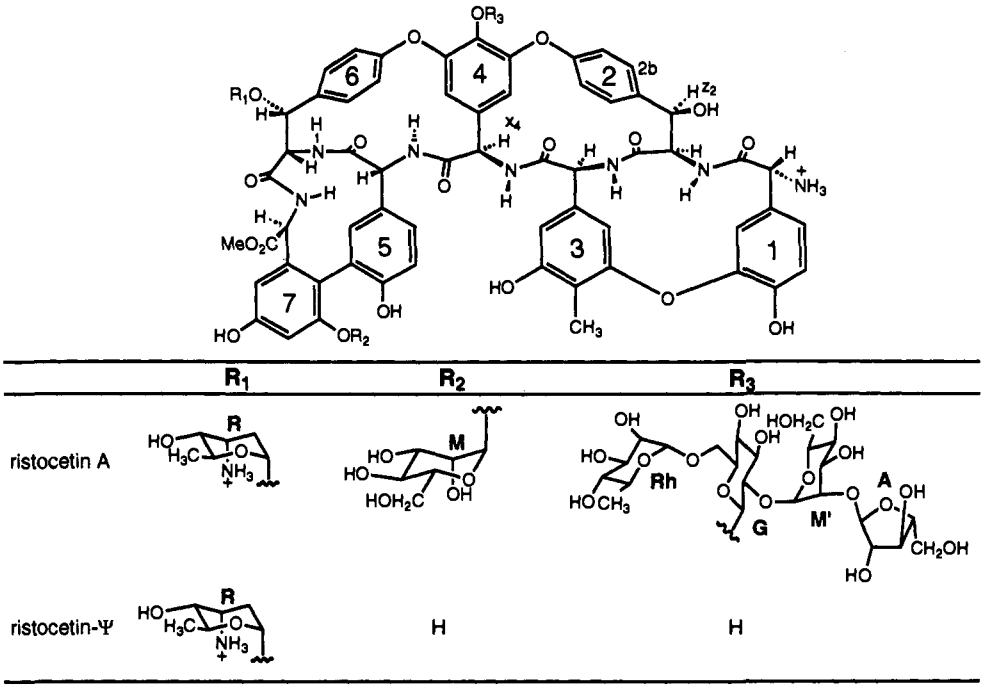

Figure 2. The structures of ristocetin A and ristocetin-Ψ (G = glucose; M = M′ = mannose; Rh = rhamnose; A = arabinose; R = ristosamine).

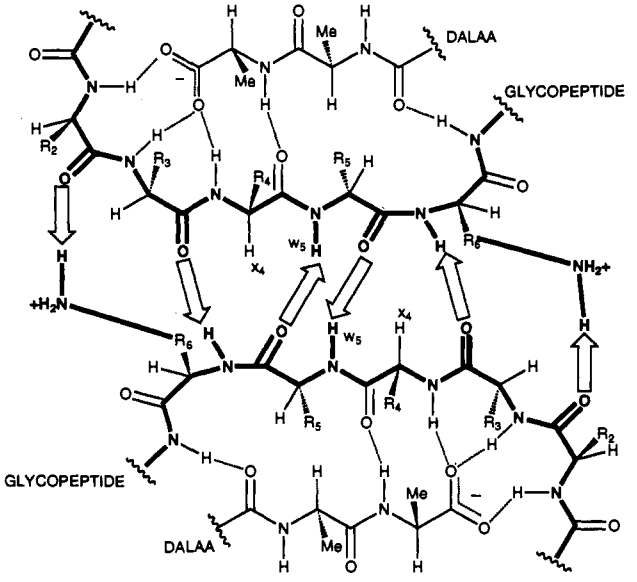

Figure 3. The hydrogen bonding network of the dimer formed between two molecules of ristocetin A when bound to DALAA. The arrows represent hydrogen bonds formed between the two ristocetin A molecules, and the broken lines indicate hydrogen bonds made to the ligand.

that is, the faces not directly involved with ligand binding (Figure 3). A substantial amount of hydrophobic surface area is also buried on dimerization (*e.g.*, *ca.* 290 Å^2 for dimerization of deglycosylated vancomycin).[6] Subsequently, dimerization has been reported and quantified for a number of other glycopeptide antibiotics with dimerization constants of up to 10^6 M^{-1}.[9,10]

It appears that molecular engineering of considerable sophistication is required to ensure complementarity of the two halves of the dimer, and it has been shown in the previous paper in this issue[10] and previously[9] that antibiotic activity appears to be determined by more than simply the affinity of these glycopeptides for cell wall precursor analogues such as DALAA. In the present study, it is demonstrated that dimerization and cell wall analogue binding are thermodynamically linked processes; formation of the dimer influences ligand affinity. In all cases studied but one, dimerization is shown to enhance the binding of ligand. These findings may suggest that dimerization plays a physiological role, and a model for the mode of action of the glycopeptide antibiotics which takes into account formation of homodimers is presented. This model rationalizes several unusual results concerning the *in vitro* activity of a recently isolated member of the group, eremomycin.[11] The possible implications of these results for the mechanism of biological information transmission (*e.g.*, transmembrane signaling and gene expression) are also discussed.

Materials and Methods

Eremomycin and vancomycin were donated by SmithKline Beecham and Eli Lilly, respectively, as their hydrochloride salts. Eremomycin-Ψ and A82846B were donated by SmithKline Beecham and Eli Lilly, respectively, as their acetate salts. Ristocetin A was donated by both Abbott Laboratories and Lundbeck as the sulfate salt. These compounds were all used without further purification. Ristocetin-Ψ was prepared as described previously.[9]

NMR Spectroscopy. Samples for ^{1}H NMR were prepared as solutions in deuterated phosphate buffer as described previously.[10] All pH and pD sample readings were measured with a Corning pH meter 125 equipped with a combination glass electrode. The pD readings quoted throughout are pH meter readings, and no corrections have been made for isotope effects or for the variation of pH with temperature.

NMR spectra were recorded on Bruker AM 400, AM 500, and AMX 500 spectrometers. One-dimensional spectra were recorded using either 8K or 16K data points. Chemical shifts were measured with respect to internal dioxan (δ = 3.74 ppm). For variable-temperature NMR work, the temperature of the probe was calibrated using samples of either methanol or ethylene glycol, using the method of van Geet.[12]

Two-dimensional ROESY spectra were recorded in phase-sensitive mode, using time proportional phase incrementation (TPPI)[13] to give quadrature detection in f_1. Routinely, 2048 data points were recorded in f_2 and 512 in f_1, with either 16 or 32 transients collected at each value of t_1. Mixing times of between 10 and 300 ms were used, with a continuous 5-kHz spin locking pulse. NOESY and DQFCOSY spectra were recorded as described previously.[10]

The dimerization constants of glycopeptide–cell wall analogue complexes were measured using either of the methods described previously.[10] Where indicated, the change in the chemical shift of the proton x_4 (see

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Figures 1 and 2) was monitored over a range of concentrations of the complex, and a simplex least-squares curve-fitting program was used to derive dimerization constants.[14] Values for ΔH_{dim} and ΔS_{dim} were calculated by determining K_{dim} over a range of temperatures and then constructing van't Hoff plots; the ligand concentrations were such that the glycopeptide remained >95% complexed at the temperatures studied.

Samples at pD 3.7 for hydrogen-deuterium exchange measurements for derivation of dimerization constants were prepared by first adjusting the pD of a solution of KD_2PO_4 (50 mM) to *ca.* pD 3 using solutions of NaOD and DCl. Following addition of the glycopeptide, the pD as adjusted to pD 3.7. For the determination of the amide proton exchange rate of w_5 of eremomycin, MM47761, and A82846B in the presence of DALAA, the ligand was first dissolved in D_2O containing KD_2PO_4 (50 mM), and this solution was added to the solid glycopeptide. The final concentrations of antibiotic and ligand were 10 and 11 mM, respectively, corresponding to at least 98% complex formation.

The spectra recorded for the observation of amide proton exchange used a Jump and Return selective excitation sequence[15] to ensure no attenuation of the amide proton resonances took place through transfer of magnetization during suppression of the residual HOD signal. Samples were equilibrated for *ca.* 10 min at the temperature of the probe (298 K) before the acquisition of the first spectrum. Typically, spectra were acquired with spectral widths of 10 ppm and 16 K data points. Spectra were recorded in *ca.* 1 min (24 transients). The delay between successive transients was 1.5 s and that between successive spectra was 5–120 min. Data sets were multiplied by an exponential broadening function of 2 Hz prior to Fourier transformation.

The decrease in intensity of the NMR signal with time for w_5 was monitored. In each case, the entire experiment took place in the NMR spectrometer probe and typically 25–40 spectra were collected in sequence at suitable intervals such that, where possible, spectra were recorded over at least 3 half-lives of decay. The loss in amide proton intensity during the acquisition of the spectra was assumed to be linear as the time taken to record each spectrum was considerably shorter than the half-life for amide proton exchange. The intensities of the resonances, measured as peak heights from the spectra relative to a nonexchangeable signal, were plotted *vs* time, and exponential functions were fitted to the resulting data sets. The time increments between successive data points were taken as the interval between the middle of acquisition of each spectrum.

UV Spectrophotometry. Affinity constants for the association of glycopeptides with DALAA were determined by UV difference spectrophotometry using the method described previously,[10] and binding constants were extracted by fitting the data to a simple 1:1 binding isotherm using a nonlinear least-squares curve fitting routine.[14]

Results and Discussion

The Interplay between Ligand Binding and Dimerization. It has been proposed that dimerization may play a part in the mode of action of vancomycin antibiotics.[8-10] Indeed the sophistication of the interactions within the dimer and the expenditure of metabolic energy presumably involved in engineering these apparently precise interactions are consistent with such a conclusion. For example, a function of the modified ring 6 amino sugar of both eremomycin and A82846B (Figure 1) appears to be to promote dimerization; the presence of this sugar does not favor ligand binding.[10] Initially, it was proposed that the binding of a single ligand to one-half of an antibiotic dimer may enhance the binding of a second ligand to the other half of the dimer. Such positive cooperativity is frequently observed in enzymatic systems where the enzyme comprises two or more polypeptide chains which are linked noncovalently and/or by disulfide bonds.[16,17] For example, the binding of molecular oxygen to subunits of the tetrameric enzyme hemoglobin induces conformational changes in the other three domains which successively increases their affinity for binding of O_2.[18] In such systems, binding isotherms are generally sigmoidal in shape[19] or at least deviate from the rectangular hyperbolic form characteristic of simple noncooperative 1:1 associations. Binding data for the addition of DALAA to both of the strongly dimerizing antibiotics eremomycin (Figure 4) and A82846B were well fitted by a simple 1:1 model, and there is consequently no evidence for cooperative binding to the glycopeptide dimer. This is perhaps surprising, given the physical model proposed below for the involvement of the dimer. It is possible that a small effect could be masked by the limitations of the method; the total absorbance change for the experiment is rather small.

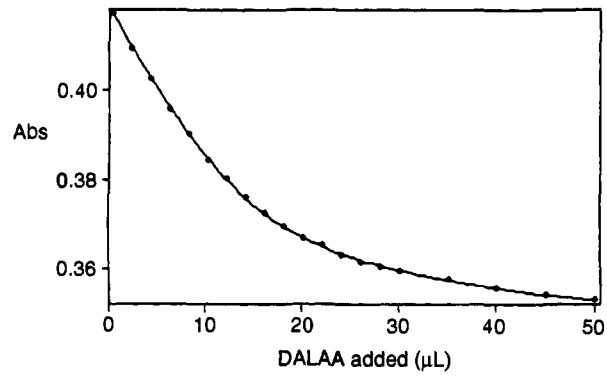

Figure 4. UV binding isotherm for the addition of DALAA to eremomycin. The curve shown is a nonlinear least-squares curve fit of the data based on a simple noncooperative model of binding. The experimental conditions are given in the Materials and Methods section.

Despite this, a variety of data concerning the binding of cell wall analogues to vancomycin group antibiotics and their derivatives has been published which indicates that the binding affinity of cell wall analogues for monomeric antibiotic is not the sole determinant of antibiotic activity.[9] For instance, eremomycin has been found to be more active *in vitro* than vancomycin[11,20] by a factor of 2–5; this was quite unexpected as vancomycin has the larger binding constant to the cell wall analogue DALAA by a factor of 23.[11] Further, it was demonstrated[11] that the addition of DALAA to *in vitro* tests of eremomycin activity actually *increased* the activity of the antibiotic for low concentrations of DALAA. This also was unexpected since the DALAA presumably competes for the same glycopeptide binding sites as the bacterial cell wall target. Indeed, no such potentiation was observed in a similar experiment using vancomycin; vancomycin activity decreased monotonically with increasing DALAA concentration.[11] Similarly, A82846B is around five times more active than vancomycin, despite sharing the same affinity for DALAA. Both eremomycin and A82846B dimerize more strongly than vancomycin.

In order to probe the possible role of dimerization in these findings, it was proposed to determine whether or not the formation of the dimer induced any change in ligand binding. However, attempts to measure the ligand binding constant for vancomycin to DALAA at concentrations higher than *ca.* 0.2 mM (corresponding to around 18% dimer) resulted, in agreement with previous findings,[3] in sigmoidal binding curves which complicated the analysis. Ligand binding studies conducted at lower glycopeptide concentrations give straightforward 1:1 binding isotherms (see, for example, ref 3). These results are an indication that the ligand binding properties of the dimer do differ from those of the monomeric glycopeptide. Expressions could be derived which take this into account, but the large number of variables involved may compromise their usefulness in curve fitting procedures. Thus, the converse experiments were considered; that is, what is the effect of ligand binding on glycopeptide dimerization? The dimerization constants of eremomycin-Ψ, ristocetin-Ψ, and vancomycin were consequently determined in the presence of the ligand acetyl-D-Ala (ADA) (Table 1). The change in the chemical shift of the antibiotic proton χ_4 with concentration was monitored.[9] A large excess of ADA was added such that ≥95% of the antibiotic

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Table 1. Dimerization Constants for a Number of Glycopeptides in the Presence and Absence of Ligands and the Effect on ΔG_{lig}

glycopeptide	ligand	K_{dim}[a] (M^{-1})	K_{ligD}/K_{lig}[b]
ristocetin-Ψ		50	
ristocetin-Ψ	ADA	300	2
ristocetin-Ψ	ADADA	240	2
ristocetin-Ψ	DALAA	700	4
vancomycin		700	
vancomycin	ADA	1300	1.4
eremomycin-Ψ		2.2×10^3 [c]	
eremomycin-Ψ	ADA	1.3×10^4 [d]	2
eremomycin		3×10^6	
eremomycin	DALAA	3×10^8	10
MM47761		5.0×10^6	
MM47761	DALAA	2.0×10^8	6
A82846B		1.8×10^5	
A82846B	DALAA	1.1×10^7	8

[a] Measured at 298 K unless otherwise indicated. [b] K_{lig} represents the binding constant between ligand and monomeric glycopeptide; K_{ligD} is the binding constant between the corresponding dimer and the same ligand. Note that this analysis assumes that the binding constant of the second equivalent of ligand to the dimer is the same as that of the first equivalent of ligand. [b] Dimerization constant at 352 K. [c] Determined at 352 K due to large line widths at lower temperatures.

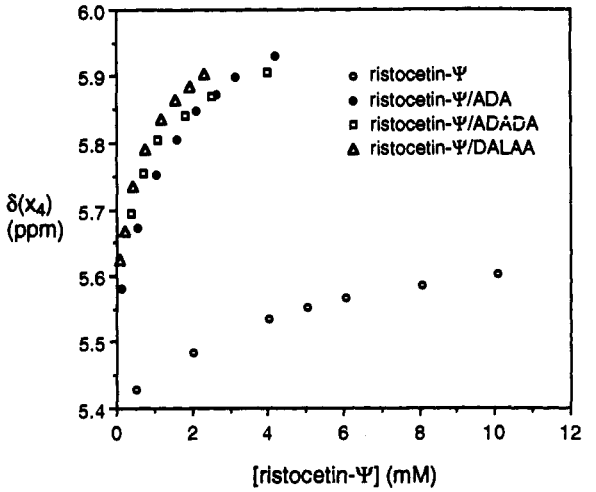

Figure 5. Plots of the chemical shift of the proton x_4 (δ, ppm) against the concentration of ristocetin-Ψ (mM) in the presence of the ligands ADA, ADADA, and DALAA, and in the absence of any ligand.

was bound to ligand at all temperatures studied. The dimerization constant K_{dim} was also determined for ristocetin-Ψ both in the presence of N-acetyl-D-Ala-D-Ala (ADADA) and in the presence of DALAA (Figure 5).

In the case of more strongly dimerizing antibiotics, the ^1H NMR amide NH exchange method described in the preceding paper in this issue[10] was used to measure K_{dim} in the presence of DALAA. The proton w_5 in the ligand-bound antibiotic dimers was assigned using a combination of ROESY and DQFCOSY experiments. The dimerization constants thus derived for eremomycin, MM47761, and A82846B in the presence of DALAA are also given in Table 1.

It can be seen that the presence of a ligand gives rise to an increase in K_{dim} for *all* of the glycopeptides listed in Table 1. A consideration of the relative free energies of monomeric and dimeric antibiotic and of these species bound to ligand reveals that if the binding of a ligand to monomeric glycopeptide enhances dimerization, then the formation of a non-ligand-bound dimer *must* favor the binding of ligand; the two processes are thermodynamically linked (Figure 6). Using Figure 6, the observed increases in K_{dim} may be converted into effects on K_{lig} (the ligand binding constant). Note that this approach cannot address possible free energy differences between the binding of the first and the second ligand to the dimer; only the total free energy change for binding both ligands can be inferred. However, the UV data discussed above for the binding of DALAA to strongly

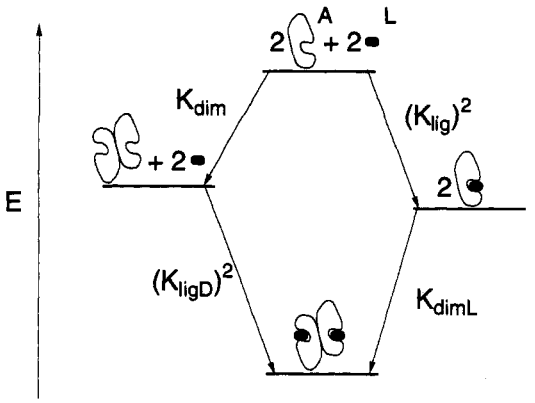

Figure 6. Representation of the relationship between equilibrium constants for dimerization in the absence and the presence of ligand. A = antibiotic; L = ligand; K_{dim} = equilibrium constant for dimerization; K_{lig} = equilibrium constant for ligand binding to monomeric antibiotic; K_{dimL} = equilibrium constant for dimerization of two antibiotics bound to ligand; K_{ligD} = equilibrium constant for binding a ligand to dimeric antibiotic. It can be seen that if $K_{dimL} > K_{dim}$ then K_{ligD} must be greater than K_{lig}. Note that these quantities could equally well be the corresponding ΔH or ΔS values.

dimerizing glycopeptides suggests that the two binding constants are equal or very similar, and they are consequently assumed to be equal throughout the remainder of this analysis. The ratios of K_{ligD}/K_{lig} (the ligand binding constant to dimer and monomer respectively) are given in Table 1. They show that these ligands bind more strongly to the dimeric glycopeptides than to the corresponding monomers. Thus there exists a positive cooperativity between glycopeptide dimerization and ligand binding. We next address possible molecular details and biological consequences of this cooperativity.

Mechanism and Biological Consequences of Dimerization/Ligand Binding Cooperativity. We consider two effects which may lead to cooperativity between ligand binding and dimerization. First, dimer formation appears likely to impart motional restriction to the glycopeptide. When the amide functional groups of the glycopeptide backbone are hydrogen bonded to water in the monomer, they will probably have somewhat more motional freedom than when hydrogen bonded to each other as in the dimer. This ordering of the amide functional groups should promote ligand binding since the magnitude of hydrogen bond fluctuations at the ligand–glycopeptide interface will be reduced (the electrostatic energy of a hydrogen bond is critically dependent on the average length of the bond). Second, the ring 6 amino sugar, where present, is proposed to form an ammonium ion-amide carbonyl hydrogen bond in the dimer to residue 2 of the complementary subunit (Figure 3). This may polarize the NH proton of this residue 2–3 amide linkage so that it forms a stronger hydrogen bond to the ligand carboxylate ion in a complex. Looked at in another way, in the ligand bound dimer, the alkylammonium ion of the amino sugar on residue 6 of the antibiotic is able to form an indirect salt bridge to the carboxylate anion of the bacterial cell wall analogue (mediated through the polarizable amide bond which connects residues 2 and 3 of the antibiotic—Figure 3).

Thus, it has been shown that ligands such as DALAA bind more strongly to glycopeptide dimers than to the corresponding monomers, providing evidence that the dimer may be implicated in the biological mode of action of the glycopeptide antibiotics. However, this evidence alone does not explain the greater activity of eremomycin compared to vancomycin nor the potentiation of eremomycin activity following the addition of DALAA.[11] The binding constants to DALAA of eremomycin[11] (3.9×10^4 M^{-1}) and vancomycin[21] (1.5×10^6 M^{-1}) were measured at concentrations of 0.14 and 0.025 mM, respectively. At these concentrations, eremomycin is 97% dimerized, while vancomycin is less than 5%

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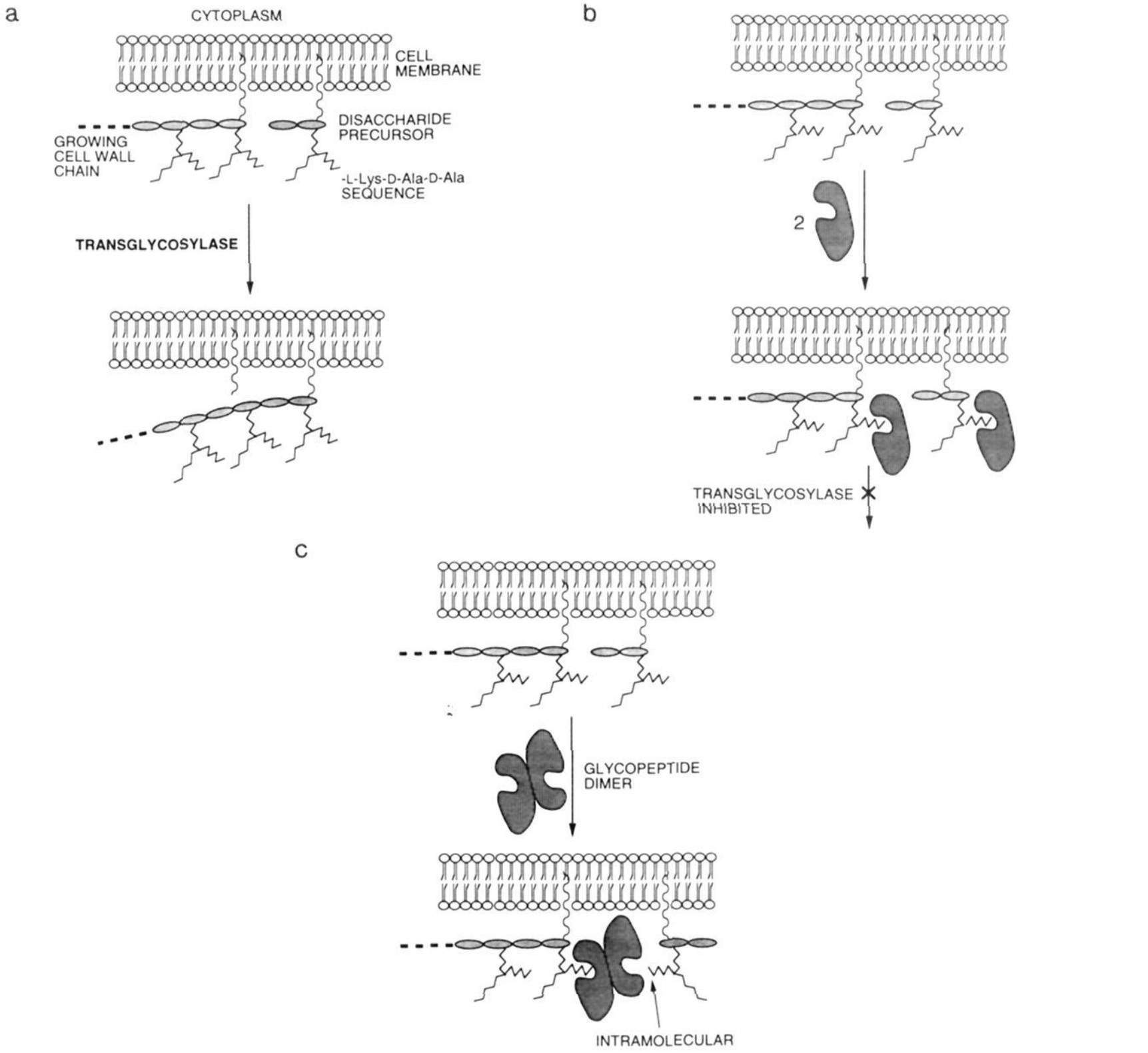

Figure 7. (a) Representation of the transglycosylation stage of bacterial cell wall biosynthesis. A disaccharide precursor which bears a peptide side chain terminating in -L-Lys-D-Ala-D-Ala is exported from the cytoplasm attached to a C_{55} carrier lipid and then incorporated into the growing polysaccharide chain by a transglycosylase enzyme. This is the first stage of cell wall synthesis at which glycopeptides are thought to act. (b) One of the previously accepted mechanisms for glycopeptide action. The antibiotic binds to the -L-Lys-D-Ala-D-Ala sequence and prevents the approach of the transglycosylase, thus preventing further growth of the cell wall polysaccharide. (c) Proposed mechanism incorporating the glycopeptide dimer. If the antibiotic binds as a dimer, then the second binding (such -L-Lys-D-Ala-D-Ala sequences are likely to be concentrated locally at this site) is effectively intramolecular, and a chelate-type enhancement of binding may be seen.

dimer. That is to say, although the dimer of a particular glycopeptide binds ligand more strongly than the corresponding monomer, the vancomycin *monomer* still binds to DALAA significantly better than does the eremomycin dimer. Therefore the cause of the enhanced activity of eremomycin relative to vancomycin cannot be probed using these simple abiotic experiments with cell wall precursor analogues such as DALAA. The solution must lie in a consideration of the biological site of action of the glycopeptides.

The cytoplasmic membrane of Gram-positive bacteria is encapsulated by a peptidoglycan cell wall comprising polysaccharide chains cross-linked by oligopeptides. Vancomycin antibiotics are of sufficiently low molecular weight to be able to diffuse through the peptidoglycan layer[22] and thus gain access to the outer surface of the cytoplasmic lipid membrane, which is the site of construction of polymeric peptidoglycan cell wall[23] (vancomycin cannot pass through the cytoplasmic membrane[24]). Figure 7a illustrates the transglycosylation step of peptidoglycan synthesis; a cell wall precusor disaccharide carrying a peptide which terminates in -L-Lys-D-Ala-D-Ala is transported from the cytoplasm attached to a C_{55} carrier lipid. This precursor is then transferred to the growing polysaccharide by a transglycosylase. This transglycosylation step is inhibited by vancomycin as the antibiotic binds to the -L-Lys-D-Ala-D-Ala sequence and prevents the approach of the transglycosylase[25] (Figure 7b). This

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mechanism is consistent with the observed buildup of cytoplasmic disaccharide precursor.[26]

If instead the glycopeptide approached the transglycosylation site as a dimer, binding of the second equivalent of antibiotic to nascent cell wall would then be effectively intramolecular (Figure 7c).[9] This has the consequence that little or no translational or rotational entropy (ΔS_{T+R}) need be lost in this second association, and a significant enhancement of binding should result (intramolecular reactions can be accelerated by up to 10^9 relative to their intermolecular counterparts[27]). The overall equilibrium constant for the binding of the dimer should thus be significantly greater than the product of the equilibrium constants for binding two monomers to the same sites. This, the chelate effect, constitutes a mechanism through which a strongly dimerizing antibiotic such as eremomycin (K_{dim} for eremomycin is over 4×10^3 times larger than that for vancomycin) could be more active *in vitro* than suggested by its UV-determined binding constant to cell wall precursor analogues. In the UV binding experiments, where the two peptide ligands are discrete entities, two quantities of ΔS_{T+R} must be lost for both ligands to associate, and the chelate effect outlined above cannot operate.

The chelate effect has been shown to operate in many situations where molecular recognition is important. Immunoglobulins are often active as dimers; each half of the dimer comprises two polypeptide chains and carries one antigen binding site.[28] Thus, when the antibody recognizes the surface of a target cell or virus, it can bind to the cell in two places. This strengthens the interaction between the antibody and the target cell significantly through the chelate effect as outlined above. Gene expression in both prokaryotes and eukaryotes is controlled by DNA-binding proteins which are commonly dimeric. Repressors, such as the prokaryotic phage 434,[29] *trp*,[30] and λ repressors[31] are noncovalent homodimers in which the two constituent polypeptide chains bear both a dimerization domain and a DNA-binding domain. The two DNA binding domains of the dimer recognize symmetry related DNA sequences, and the resulting protein–DNA complex is again stabilized by the chelate effect. In eukaryotes, dimerization among transcription factors has become a recurring theme,[32] exemplified by the yeast protein GAL4,[33] which carries two zinc-containing DNA-binding motifs in the dimer. In the case of the transcription factor hepatocyte nuclear factor-1α (HNF-1α), a polypeptide cofactor has also been characterized[32] which binds to the HNF-1α protein and stabilizes dimeric HNF-1α. The functional dimeric protein binds to DNA, controlling gene expression. This is analogous to the glycopeptide/cell wall peptide system of the present work, where the binding of DALAA to a glycopeptide promotes glycopeptide dimerization.

The above mechanism for the mode of action of the glycopeptides can also explain the potential of eremomycin activity *in vitro* following the addition of DALAA. The binding of DALAA to eremomycin has been shown above to increase the dimerization constant by a factor of 100. Thus, a greater proportion of dimer will exist in the presence of DALAA, and delivery of dimer to the site of action of the antibiotic will be facilitated. So long as the concentration of DALAA is not too high, subsequent debinding of the exogenous DALAA will alow the preferred association (where the binding of the second -LAA residue is intramolecular) described above. In this fashion, the activity of eremomycin may be increased by small amounts of DALAA.

Why is a similar effect not observed for vancomycin, which also forms a dimer? First, K_{dim} for vancomycin is much smaller and the cooperative increase in dimerization with ligand binding is also much smaller (a factor of 2 for the ligand ADA), so that the change in dimer population on the addition of DALAA would be less significant than for eremomycin. Second, vancomycin binds DALAA much more tightly than does eremomycin and both binding sites of the dimer would become saturated at much lower concentrations of DALAA. In fact, the potentiation experiment has recently been repeated using a more sensitive assay (D.A.B., unpublished results), and a small potentiation of vancomycin activity was observed. This model for the participation of glycopeptide dimers in the mode of action of the antibiotics is therefore consistent with the available data correlating ligand binding affinities, dimerization potentials, and *in vitro* antibiotic activities. Differences in dimerization potential have been invoked previously to explain trends in *in vitro* activity.[9]

The Residue 6 Amino Sugar. It is notable that the magnitude of the ligand binding/dimerization cooperativity is smallest in the case of vancomycin (Table 1) and that this is the only compound listed in Table 1 which lacks a residue 6 amino sugar. It has been calculated that this sugar promotes dimerization by a factor of 250[10] and suggested that the sugar plays little direct role in ligand binding (other than that exerted through dimerization). In fact, it can be shown that this sugar actually opposes ligand binding in the absence of dimerization. The UV binding constant between A82846B and DALAA is 1.0×10^6 M^{-1}, measured at [A82846B] = 0.11 mM.[10] This corresponds to 85% dimer (K_{dim}(A82846B) = 1.8×10^5 M^{-1}). The binding of DALAA to the A82846B dimer is 8 times more favorable than binding it to the monomer (Table 1), and therefore the affinity of DALAA for the A82846B monomer is probably closer to $(1–2) \times 10^5$ M^{-1} (that is, $1 \times 10^6/8$). This is around a factor of 10 lower than the corresponding affinity constant for vancomycin monomer + DALAA (1×10^6 M^{-1} at <5% dimer). It is difficult to pinpoint the precise molecular nature of this negative effect on ligand binding; however the salient point is that overall this amino sugar promotes the *in vitro* antibacterial activity of A82846B relative to vancomycin by a factor of 5.[20] This serves to emphasize the considerable subtlety displayed by such systems which appear to have evolved under the pressures of natural selection.[34]

Teicoplanin. One member of the glycopeptide group of antibiotics, teicoplanin[35–37] (Figure 8a), does not appear to form a dimeric structure of the type described in this work (by comparison of ^{1}H NMR spectra of teicoplanin with those of vancomycin, eremomycin, and ristocetin A; data not shown). However, teicoplanin bears a C_{10} alkyl side chain attached to the residue 4 sugar substituent, and this chain may itself oppose dimerization. It also seems possible, or even probable, that this hydrophobic chain would interact with the lipid-containing cell membrane of the target bacterium, thereby anchoring the glycopeptide antibiotic. Thus, the interaction of the peptide portion of the antibiotic with nascent cell wall peptide would be effectively intramolecular (Figure 8b) and hence more favorable. This is somewhat analogous to the mechanism proposed above for the involvement of dimerization in antibiotic action and is consistent with the hypothesis that the mode of action of these antibiotics may consist of more than simply the binding of D-Ala-D-Ala terminating peptides to monomeric glycopeptide molecules in solution. It is striking that in precisely the case that dimerization is not observed (teicoplanin), a structural variant (the C_{10} alkyl chain) is present which allows in principle a similar functional intramolecular event.

This hypothesis is supported by recent findings that teicoplanin

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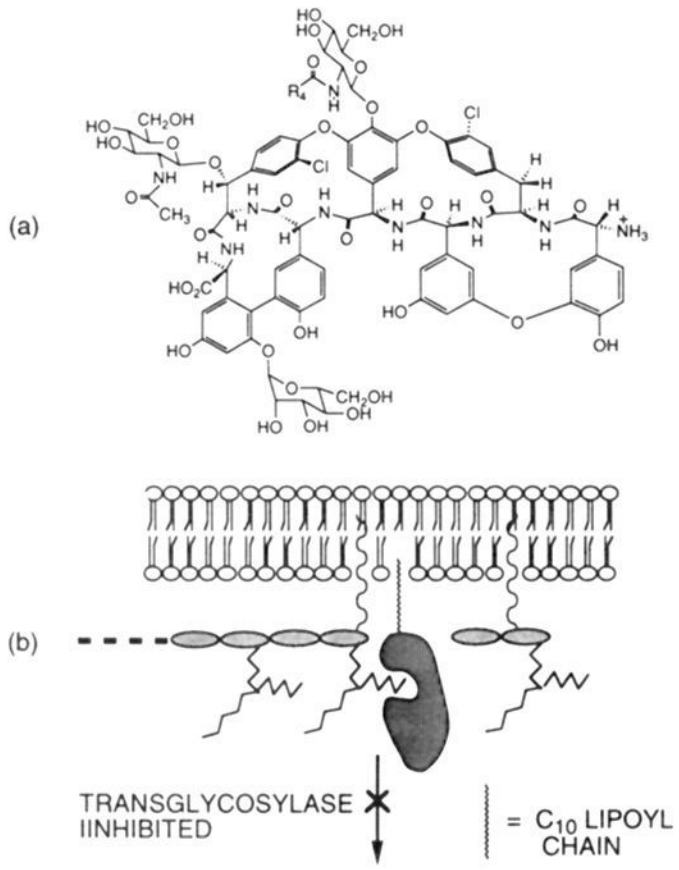

Figure 8. (a) The structure of teicoplanin. R_4 = the C_{10} lipoyl side chain. (b) Putative model for the involvement of the hydrophobic side chain of teicoplanin in its mode of action. The lipoyl chain may interact with the lipid bilayer of the cell membrane, and thus the binding of the peptide portion of the antibiotic to nascent peptidoglycan would again be effectively intramolecular.

is active against bacterial strains possessing low-level (vanB phenotype) vancomycin resistance.[38] This resistance arises from the substitution of approximately 90% of the -D-Ala-D-Ala terminating peptidoglycan precursors for -D-Ala-lactate sequences. The affinity of glycopeptides for such modified peptides is much diminished,[39] which alone should significantly reduce their activity. In the case of vancomycin, however, a further factor may act to decrease its activity. If 90% of the -D-Ala-D-Ala containing precursors are transformed into -D-Ala-lactate, then the chance of *two* of the remaining -D-Ala-D-Ala peptides being adjacent to each other is reduced by a factor of 100. Thus the intramolecular advantage of dimerization presented above would be largely negated. However, teicoplanin in the analogous case does not require two adjacent precursors to display an intramolecular rate acceleration as proposed above and could therefore retain antibiotic activity, as long as some -D-Ala-D-Ala containing precursors were present. Further, semisynthetic derivatives of vancomycin, in which the amino group of the residue 4 disaccharide has been acylated or alkylated with C_8–C_{10} lipids,[40,41] were found to possess excellent activity against vancomycin-resistant enterococci.[42] Interaction of the modified vancomycin with the cytoplasmic membrane, analogous to that proposed above for teicoplanin, may result in the enhanced activity.

Anticooperativity in Ristocetin A. Ristocetin A (Figure 2) also forms a dimer in aqueous solution of the type discussed above.[8] The ristocetin A dimer exists in two conformers which, in the ^{1}H NMR spectrum, are in slow exchange on the chemical shift time scale; the two forms of the dimer are also in slow exchange with the monomer.[8] The largest chemical shift differences between the two dimeric conformers are found in the tetrasaccharide of residue 4 with smaller changes in the aglycon protons adjacent to the tetrasaccharide. This situation is similar to that of eremomycin, where the two conformers have been shown to arise,

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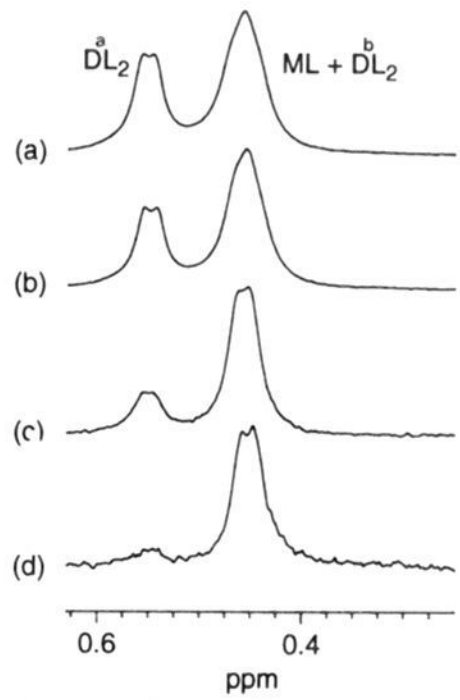

Figure 9. Upfield region of the 1D ^{1}H NMR spectra of ristocetin A bound to DALAA, showing the effect on the proportions of monomer (ML) and the signals due to two forms of dimer (D^aL_2 and D^bL_2) on dilution of the mixture. The concentrations of ristocetin A are (a) 9.5, (b) 2.4, (c) 0.48, and (d) 0.12 mM. The ratio of ristocetin A to DALAA is *ca.* 1:1.1 in all cases.

at least in part, from 180° rotations of the residue 4 disaccharides.[10] The same explanation may apply to ristocetin A, but this has not been proven. Because the ristocetin A dimer is in slow exchange with the monomer on the chemical shift time scale, K_{dim} cannot be measured *via* the chemical shift titration method which has been used for other members of the group (see above). A value for K_{dim} of *ca.* 500 M^{-1} has been estimated from integration of the monomer and dimer signals of one of the tetrasaccharide protons.[43]

The effect of added ligand on ristocetin A dimerization was probed by examining the ^{1}H NMR spectrum of ristocetin A in both the absence and presence of DALAA. The C-terminal alanine methyl (A_CMe) group of ligands such as DALAA experiences a very characteristic chemical shift change on binding to vancomycin group antibiotics; it shifts upfield from 1.42 ppm (for the free ligand) to around 0.5 ppm (bound). In the spectrum of the ristocetin A/DALAA complex (Figure 9a; 9.5 mM in each component), two clearly distinct signals are seen in this region, at 0.46 and 0.55 ppm, corresponding to DALAA bound to monomeric and to dimeric ristocetin A. Note however that the signal at 0.46 ppm does not have the doublet multiplicity expected for an alanine methyl group but instead comprises two overlapping signals (the likely cause of this is discussed below). Assignment of these two signals was made from DQFCOSY and ROESY experiments; the two signals share a chemical exchange cross peak in the ROESY spectrum, indicating that they arise from the same proton(s) in two different environments. ROEs and scalar couplings were used to confirm that the two signals both arise from the A_CMe group.

The solution was then diluted in steps to 0.12 mM (Figure 9b–d). Dilution decreases the proportion of dimer relative to monomer, and in accord with this one of the two bound methyl group signals clearly decreases in intensity relative to the other. At 0.12 mM there is essentially a single signal, at 0.46 ppm, which corresponds to the methyl group bound to monomeric ristocetin A (Figure 9d). This monomer-bound ligand signals is now clearly a doublet. This change suggests that the resonance overlapping with this at higher concentrations corresponds to a second dimer-bound ligand signal. This appears probable as the glycopeptide dimers have been shown to exist in two forms,[8,9] and the same A_CMe group in the eremomycin-Ψ/DALAA complex exhibits two signals (Figure 10). Unfortunately, overlap of the C-terminal alanine $C^\alpha H$ resonances for the monomer- and dimer-bound ligand prevents unambiguous identification of the over-

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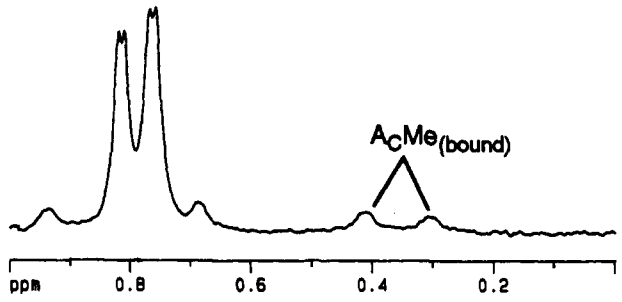

Figure 10. Partial ^{1}H NMR spectrum of eremoycin-Ψ bound to DALAA, illustrating the two signals for the bound C-terminal alanine methyl group of DALAA ($A_CMe_{(bound)}$).

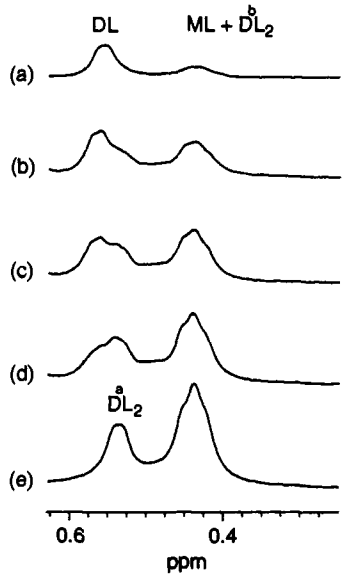

Figure 11. Series of 1D ^{1}H NMR spectra following the addition of DALAA to a solution of ristocetin A (10.3 mM): (a) 0.24 equiv of DALAA, (b) 0.48 equiv of DALAA; (c) 0.60 equiv of DALAA, (d) 0.72 equiv of DALAA, and (e) 1.0 equiv of DALAA. DL = dimeric ristocetin A with one binding site occupied by DALAA; D^aL_2 and D^bL_2 = the two dimeric forms of ristocetin A with both binding sites occupied by DALAA; ML = monomer bound to DALAA. Spectra were recorded using a Bruker AMX 500 at a probe temperature of 298 K.

lapped signal in 2D spectra of the ristocetin A/DALAA complex. Assuming that the monomer-bound resonance masks a second dimer signal of similar intensity to that at 0.55 ppm, a dimerization constant for the complex of *ca.* 350 M^{-1} was calculated (*cf.* 500 M^{-1} for ristocetin A alone). It is also of note that one of the two dimer forms binds to DALAA more strongly than the other. DALAA was titrated into a ristocetin A solution, and ^{1}H NMR spectra were recorded (Figure 11a–e). Initially, only one of the two possible DL (that is, dimer bound to one ligand) species is seen, indicating that the affinities of the two dimer forms for DALAA differs.

Thus, the presence of the cell wall analogue DALAA opposes ristocetin A dimerization. A similar conclusion has recently been reached using isothermal microtitration calorimetry.[44] The data indicate that the free energy of dimerization of the monomer–DALAA complex is less negative than for the antibiotic monomer is the absence of ligand. This result was unexpected, as it contrasts directly with the data obtained above for the other members of the vancomycin group, and could be taken to invalidate the model proposed above for the biological role of dimerization. However, it should be noted that the pseudoaglycon of ristocetin A (ristocetin-Ψ, Figure 2), which exhibits *positive* cooperativity (see above), is a factor of 10 *more* active than ristocetin A *in vitro* for a range of bacterial strains,[45] despite binding DALAA *ca.* 10 times less well than ristocetin A itself.[46] Thus, the lower antibacterial activity exhibited by ristocetin A is consistent with

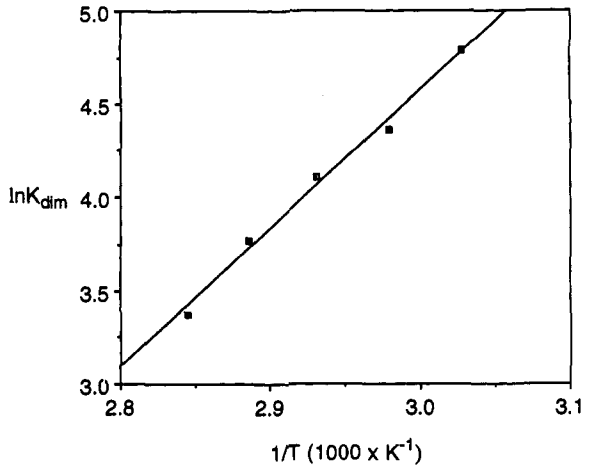

Figure 12. Van't Hoff plot (ln K_{dim} *vs* $1/T$) for the dimerization of vancomycin in the presence of the ligand ADA. The concentration of ADA was maintained such that the vancomycin was ≥95% bound at all concentrations and temperatures.

its anticooperativity, and this is in accord with the proposed model of positive cooperativity between dimerization and ligand binding being influential in the mode of action of the glycopeptides. The role of the tetrasaccharide, however, remains unclear, since although it promotes dimerization in the free antibiotic (K_{dim} = 500 M^{-1} for ristocetin A, K_{dim} = 50 M^{-1} for ristocetin-Ψ), its presence triggers a transition from cooperative ligand binding/dimerization (for ristocetin-Ψ) to anticooperative behavior (ristocetin A).

Enthalpy–Entropy Compensation in Ligand Binding. In an attempt to quantify further the cooperativity effect, the dimerization of vancomycin in the presence of ADA was measured at a number of temperatures in the range 330–350 K, and the data were used to construct a van't Hoff plot (Figure 12). The derived values for the enthalpy and entropy of dimerization were

$$\Delta H_{dim} = -62 \text{ kJ mol}^{-1}$$

$$T\Delta S_{dim} = -44 \text{ kJ mol}^{-1} \text{ (at 298 K)}$$

These are substantially larger than the corresponding values for the dimerization of vancomycin in the absence of ligand (ΔH_{dim} = −36 kJ mol^{-1}, $T\Delta S_{dim}$ = −20 kJ mol^{-1} at 298 K).[9] However, there is only a small net increase in ΔG_{dim} for dimerization (2 kJ mol^{-1}; a factor of 2 in K_{dim}) despite the large increase in electrostatic binding energy ($\Delta\Delta H$ = −26 kJ mol^{-1}), as the entropic cost of forming the tighter association counterbalances the more favorable enthalpy term (enthalpy–entropy compensation).[5] Recent isothermal microtitration calorimetric data[44] concludes that, although the increase in ΔG_{dim} for vancomycin accords well with that measured by ^{1}H NMR, the change in ΔH_{dim} is considerably smaller ($\Delta\Delta H_{dim}$ = −3 kJ mol^{-1}). A recent study of DNA triplex melting[47] yielded differences in values for melting enthalpies when determined by either differential scanning calorimetry (DSC) or van't Hoff plots of variable temperature UV spectrophotometric data compared to values determined by isothermal calorimetry. Similar analyses of duplex melting showed good agreement between the different methods.[47]

These results emphasize that when dealing with multisubunit associations (that is, complexes which are not simply 1:1, as in a triplex or a ligand-bound glycopeptide dimer), there can be significant discrepancies between enthalpies determined by

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different methods. These discrepancies may arise because of a fundamental difference in the nature of the techniques—in both DSC and van't Hoff analyses the enthalpy is obtained by varying the temperature of the sample, while in isothermal calorimetry the temperature is necessarily held constant through the course of the experiment. Thus, in the first two techniques, altering the temperature not only will alter the equilibrium for the process under investigation (*i.e.*, dimerization in the vancomycin–ADA system and melting of a triplex to give a duplex and a single DNA strand in the other case) but also will affect the strength of the other association present (the vancomycin–ligand complex and the DNA duplex). Considering just the glycopeptide case, if ligand binding is exothermic, then raising the temperature will reduce the strength of the vancomycin–ADA interactions (resulting in a falling K_{lig}). The cooperative effect exerted by the binding of ADA on dimerization may therefore be reduced, and the dimerization constant would be seen to drop more sharply with temperature (a more negative ΔH_{dim}) than would be predicted from an enthalpy obtained isothermally. Consequently, the van't Hoff analysis of vancomycin–ADA dimerization may overestimate ΔH_{dim}, and the result obtained by isothermal microtitration calorimetry may reflect the parameter more accurately.

However, the isothermal calorimetric data still reveals an increase in the exothermicity of vancomycin dimerization in the presence of ligand and, as was detailed earlier (Table 1), ΔG_{dim} is significantly greater for a range of glycopeptides when bound to ligand. A thermodynamic linkage diagram similar to that depicted in Figure 6 can be constructed to relate the enthalpies of these processes. The increase in the magnitude of ΔH_{dim} for vancomycin in the presence of ADA can thus be converted to an effect on ΔH_{lig} when ligand is bound to a dimer rather than a monomer. Thus, ΔH_{ligD} (the enthalpy change for binding one ligand to a dimer) is 1.5 kJ mol^{-1} more exothermic than ΔH_{lig} for the binding of monomeric vancomycin to ADA. Although this is a small difference, it suggests that the extension of an aggregate (from two molecules to four in this case) may improve bonding throughout the whole aggregate, not just at the particular interface in question. In addition, for the dimerization of ristocetin-Ψ in the presence of cell wall analogues, a much larger limiting downfield chemical shift is observed for x_4 (Figure 5), consistent with a tighter complex than in the dimerization of ristocetin-Ψ alone.

Biological Signaling. The promotion of noncovalent associations observed above on shifting from a two molecule aggregate to a four molecule one (as expressed by more negative ΔG_{dim} for many glycopeptides in the presence of ligand) may be an important and general phenomenon. Processes in which the binding of one entity to another transmits some type of information through space (biological signaling) are ubiquitous in living organisms. The cooperative binding of ligands to an allosteric protein or enzyme (that is, one which comprises more than one polypeptide chain, each with a ligand binding site[48]) can be envisaged as a form of signaling. Binding of the first ligand induces a conformational change in the protein subunit to which it has bound. This change is transmitted (the signal) to a second subunit, which alters the affinity of that subunit for ligand (*e.g.*, hemoglobin[18]). In a similar fashion, the glycopeptide/cell wall analogue cooperativity reported herein can be likened to a signaling process. Thus, the formation of a glycopeptide dimer induces some change in the antibiotic (the signal) which causes the antibiotics listed in Table 1 to bind ligand more strongly. Associated with the increase in ligand binding affinity observed for vancomycin on dimerization is a small increase in the *exothermicity* of binding. This is indicative of a tighter complex, exhibiting improved electrostatic bonding.

Similarly, the structure of hemoglobin becomes more compact on oxygenation,[49] indicating better packing interactions in the oxygenated form. A recent ^{1}H NMR hydrogen/deuterium exchange study of an engineered insulin dimer (the insulin monomer consists of an α and a β chain, linked by two disulfide bonds, several salt links, numerous nonpolar interactions, and two hydrogen bonds) revealed that, on formation of the $(\alpha\beta)_2$ dimer, the two intramonomer (but *inter*chain) α-β hydrogen bonds were stabilized relative to monomeric insulin.[50] These hydrogen bonds are distant from the dimerization interface, so that their stabilization is indicative of an increase in the strength of interactions throughout the aggregate. It is notable that the hydrogen bonds *within* each chain were essentially unaffected by dimerization (as judged from hydrogen–deuterium exchange rates), consistent with a global tightening of the *inter*chain interactions in the dimer.

Thus, conformation tightening, which can be associated with transmission of information between subunits in extended aggregates, indicates that favorable free energy changes (and perhaps increased exothermicities) have occurred within the system. In simpler systems, it may be shown that the extension of an aggregate can result in a tightening of the interactions within the aggregate. For example, the dissociation into single strands (melting) of short duplexes (≤*ca.* 12 base pairs) of poly(A+U) RNA occurs over a broad temperature range (*ca.* 20 °C).[51] As the number of base pairs is increased, the melting transition sharpens substantially and, for very long duplexes, resembles a phase transition.[52] The extension of an aggregate (for example, the binding of a ligand to a dimeric receptor) is a common feature of signaling processes, possibly because induction of conformational tightening of intersubunit contacts promotes the process of signal transmission.

Transmembrane signaling has been the focus of much research recently,[53-56] but the precise mechanism by which information is transferred remains obscure. Transmembrane receptors may be classified according to the nature of the membrane-spanning region. One class forms ion channels, and a signal is transmitted through the passage of ions.[57] A second class contains seven transmembrane regions and is exemplified by the G-protein coupled receptors such as rhodopsin and the β-adrenergic receptor.[58] Finally, the third class contains either one or two highly hydrophobic transmembrane domains per polypeptide chain; the epidermal growth factor (EGF) receptor[59] and the bacterial aspartate receptor[60] fall into this category. The ion transport class is distinct from the other two as material passes through the membrane in the course of the signaling event, and we shall focus on the other two classes.

It has been noted that agonists (compounds which bind to a receptor and elicit in it a conformational response) to β-adrenergic receptors tend to bind with large negative enthalpies (indicating a relatively ordered complex), while antagonists, which bind but do not transmit a signal, display significantly less exothermic enthalpies of association.[61,62] Further, the exothermic agonist binding is accompanied by an unfavorable change in entropy,

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while the antagonists generally bind with an overall positive entropy change. This balance of ΔH and ΔS means that the differences in binding constant between the two types of ligand are often rather small, but are the result of widely differing thermodynamic contributions. Thus we see that the agonist–receptor complexes have relatively good electrostatic interactions and suffer more motional restriction than in the case of the complexes formed to the antagonists. It may be these properties of the agonists which allow the transduction of a conformational change in the receptor.[5] For example, one model which accommodates the experimental facts proposes two states, say A and B, of the receptor; where A is the resting state, and B the active conformation which is poorly populated. The agonist may bind well to the B state in a relatively exothermic process and therefore with a relatively unfavorable entropy change. In this model, the antagonist binds preferentially to the A state in a process in which electrostatic interactions in the complex are relatively little improved and therefore with relatively less ordering.

Members of the third class of receptor listed above frequently exist as noncovalent or disulfide-linked dimers. This class can be further divided according to whether a receptor monomer displays one or two transmembrane sequences. Those which induce activity in the protein tyrosine kinase (*e.g.*, the EGF receptor) carrying one transmembrane domain per polypeptide chain and appear to function exclusively as dimers.[53] In the case of the EGF receptor, it has been shown that dimerization is the rate-limiting step for tyrosine kinase activation following the addition of the ligand EGF.[63] The bacterial aspartate receptor bears two transmembrane regions per monomer and also forms a noncovalent dimer. The incorporation of engineered disulfide linkages between the subunits of the dimer has revealed that dimer association/dissociation is not necessary for signal transmission.[64] Further, the effective removal of both the cytoplasmic and the transmembrane domains of one subunit does not prevent signaling, suggesting that intersubunit interactions in either of those regions are not necessarily implicated in the signaling process.[65] However, an X-ray crystal structure of the dimeric extracellular domain has been carried out in both the presence and the absence of bound aspartate,[66] and significant differences between the two structures were found in the relative orientation of the two subunits (but not in the conformation of each individual subunit). The aspartate-bound dimer was also tighter and more compact than the ligand-free dimer. This finding, together with the data discussed above from a variety of systems, supports the hypothesis that signaling can be induced by an increase in the exothermicity of formation of an extended aggregate (relative to that for formation of its monomeric counterparts).[5]

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Conclusion

It has been shown that ligand binding and homodimerization of glycopeptide antibiotics is, in most cases examined, cooperative. It is remarkable that compounds of such modest molecular weight (*ca.* 1500–2000 g mol^{-1}) can exhibit **two** sophisticated recognition sites, one for the binding of nascent bacterial cell wall and the other for the formation of a specific homodimer. This cooperativity, together with the anomalous antibacterial activity of a number of members of the group (*e.g.*, eremomycin and A82846B), has led to a model for the possible involvement of glycopeptide dimers in the mechanism of action of these antibiotics. In this model, the dimer binds two adjacent molecules of cell wall precursor, and the resulting complex is stabilized by the chelate effect, thereby increasing the activity of the glycopeptide. The cooperativity between the two processes involved, which probably arises from subtle conformational changes or perhaps from the restriction of motion of the peptide backbone, reinforces the strength of the dimer–ligand complex. The only exception to cooperative behavior was that of ristocetin A, which bears a bulky tetrasaccharide substituent on residue 4, and dimerization is anticooperative in the presence of ligand. Removal of the tetrasaccharide to generate ristocetin-Ψ results in cooperative ligand binding/dimerization behavior, and this is consistent with the higher antibacterial activity of ristocetin-Ψ. The model was extended to include teicoplanin, a nondimerizing member of the group. The lipoyl side chain of teicoplanin may interact with the cell membrane, so that its binding to cell wall precursors is effectively intramolecular.

A number of examples have been given which suggest that more extended noncovalent aggregates may allow the formation of improved electrostatic interactions, and this idea has been related to a possible mechanism for at least some biological signaling whereby tighter (that is, more exothermic) binding may aid a conformational change. Indeed a small change in ΔG may, as seen for the β-adrenergic receptor complexes, be the balance of larger changes in ΔH and ΔS and may therefore result in quite different physical effects.

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