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Cooperative Binding Interactions of Glycopeptide Antibiotics

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Abstract: Glycopeptide antibiotics of the vancomycin group bind to bacterial cell wall analogue precursors, and typically also form dimers. We have studied the interplay between these two sets of noncovalent bonds formed at separate interfaces. Indole-2-carboxylic acid (L) forms a set of hydrogen bonds to the glycopeptide antibiotic chloroeremomycin (CE) that are analogous to those formed by *N*-Ac-D-Ala. The ligand/CE dimer interactions (in L/CE/CE/L) are shown to occur with positive cooperativity and structural tightening at the dimer interface. From theoretical considerations and from other data, it is inferred, but not proven, that in the exercise of positive cooperativity, the interface that will be tightened to the greatest degree is the one that lies in the shallowest free energy well.

Introduction

Noncovalent interactions are most frequently the basis for the multitude of recognition processes that occur in Nature. In the formation of such noncovalent interactions, cooperativity is a common feature. When noncovalent binding interactions occur with positive cooperativity, then the observed binding energy is greater when the interactions occur together than when they occur in isolation from each other. Conversely, when the interactions occur with negative cooperativity, then the observed binding energy is less when the interactions occur together than when they occur in isolation.

The occurrence of positive cooperativity among structures that are important in Nature causes changes in the dynamics of the structures. For example, in DNA duplexes it is observed that the structures are frayed at the ends of the duplex to a greater degree than they are in the middle. The reduced dynamics of the central base pairs of the DNA duplex are demonstrated by their slower NH exchange rates.[1] The dissociation constant of the second base pair of the 5′-di(CGCGATCGCG) self-complementary duplex is close to the square of the constant for the terminal pair; which is an indication of positively cooperative binding.[2] In a similar manner, amide proton exchange data demonstrate that the ends of an alanine-based peptide helix are frayed.[3] Positive cooperativity is also important in the formation of noncovalently bound aggregates of synthetic materials. For example, a series of conjugated zinc porphyrin oligomers, from the dimer through to the hexamer, form stable ladder complexes with linear bidentate ligands.[4] The proton NMR spectra of these ladders show how upon addition of excess ligand the terminals of the ladder structure fray, analogous to the DNA studies above. Ladder formation and dissociation exhibit many indications of positive cooperativity.[4] Collectively, these examples indicate that the center portions of such structures are formed with positive cooperativity relative to the ends.

In this paper, we explore by proton NMR spectroscopy the tightening of noncovalent interfaces formed by glycopeptide antibiotics due to positive cooperativity. We also consider the role, in structural tightening, of kinetic vs thermodynamic stabilities of noncovalent interfaces.

Glycopeptide antibiotics of the vancomycin group bind to bacterial cell wall analogue precursors, and typically also form dimers. Thus, two sets of noncovalent interactions can be simultaneously made and studied to see if they are made with positive or negative cooperativity. Indole-2-carboxylic acid (ligand, L) can form 4 hydrogen bonds to the glycopeptide antibiotics (Figure 1).[5] These hydrogen bonds are analogous to those formed by the bacterial cell wall precursor analogue *N*-Ac-D-Ala. As in the case of natural ligands, indole-2-carboxylic acid can bind into two faces of the dimer (Figure 2). Previous work has shown that when indole-2-carboxylic acid binds to ristocetin A, the binding of this ligand is negatively cooperative with respect to dimerization of the antibiotic.[5] We therefore decided to study the binding of indole-2-carboxylic acid to the antibiotic chloroeremomycin (CE, Figures 1 and 2), in the expectation that this binding would also occur with negativey cooperativity with respect to dimerization of the antibiotic. In this event, the binding is shown to be positively cooperative.

Results and Discussion

The proton NMR spectrum of 20 mM CE in the presence of 50 mM ligand at pH 6 in the region from 8 to 12 ppm is reproduced in Figure 3a. It is evident from this spectrum, in

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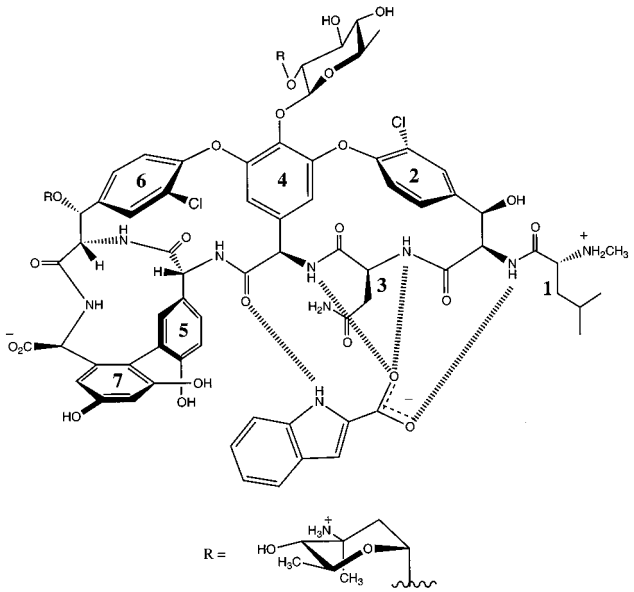

Figure 1. Chloroeremomycin monomer bound to indole-2-carboxylic acid.

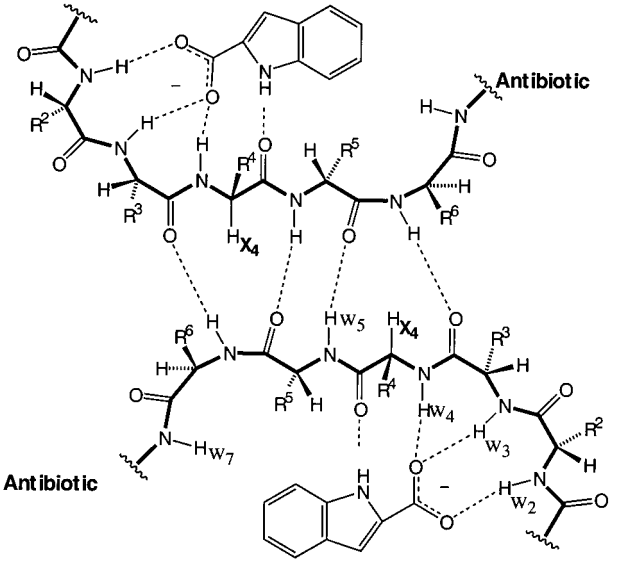

Figure 2. Representation of the 2:2 complex formed by the dimer of chloroermomycin and two molecules of indole-2-carboxylic acid. Hydrogen bonds are indicated by dashed lines and proton x_4 is labeled, as are the NH-protons w_2, w_3, w_4, w_5, and w_7 in one-half of the dimer.

conjunction with the spectra obtained at lower concentrations of ligand, that the signals due to the NH protons w_2,w_3,w_4,w_5, and w_7 occur in each case as two resonances. This is because the chloroeremomycin dimer is asymmetric.[6] The two halves of the dimer interface are nonequivalent, and the two environments (annotated as w and w* where assigned) are in slow exchange on the NMR time scale. As the concentration of the ligand is gradually lowered from that used to obtain Figure 3a, to give Figures 3b to 3e, the w_2 resonance initially moves toward higher field at a faster rate than does the w_2* resonance. This is because the two binding sites have different affinities, and the low affinity site initially becomes less occupied to a larger extent than the high affinity site as the dilution proceeds. The chemical shift change of the w_2 and w_2* resonances as a function

(6) Prowse, W. G.; Kline, A. D.; Skelton, M. A.; Loncharich, R. J. *Biochemistry* **1995**, *34*, 9632−9644.

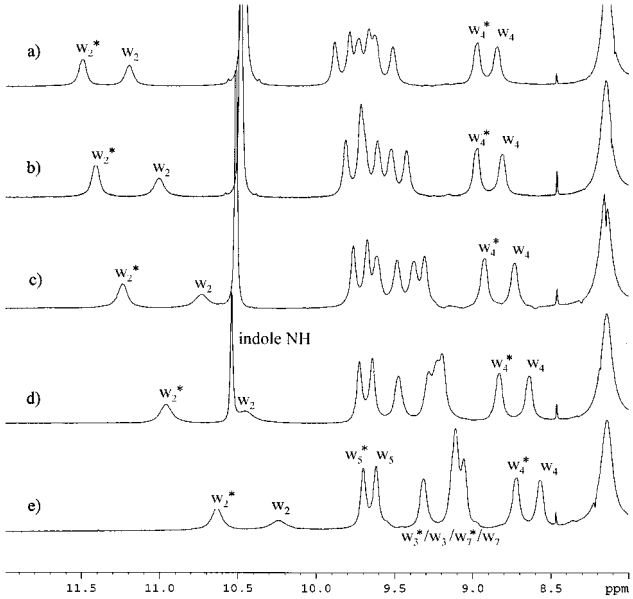

Figure 3. Amide region of the 1-D proton NMR spectra of chloroeremomycin (CE) plus ligand (L) at relative CE:L concentrations of (a) 20 mM:50 mM, (b) 20 mM:25 mM, (c) 20 mM:10 mM, (d) 20 mM:4 mM, and (e) 20 mM:0 mM in 9:1 H_2O:D_2O measured at pH 6. The resonances due to w_2, w_2*, w_4, and w_4* can be seen to move upfield as the ligand concentration decreases. The ligand NH is indicated, as is the more clustered region in part e, which contains the w_3, w_3*, w_5, w_5*, w_7, and w_7* resonances.

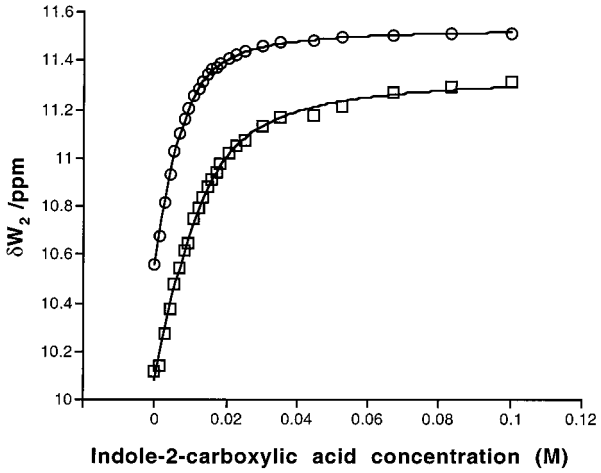

Figure 4. Plot of chemical shifts vs concentration of added indole-2-carboxylic acid for protons w_2* (circles) and w_2 (squares) of chloroeremomycin (20 mM) measured at pH 6. Binding constants are calculated by curve-fitting using Kaleidagraph 3.0 (Abelbeck software).

of ligand concentration can be used to obtain binding curves (Figure 4). Using curve-fitting methods, indole-2-carboxylic acid was found to bind into the higher affinity site with $K^*_{\text{lig/dimer}} = 600 \pm 150\ \text{M}^{-1}$, and into the lower affinity site with $K_{\text{lig/dimer}} = 100 \pm 25\ \text{M}^{-1}$.

The pairs of w and w* signals in the two halves of the ligand-bound dimer can only become equivalent through dissociation of the asymmetric antibiotic dimer (and its recombination in the alternative manner). Since these pairs of signals are not time-averaged, there must be a considerable barrier to dissociation of the ligand-bound dimer. From the coalescence temperature (285 K) of the x_4 pair of signals (Figure 5), this barrier is 62 ± 4 kJ mol^{-1}. In contrast, the two indole-2-carboxylic acid ligands can exchange between the two halves of the dimer at a rate that is fast on the NMR time scale. This is evidenced by the

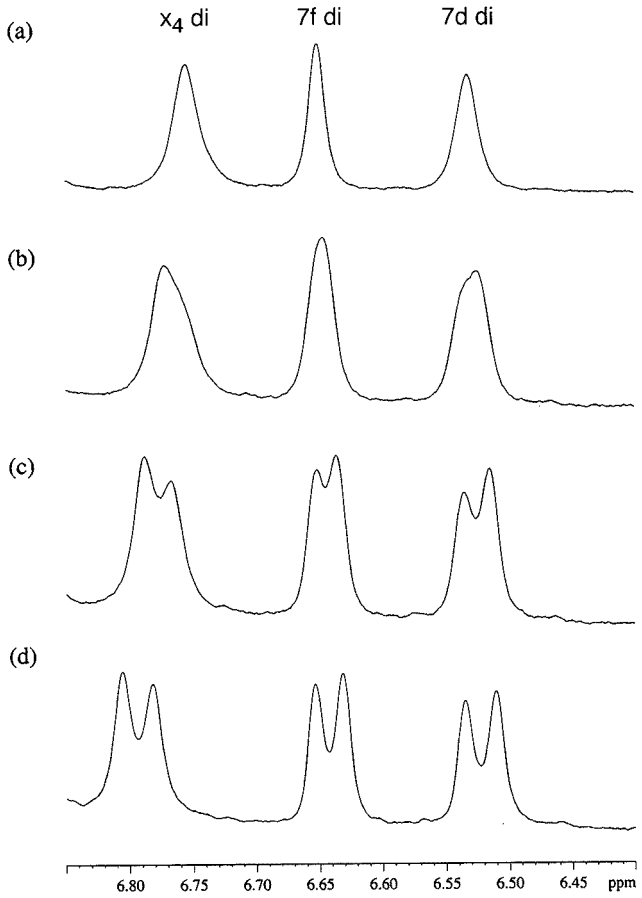

Figure 5. Splitting of the resonances due to the asymmetric dimer into two signals: 2 mM chloroeremomycin, 100 mM indole-2-carboxylic acid in D_2O at pD 6 at (a) 290, (b) 285, (c) 280, and (d) 275 K.

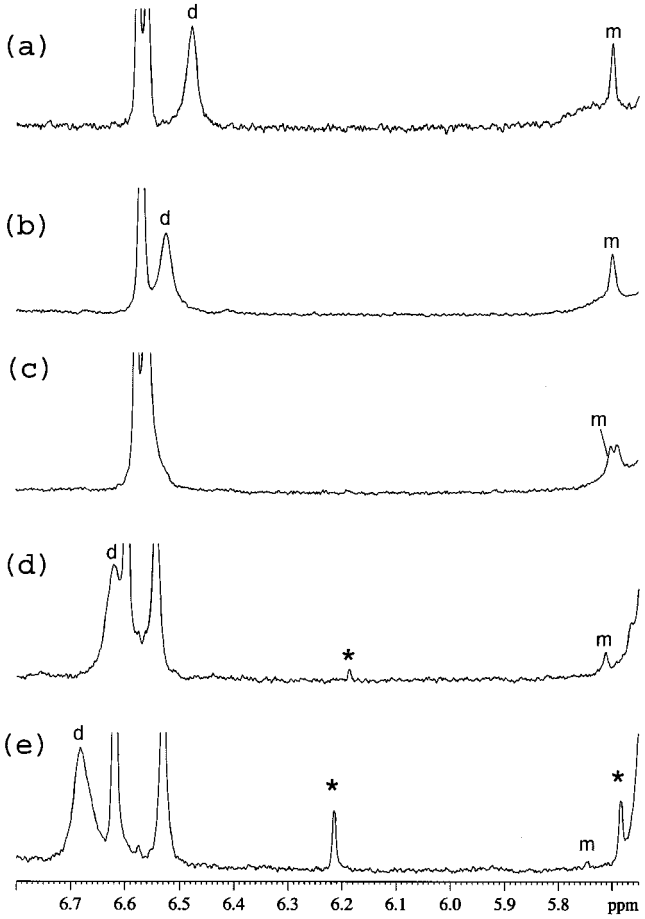

Figure 6. Relative populations of the x_4 monomer and dimer peaks as a function of indole-2-carboxylic acid concentration. Chloroeremomycin (0.25 mM) with (a) 0, (b) 0.2, (c) 0.5, (d) 2, and (e) 10 mM of indole-2-carboxylic acid in D_2O at pD 6 and 290 K. m: x_4 monomer, d: x_4 dimer, asterisked signals are derived from impurity of the indole-2-carboxylic acid. In part c, the signal for the x_4 dimer is under the two dimer signals for 7d and 7f. In parts a and b, the x_4 monomer signal was overlapped with another signal due to the monomer, from which it is separated in part c and subsequent parts of the titration.

fact that the signals due to ligand give only one (time-averaged) set of resonances (see, for example, the sharp indole NH resonance at 10.48 ppm in Figure 3a). This fast exchange of the ligand is confirmed by the behavior of the w and w* resonances. Since these resonances move gradually from the chemical shifts observed in free antibiotic to those observed in ligand-bound antibiotic (Figure 3), the chloroeremomycin dimer is in fast exchange on the NMR time scale with the chloroeremomycin dimer bound to indole-2-carboxylic acid. Thus, the depth of the free energy well for removal of either of the indole-2-carboxylic acid ligands from their respective binding sites is less than the depth of the free energy well for dissociation of the dimer interface of the ligand-bound dimer. Indeed, the difference in the relative depths of these wells is likely to be large since the dimerization constant is much larger than the ligand binding constants (see following sections).

The dimerization constant of chloroeremomycin in aqueous solution at pD 6 and 290 K, as determined in the present work from the relative intensities of several peaks due to monomer and due to dimer, is $(1.0 \pm 0.4) \times 10^5\ M^{-1}$. We have also measured this dimerization constant under the same conditions, in the presence of a high concentration (100 mM) of indole-2-carboxylic acid, i.e., of the complex shown in Figure 2. The measurement was made by direct observation of the relative intensities of the x_4 resonance in the ligand-bound dimer and in the ligand-bound monomer. The assignment of these signals was aided by noting that where the signals due to the two asymmetric halves of the dimer are separated by a relatively small chemical shift, they split from singlet to doublet signals on lowering the temperature (Figure 5). The x_4 resonance of the dimer was recognized through its NOESY cross-peak to the w_5 resonance of the dimer, and was then correlated to the x_4 resonance of the monomer by a transfer of saturation experiment.

Indole-2-carboxylic acid was titrated into a solution of the antibiotic at a sufficiently low concentration such that at the beginning of the titration, a population of the x_4 monomer signal could be observed (Figure 6). As the titration proceeds, the population of the x_4 monomer signal decreases (Figure 6), showing that ligand binding is positively cooperative with respect to dimerization. The relative populations of the antibiotic dimer and monomer signals at 100 mM concentration of indole-2-carboxylic acid and 0.05 mM antibiotic (spectrum obtained at 800 MHz to optimize sensitivity) give the dimerization constant of the ligand-bound dimer as $(6 \pm 2) \times 10^5\ M^{-1}$. Since the dimerization constant of the antibiotic in the absence of ligand is $(1.0 \pm 0.4) \times 10^5\ M^{-1}$, the binding of ligand is positively cooperative with respect to dimerization by a factor of about 6.

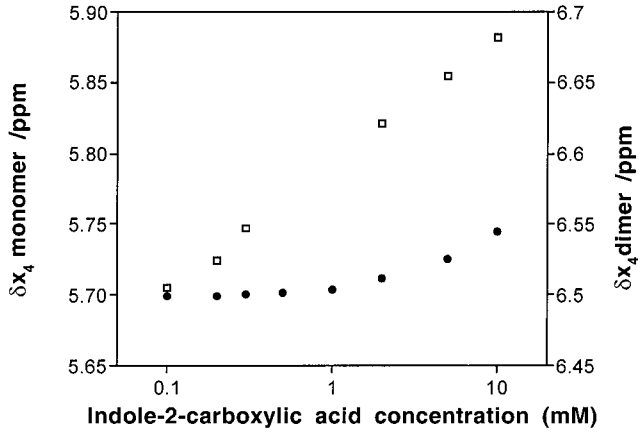

Figure 7. Plot of chemical shifts vs concentration of added indole-2-carboxylic acid for protons x_4 dimer (squares, right scale) and x_4 monomer (circles, left scale) of 0.25 mM chloroeremomycin in D_2O at pD 6 and 290 K.

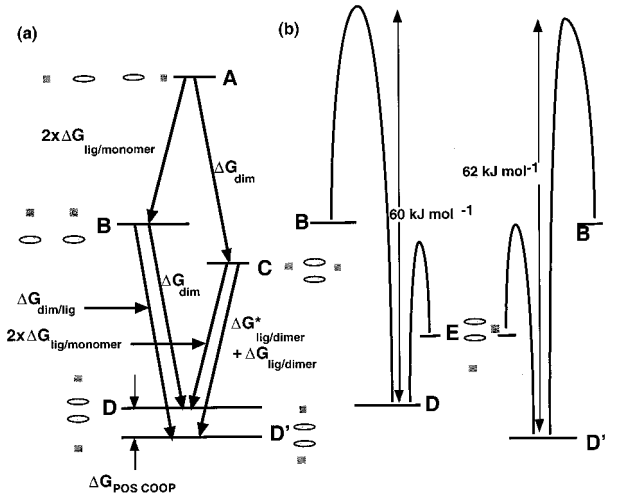

Figure 8. Representation of (a) the thermodynamics of formation and (b) the kinetics of dissociation of the 2:2 complex of chloroeremomycin and indole-2-carboxylic acid. In the absence of cooperativity, **D** would be formed and not **D′**. Under such circumstances, by definition both **A** to **C**, and **B** to **D**, correspond to ΔG_{dim}, and both **A** to **B**, and **C** to **D** correspond to $2 \times \Delta G_{lig/monomer}$. In the case of positive cooperativity **D′** is formed and not **D**. Under these circumstances, the free energy of dimerization of the antibiotic when bound to ligand ($\Delta G_{dim/lig}$) is, relative to ΔG_{dim}, increased by the value of the positive cooperativity. Similarly, the sums of the free energies of ligand binding to the two sites of the dimer ($\Delta G^*_{lig/dimer} + \Delta G_{lig/dimer}$), relative to $2 \times \Delta G_{lig/monomer}$, are also increased by the value of the positive cooperativity. In part b, the barriers to be surmounted for **D** to **E** and for **D′** to **E** are arbitrary except insofar as they are known to be much less than the corresponding barriers for dimer dissocation.

Additionally, although the x_4 dimer signal shows a significant downfield shift during the whole of the titration, the x_4 monomer signal shifts downfield only toward the end of the titration (Figure 7). This observation implies that indole-2-carboxylic acid has a lower affinity for the monomeric form of the antibiotic than for the dimeric form. In light of the known binding constants of the ligand into the two halves of the dimer (see above), the binding constant of indole-2-carboxylic acid to monomeric antibiotic can be calculated. Such a calculation makes use of a thermodynamic cycle (Figure 8). In this figure, the ovals represent molecules of chloroeremomycin, and the squares represent molecules of indole-2-carboxylic acid. Free energy is represented vertically, and is more negative at lower levels. Where the molecules are shown spread out horizontally, they are not associated; where they are stacked vertically, they are associated. Thus, two molecules of ligand-bound monomer are represented by level **B**, one of antibiotic dimer by level **C**, and one molecule of ligand-bound dimer by level **D** in the (hypothetical) circumstances that this ligand-bound dimer could be formed without (positive) cooperativity. Since positive cooperativity operates in practice, this means that **D′** is produced instead of **D** and the free energy of the former is more negative than the free energy of the latter.

In Figure 8, the hypothetical ligand-bound dimer **D** that would be formed in the absence of positive cooperativity is a very useful reference point in understanding the origin of the positive cooperativity. In **D**, each ligand molecule would be able to bind as strongly to dimer as it does to monomer (i.e., the free energy benefits **A** to **B** and **C** to **D** would be equal). In practice, the dimerized states of the antibiotics (either ligand-bound, or ligand-free) have the two disaccharide units (Figure 1) arranged in a head-to-head manner, but the two peptide backbones arranged in a head-to-tail manner.[6−8] This is why the dimers are asymmetric, and can exhibit two ligand-binding sites with different affinities.

It is evident from Figure 8 that:

$$2\Delta G_{lig/monomer} + \Delta G_{dim} + \Delta G_{pos\ coop} = \Delta G_{dim} + \Delta G^*_{lig/dimer} + \Delta G_{lig/dimer}$$

Therefore:

$$(K_{lig/monomer})^2 \times 6 = K^*_{lig/dimer} K_{lig/dimer} = 600 \times 100$$

Thus, $K_{lig/monomer}$, the binding constant of indole-2-carboxylic acid to the antibiotic monomer, can be calculated as 100 ± 25 M^{-1}. This value allows the conclusion that upon titration of indole-2-carboxylic acid into a solution of the antibiotic as described above, the ligand will first bind into the higher affinity site of the dimer (mainly in the concentration range of ligand from 0 to 3 mM). Subsequently it will bind largely into the lower affinity site of the dimer and to the antibiotic monomer (which have very similar affinities). It is for this reason that the chemical shift of x_4 in the dimer is affected throughout the titration, whereas that of x_4 in the monomer changes largely in the latter part of the titration (Figure 7).

Importantly, the thermodynamic cycle (Figure 8) illustrates that the positive cooperativity is the same whether measured as (i) the increase in binding affinity of two molecules of ligand to dimer over their affinity for two molecules of monomer or as (ii) the increase in dimerization constant in the presence of ligand vs its absence. The positive cooperativity is a property of the whole bound system and not of any particular interface. For example, in the case of the system shown in Figure 2, it is the increase in binding energy of the bound system as it exists in reality over that expressed by $2\Delta G_{lig/monomer} + \Delta G_{dim}$ (Figure 8).

The thermodynamics of binding (Figure 8a, see also the summary of data in Table 1) are determined by the difference in free energies of free and bound states. However, it appears likely that the tightness of binding is a measure of the depth of

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Table 1. Thermodynamic Parameters of the Various CE:L Complexes

change of states[a]	equilibrium constant (M^{-1})		free energy change (kJ mol^{-1})[b]	
A to **C**	K_{dim}	$(1.0 \pm 0.4) \times 10^{5c}$	ΔG_{dim}	−27.8
1/2 (**A** to **B**)	$K_{lig/monomer}$	100 ± 25^{d}	$\Delta G_{lig/monomer}$	−11.1
B to **D′**	$K_{dim/lig}$	$(6 \pm 2) \times 10^{5e}$	$\Delta G_{dim/lig}$	−32.1
C to **E**	$K^{*}_{lig/dimer}$	600 ± 150^{f}	$\Delta G^{*}_{lig/dimer}$	−15.4
E to **D′**	$K_{lig/dimer}$	100 ± 25^{f}	$\Delta G_{lig/dimer}$	−11.1
D to **D′**			$\Delta G_{POS\ COOP}$	−4.3

[a] The states written in bold are described in Figure 8. [b] Calculated from the equilibrium constants. [c] Data obtained in aqueous solution at pD 6.0 and 290 K; 0.02 mM CE. [d] Calculated from known equilibrium constants (see text). [e] Data obtained in aqueous solution at pD 6.0 and 290 K; 0.05 mM CE and 100 mM indole-2-carboxylic acid. [f] Data obtained in aqueous solution at pH 6.0 and 290 K; 20 mM CE and varying concentrations of indole-2-carboxylic acid (see Figures 3 and 4).

the free energy well in which the ligand lies.[9] To understand why this might be the case, we extend the analysis of Figure 8. We now consider the relative depths of the free energy wells in which the isolated dimer and the ligand bound monomer lie, with respect to dimer dissociation and ligand dissociation, respectively (Figure 8b). If these interfaces are tightened through the expression of positive cooperativity, which one is likely to be tightened to the greater degree?

We have earlier established from the NMR data that the depth of the well for the dissociation **D′** to **E** (loss of a ligand from the dimer) is less (and probably much less) than that of the well for the dissociation **D′** to **B** (dissociation of the ligand-bound dimer). For the case of the absence of positive cooperativity, we can also be confidant that the depth of the well for loss of ligand ($K = 100 \pm 25\ M^{-1}$, **D** to **E**) should be much less than that for dissociation of dimer [$K = (1.0 \pm 0.4) \times 10^5\ M^{-1}$, **D** to **B**]. Indeed, we have established the barrier to the latter process to be very large (60 ± 4 kJ mol^{-1}) through coalescence of nonequivalent proton resonances in different halves of the asymmetric dimer. The situation is summarized in Figure 8b.

In the absence of positive cooperativity, the available thermal energy will result in the population of states much closer to the transition state for loss of a ligand (**D** to **E**, Figure 8) than for dissociation of the dimer (**D** to **B**, Figure 8). Thus, the ligand/antibiotic interfaces will be more dynamic in their behavior than will the dimer interface. When the positive cooperativity is expressed (**D** to **D′**), we must consider the templating effects of one set of noncovalent interactions upon another. It seems a physically reasonable conclusion that the more ordered dimer interface will act as a better template to improve bonding at the ligand/antibiotic interfaces, rather than vice versa. In summary, the more strongly bound interactions can be used to limit the motion of, and improve the bonding at, the set of interactions that was the weaker set when made in isolation. Therefore, in this system we should expect a ligand interface to have the greater potential to tighten than the dimer interface.

A sensitive probe for structural tightening is available from chemical shift changes at the various interfaces. It is well established that in the association of two peptide backbones through the formation of hydrogen bonded networks (e.g., in the formation of β-sheets), increased downfield shifts of α-CH and NH protons indicate shorter distances between the two peptide backbones.[10−14] Therefore, changes in tightness at specified points of the three interfaces can in principle be assessed from changes in δ values for the α-CH and NH proton probes. The changes in δ values that are of interest are (i) the dimer interface formed in the presence or absence of ligand and (ii) the ligand interfaces formed in the monomer vs the dimer.

Table 2. Chemical Shifts (ppm) of the Protons x_4, in Monomeric and Dimeric Chloroeremomycin (CE), Both in the Absence and in the Presence of Indole-2-carboxylic Acid (L)[a]

$\Delta\delta x_4\,(CE)_{monomer}$	5.70	$\delta x_4\,(CE{:}L)_{monomer}$	5.79
$\Delta\delta x_4\,(CE)_{dimer}$	6.48	$\delta x_4\,(CE{:}L)_{dimer}$	6.75
$\Delta\delta x_4$	0.78	$\Delta\delta x_4$	0.96

[a] Data obtained in aqueous solution at pD 6.0 and 290 K.

The tightness of the dimer interface in the absence and presence of the ligand can be estimated from the chemical shift change of the x_4 resonance in the absence and presence of the ligand. The data for the x_4 resonance (Table 2) indicate that positive cooperativity causes tightening of the dimer interface (0.96 > 0.78 ppm), in agreement with earlier work.[15,16] We note that the ligand (indole-2-carboxylic acid) is aromatic. It is therefore possible that a ring current effect from the indole could cause a larger chemical shift difference for the x_4 resonance than that due to structural tightening alone. However, the slightly greater barrier to dissociation of the dimer in the presence (62 ± 4 kJ mol^{-1}) compared to absence of the ligand (60 ± 4 kJ mol^{-1}) indicates that there is indeed some tightening of the dimer interface due to the positive cooperativity. Although the change in well depth is relatively small (2 kJ mol^{-1}), it is reliable since it is unambiguous that the coalescence temperature of the dimer resonances in the absence of the ligand (280 K) is slightly lower than in the presence of the ligand (285 K).

However, it is not possible to establish whether the ligand/antibiotic interfaces are tighter in the dimer than in the monomer since the appropriate reference points for the ligand/monomer interface are not available. This is because the dimerization constant of the ligand-bound CE is very large [$(6 \pm 2) \times 10^5\ M^{-1}$]. Therefore, the relatively broad amide NH resonances are not of sufficient intensity to be observed at the very low concentrations of antibiotic (0.05 mM) necessary to populate them to even a small extent in the ligand/monomer complex.

Since the CE/indole-2-carboxylic acid system is not suitable to assess the effects of relative barriers upon structural tightening, we note other experimental data.[9,16] In the cases of strongly dimerizing antibiotics, the greater dimerization constants of these antibiotics in the presence of the aliphatic ligand *N*,*N*-di-Ac-Lys-D-Ala-D-Ala vs its absence (e.g., ca. 10^7 vs ca. $10^5\ M^{-1}$ for eremomycin) is associated with only a small tightening of the dimer interface (difference in $\Delta\delta x_4$ values = 0.02 ppm). In the cases of weakly dimerizing antibiotics, the greater dimerization constants of these antibiotics in the presence of *N*,*N*-

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di-Ac-Lys-D-Ala-D-Ala vs its absence (e.g., ca. 900 vs ca. 120 M^{-1} for dechlorovancomycin) is associated with a relatively large tightening of the dimer interface (difference in $\Delta\delta x_4$ values = 0.18 ppm). That the eremomycin dimer lies in a deeper free energy well than does the dechlorovancomycin dimer is established by the observation that the former is in slow exchange with its monomeric form,[17] whereas the latter is in fast exchange with its monomeric form. It is evident that an interaction in a shallow well is tightened to a greater degree by positive cooperativity than is one in a much deeper well.

That these correlations are causally related to well depths, rather than to thermodynamic stabilities, is supported by other data. Ristocetin A and vancomycin have similar dimerization constants, but the former has a higher barrier to dissociation of the dimer and forms a tighter dimer interface.[9]

The reasons why the binding of indole-2-carboxylic acid is negatively cooperative with respect to the dimerization of ristocetin A, but positively cooperative with respect to the dimerization of chloroeremomycin, can currently only be a matter of speculation. It seems possible that the key difference lies in the fact that ristocetin A has a tetrasaccharide attached to residue 4, whereas chloroeremomycin has a disaccharide in this position. These sugars may well occupy different planes with respect to the peptide backbones in the antibiotic dimers. Thus, the ligand may clash sterically with the sugars in the ristocetin A dimer but avoid such a clash in the chloroeremomycin dimer.

Conclusions

We have investigated a system in which the nature of the binding sites is well established on the basis of previous NMR studies, and where nonspecific binding effects can therefore be excluded with some confidence. Our data give further support to the concept that positive cooperativity will be accompanied by structural tightening in a complex. Theoretical considerations, and the available experimental evidence, suggest that structural tightening is preferentially exercised at the noncovalently bound interface that lies in the shallower free energy well. The considerations emphasize how binding energy can arise remotely from a binding site. In antibodies where somatic mutations remote from the binding site for hapten increase the affinity for hapten, a long-range effect on binding site flexibility can be considered.[18] However, our present considerations indicate that the hapten affinity can be increased by structural tightening remote from the binding site.

Experimental Section

Chloroeremomycin, as the acetate salt (LY 264826), was obtained from Eli Lilly (Indianapolis) and indole-2-carboxylic acid was purchased from Aldrich. All NMR samples were made with H_2O:D_2O (9:1) or D_2O and adjusted to pH 6.0 using DCl. 3-(Trimethylsilyl)propionic-*2,2,3,3,-d*$_4$ acid was used as a reference. The 3-9-19 WATERGATE pulse sequence[19] or presaturation was used to suppress the water signal where necessary. All NMR spectra were obtained from Bruker 500 and 600 MHz or Varian Unity 500, 600, and 800 MHz spectrometers at 290 or 280 K.

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