An Enthalpic Component in Cooperativity: The Relationship between Enthalpy, Entropy, and Noncovalent Structure in Weak Associations

Christopher T. Calderone and Dudley H. Williams*

Contribution from the Cambridge Centre for Molecular Recognition, University Chemical Laboratory, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, United Kingdom

Received August 14, 2000

Abstract: Attempts to quantify binding interactions of noncovalent complexes in aqueous solution have been stymied by complications arising from enthalpy−entropy compensation and cooperativity. We have extended work detailing the relationship between noncovalent structure and free energy of binding to include the roles of enthalpy and entropy of association. On the basis of van't Hoff measurements of the dimerization of vancomycin type antibiotics, we demonstrate that positive cooperativity manifests itself in a more favorable enthalpy of association and a partially compensating less favorable entropy of association. Finally, we extend these results to rationalize thermodynamic observations in unrelated systems.

Introduction

Several researchers have developed methods to semiquantitate binding affinities based on the assumption that contributions to binding energies can be partitioned in terms of individual interactions, and that these individual binding energies are additive and independent of each other.[1] However, in general, it is impossible to study one binding interaction in isolation from the others at an interface. As an example, consider a hydrogen bond formed in a noncovalent complex. The hydrogen bond will form with a bond distance and angle and with residual motion that is determined at least partially by the overall structure of the complex. The overall structure of the complex is in turn determined by the sum total of interactions in the association. Thus, interactions in an association are highly context-dependent and concepts such as a "typical hydrogen bond" are difficult to define in a biomolecular recognition event. This and other limitations of these methods have been discussed.[2] Despite their theoretical shortcomings, the empirical methods have provided valuable—and considering their limitations, surprisingly accurate—approximations.[1]

One of the assumptions made by empirical methods is that the entropic cost of localizing the two components with respect to one another is constant, regardless of the nature of the association; that is, these methods ignore *enthalpy−entropy compensation*. Enthalpy−entropy compensation is the empirical observation that highly exothermic interactions tend to have large adverse entropy changes, whereas more thermoneutral interactions tend to have less unfavorable entropy changes.[3−9] As we often consider enthalpy to be a measure of the "strength" of an interaction and entropy to be a measure of disorder in an interaction, this accords well with our intuition that a more exothermic association leads to a more rigidly held complex, with less residual disorder in the form of intermolecular motion. A theoretical treatment of enthalpy−entropy compensation in such point interactions has been given.[9,10] Further, it seems reasonable that a compensating relationship between enthalpy and entropy should be applicable to associations of greater than one interaction as well. Experimental data show good correlation for associations in the gas phase[10] as well as in nonpolar solvents.[11] These latter observations are relevant in that enthalpy−entropy compensation is a property not only of point interactions but also of more complex associations. Importantly, the theory is good enough to correct errors that have accumulated in the literature.[11] Analogously where there is positive cooperativity, we would expect a noncovalent complex held together by a network of such point interactions in an aqueous environment also to be more tightly bound than one held together by a less extensive network of otherwise similar interactions. However, this hypothesized relationship between motion and binding energy has not yet been fully explored in these more complex cases.

Recent work from this laboratory has demonstrated a relationship between binding energy and structural tightening where the structural tightening was assayed by NMR. It appears that this is a general phenomenon for cases where positive cooperativity is expressed, as we have observed it in three distinct systems. (i) Using members of a set of glycopeptide antibiotics known to dimerize with widely varying affinities, we observed a downfield chemical shift change of a proton, designated x_4, upon dimerization. In analogy with the correlation observed between downfield chemical shift changes of β-strand α protons as interstrand distance decreases,[12,13] $\Delta\delta x_4$ ($\equiv \delta x_4^{\text{dimer}} - \delta x_4^{\text{monomer}}$) correlates with intermonomer distance

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10.1021/ja003016y CCC: $20.00
Published on Web 06/12/2001

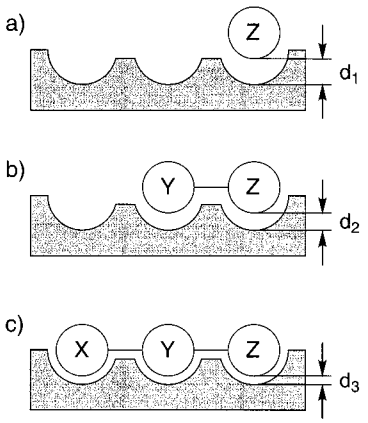
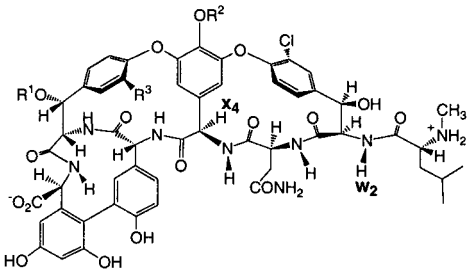


Figure 1. Abstract receptor to illustrate the relationship between binding energy and noncovalent structure. (a) The receptor possesses binding sites for ligands **X**, **Y**, and **Z**. Binding of **Z** results in a structure with intermolecular distance d_1. (b) Binding ligand **Y**−**Z** results in a tighter interface relative to **Z** alone ($d_2 < d_1$) with concomitant increase in the energetic favorability of the interactions of **Y** and **Z** with the receptor (positive cooperativity). (c) Binding ligand **X**−**Y**−**Z** results in a still tighter interface ($d_3 < d_2$) with a greater increase in the energetic favorability of the interactions **X**, **Y**, and **Z** with the receptor.

in the dimer. More importantly in this context, we also observed a correlation of free energy of dimerization with $\Delta\delta x_4$; that is, as free energy of dimerization increases, there is an increase in structural tightness.[14] (ii) Second, these glycopeptide antibiotics form complexes with peptide ligands in which an antibiotic amide proton designated w_2 hydrogen bonds with a ligand carboxylate. We have observed a similar correlation between $\Delta\delta w_2$ and increasing free energy of binding, again reflecting a structural tightening at a binding interface.[15,16] (iii) Finally, in NMR studies of carboxylic acids, we have noted an increase in the chemical shift change of the acid protons upon dimerization as a function of increasing dimerization free energy.[17]

We can expand these arguments to an abstract case. Consider a receptor that binds ligands **X**, **Y**, and **Z** with affinities ΔG_X, ΔG_Y, and ΔG_Z (Figure 1). Clearly, binding some combination of these ligands, each as separate entities, will result in an overall free energy change represented by the sum of the contributions from each ligand individually. Now consider a linker that connects **Y** and **Z** to form **Y**−**Z** (Figure 1b). In general, the free energy change for binding **Y**−**Z** will *not* equal $\Delta G_Y + \Delta G_Z$ as it does in the unlinked case. If binding the linked ligand is more favorable than binding the unlinked ligands, there is *positive* cooperativity; if binding the linked ligand is less favorable than binding the unlinked ligands, there is *negative* cooperativity. We propose where the linker is rigid and strain-free that Figure 1b is an abstract representation of binding with positive cooperativity. The increased free energy of binding **Y**−**Z** relative to **Z** results in a structural tightening of the receptor−ligand complex ($d_2 < d_1$). This, in turn, increases the overall free energy of binding **Y**−**Z** to some ΔG_{Y-Z} that is more negative than $\Delta G_Y + \Delta G_Z$. In some way, binding information is transferred between the ligands by the presence of the linker, resulting in a different free energy of binding relative to the nonlinked ligands. The binding of **Y** affects the binding of **Z** in the linked ligand, and vice versa. Similarly, addition of ligand **X** to form **X**−**Y**−**Z** leads to further structural tightening ($d_3 < d_2$) with further cooperative enhancement of binding (Figure 1c).



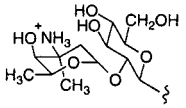
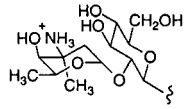
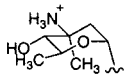
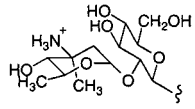
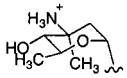
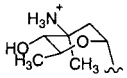
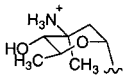
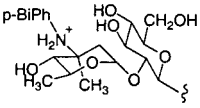
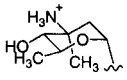
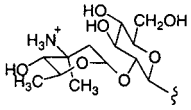
Antibiotic	R^1	R^2	R^3
1, Monodechloro-vancomycin	H		H
2, Vancomycin	H		Cl
3, Chloroeremomycin			Cl
4, Chloroeremomycin pseudoaglycone		H	Cl
5, Eremomycin pseudoaglycone		H	H
6, Phenylbenzyl-chloroeremomycin			Cl
7, Eremomycin			H

Figure 2. Structures of vancomycin type antibiotics. Protons discussed in the text are labeled. *p*-BiPh = *para*-biphenyl.

The above results show that the structure of a complex is at least partially determined by the degree of cooperativity in the association. However, we have not previously explored the consequences of the structure of noncovalent cooperative complexes for the enthalpies and entropies of association. We report here the decomposition of free energy of association into enthalpic and entropic contributions and propose a model for cooperativity that incorporates the roles of enthalpy and entropy.

Results and Discussion

We studied the dimerization thermodynamics of vancomycin type glycopeptides (Figure 2). Antibiotics were studied by van't Hoff analysis of NMR data as described previously.[18] The proton

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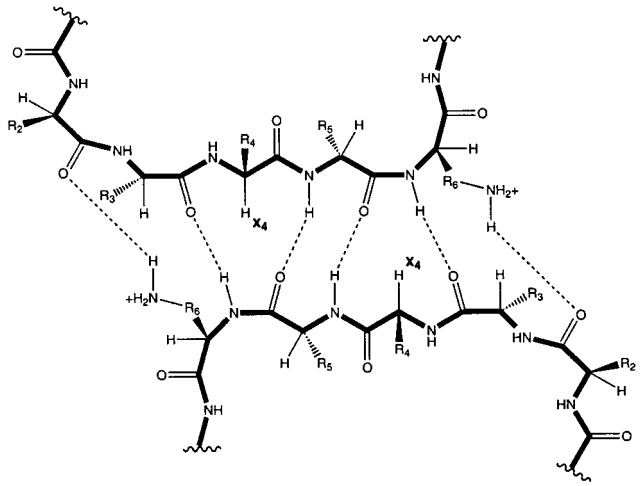

Figure 3. Representation of the dimerization interface in vancomycin type antibiotic dimers. Intermonomer hydrogen bonds are designated by dashed lines and proton x_4 is labeled.

Table 1. Thermodynamic and Chemical Shift Data for Vancomycin Group Antibiotics[a]

antibiotic	ΔG (kJ/mol)	ΔH (kJ/mol)	$300\Delta S$ (kJ/mol)	$\Delta\delta x_4$ (ppm)
1	-12 ± 1[b]	-20 ± 2	-8 ± 2	0.55 ± 0.03[b]
2	-16 ± 1[c]	-36 ± 2[c]	-20 ± 5[c]	0.70 ± 0.03[b]
3	-22 ± 2	-45 ± 5	-23 ± 5	0.78
4	-23 ± 1	-52 ± 3	-29 ± 2	0.85 ± 0.03
5	-24 ± 1[c]	-51 ± 3[c]	-27 ± 3[c]	0.85 ± 0.03

[a] Errors in free energy for **1**, **2**, **4**, and **5** (fast exchange on the NMR time scale) are taken to be ±20% in terms of K_{dim}, while for **3** (slow exchange on the NMR time scale) error is taken to be ±30% in terms of K_{dim}. Chemical shift data for the fast exchanging antibiotics are taken to be ±0.03 ppm [b] Data taken from ref 14. [c] Data taken from ref 18.

designated x_4 is located at the center of the dimer interface, and has been shown to suffer a downfield chemical shift change upon dimerization, with $\Delta\delta x_4$ in the range of ca. 0.5−0.85 ppm (Figure 3).[14] The large value of $\Delta\delta x_4$ allows us to use the NMR signal of this proton to assay dimerization. In some cases, the antibiotic monomer and dimer forms are in slow exchange on the NMR time scale and can be observed simultaneously. For these antibiotics, integration of x_4 peaks for monomer and dimer gives a direct measure of the dimerization constant K_{dim}. In other cases, the antibiotic monomer and dimer forms are in fast exchange on the NMR time scale. For these antibiotics, the chemical shift of x_4 represents a weighted average of $\delta x_4^{monomer}$ and δx_4^{dimer}. Simplex least-squares curve fitting[19] of a plot of δx_4 against antibiotic concentration allows determination of $\delta x_4^{monomer}$ and δx_4^{dimer} as well as of K_{dim} (Table 1).

Correlation of Free Energy of Association with $\Delta\delta x_4$. The limits of the NMR method restrict us to studying glycopeptide dimerization with K_{dim} < ca. 10^5 M^{-1} ($-\Delta G$ < ca. 29 kJ/mol). We measured chemical shift data and dimerization affinities for **4** and **5**, semisynthetic members of the group that had not previously been studied in this regard. These new antibiotics further confirm the correlation reported with **1**−**3**, **6**, and **7**:[14] increasingly favorable free energies of binding induce structural tightening (Figure 4).

Correlation of Dimerization Enthalpy with $\Delta\delta x_4$. Previous work from this laboratory partitioned observed binding free

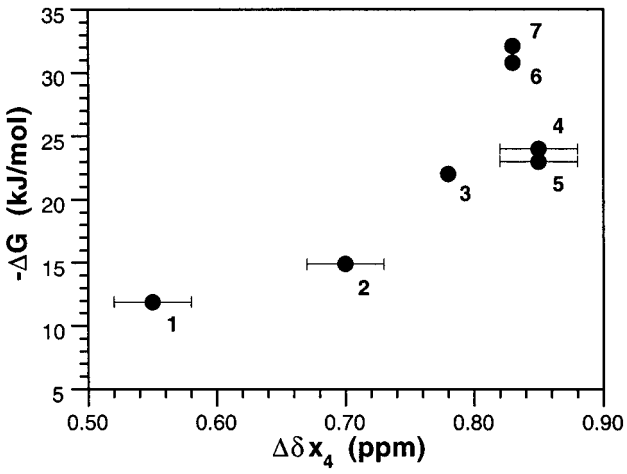

Figure 4. Plot of the free energy of dimerization of the vancomycin group antibiotics against $\Delta\delta x_4$, with $\Delta\delta x_4$ defined as in the text. Data for **1**, **2**, **6**, and **7** taken from ref 14. Chemical shift data for **3**, **4**, and **5** and dimerization free energy for **3** and **4** measured in this work. **3**, **6**, and **7** were in monomer−dimer slow exchange on the NMR time scale, and chemical shifts for proton x_4 were directly measured. The other antibiotics were in monomer−dimer fast exchange, and limiting chemical shifts for $\delta x_4^{monomer}$ and δx_4^{dimer} were determined by curve fitting. For the fast exchanging antibiotics, the error in determining $\Delta\delta x_4$ was taken to be ±0.03 ppm.

energies into several components according to eq 1:[20−23]

$$\Delta G^\circ = \Delta G_{t+r} + \Delta G_r + \Delta G_h + \sum \Delta G_p \qquad (1)$$

ΔG_{t+r} represents the entropic cost of forming a bimolecular complex from two molecules; ΔG_r represents the entropic cost of freezing bond rotations in the complex; ΔG_h represents the entropic benefit (at room temperature) of the hydrophobic effect; and $\sum\Delta G_p$ represents the enthalpic benefits of the electrostatic interactions in the complex. (Terms to reflect van der Waals interactions and conformational strain in the complex are taken to be negligible.) That this equation is a useful approximation is demonstrated by the predictive success of empirical methods based on this partitioning. Parametrization of eq 1 using a training set of 45 drugs of known binding constants gave values that could be used to predict binding constants with a standard deviation of 1.4 orders of magnitude for members of the training set and a standard deviation of 1.7 orders of magnitude for compounds outside the training set.[1]

Major contributors to binding enthalpy are the electrostatic interactions formed in the complex. Such electrostatic interactions are distance dependent. Thus, if structural tightening is a true effect of positive cooperativity exercised at a binding interface, then we should observe concomitant increases in exothermicity of association. A plot of $\Delta\delta x_4$ against dimerization enthalpy shows that this is indeed the case (Figure 5); structural tightness correlates with exothermicity of association.

Evidence for Enthalpy−Entropy Compensation in Dimerization. Plotting enthalpy of association against entropy of association for **1**−**5** reveals a compensating trend (Figure 6). It would appear that the effects of enthalpy and entropy oppose

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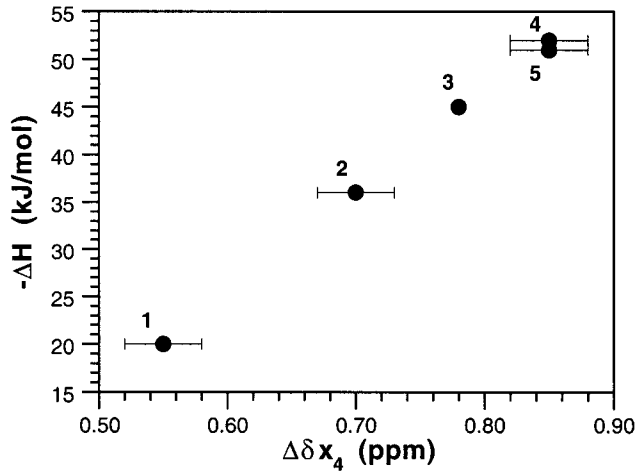

Figure 5. Plot of the enthalpy of dimerization against $\Delta\delta x_4$. The error in chemical shift difference for **1**, **2**, **4**, and **5** was taken to be ±0.03 ppm.

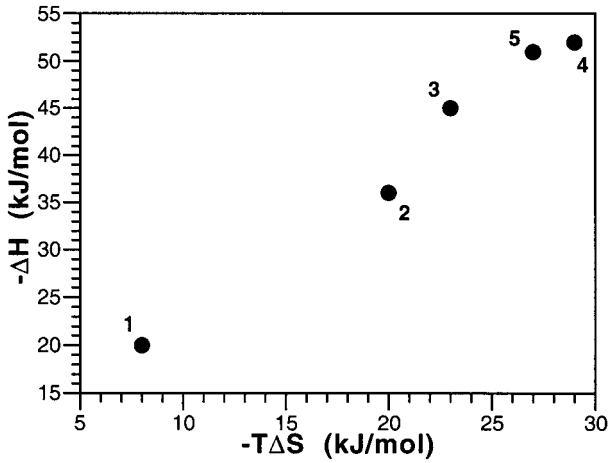

Figure 6. Plot of the enthalpy of dimerization of the vancomycin group against the entropy of dimerization calculated at 300 K. Thermodynamic data for **2** and **4** were taken from ref 18.

each other in cooperative interactions, in accord with observations for less complex associations.[10,11]

Further inspection of eq 1 shows that the main contributors to entropy of binding are the hydrophobic effect and the entropic costs of freezing bond rotations (ΔG_r) and intermolecular motion (ΔG_{t+r}). The hydrophobic effect should show little dependence on the structure of the complex formed so long as the buried hydrocarbon remains inaccessible to solvent. The other two terms should depend on the nature of the binding interactions formed; that is, the more disordered the complex, the less unfavorable these terms should be to the association.

Positive cooperativity induces two related effects: structural tightening and increased exothermicity of association; that is, the complex is described by a deeper enthalpic well. Overall, the result is a more rigidly held complex, with less residual motion (more unfavorable ΔG_{t+r}). We would expect a similar effect on those rotatable bonds restricted upon complex formation, i.e., the more exothermic association would also restrict motion within the components of the complex themselves (more unfavorable ΔG_r).

Care must be taken in interpreting this result, however: (i) The hydrophobic effect is entropy driven and approximately thermoneutral at room temperature. At higher temperatures, the hydrophobic effect tends to be enthalpy driven as opposed to entropy driven. Further, in general, heat capacity ΔC_p is nonzero (i.e., enthalpy is nonconstant as a function of temperature). The van't Hoff equation assumes a constant enthalpy and entropy over the range of temperatures studied. However, should such an effect be important, we would expect to see curvature in the van't Hoff plot. Such curvature was not observed to any significant degree for any of the antibiotics studied in this work. (ii) In the general case, there will be an entropic cost for internal conformational restrictions upon complex formation. The antibiotics studied are extensively cross-linked and relatively rigid, and we would expect entropic costs due to conformational changes to be negligible. Thus, it would appear that among the antibiotics studied, the more exothermic an association, the more adverse is the entropy change due to restriction of relative motion of the associating entities. Just as we would expect a more "strongly" held complex involving a single interaction to have less residual motion, a series of positively cooperative exothermic interactions are also associated with less disorder upon complex formation.

Consequences for Cooperative Binding. Taken together, these results suggest an enthalpic basis for positive cooperativity of binding. Several effects are operating simultaneously: (a) Addition of cooperative interactions reduces the interfacial distance in the complex. (b) The tighter interface increases the exothermicity of association. (c) The increased exothermicity reduces the residual dynamics of the association. All these effects are mutually reinforcing (for example, since kinetic energy of residual motion opposes intimate binding, a less dynamic complex will be more tightly bound). The net result is that the addition of positively cooperative interactions increases the exothermicity of binding as well as the unfavorable entropy of binding.

Enthalpy−entropy compensation dictates that this must be the case: the increased enthalpic benefits of tighter binding result in increased entropic costs of restriction of relative motion of the associating entities. In this set of experiments, the increase in favorable enthalpy brought about by structural tightening is only *partially* compensated for by a more unfavorable entropy, with the net result of a more favorable free energy of binding. Strictly speaking, the only necessary condition for this to be the case is that a plot of the form of Figure 6 have a slope greater than 1. It is important to make clear, though, that enthalpy−entropy compensation plots are in general curved for bimolecular associations in which a major contributor to adverse entropy is the loss of translational and rotational entropy.[9−11] Note, however, that enthalpy−entropy compensation plots for melting are typically linear. This is because T_m (e.g., of crystals) is determined under conditions where $\Delta G = 0$. Since $\Delta G = \Delta H - T\Delta S$, at equilibrium, $\Delta H = T_m\Delta S$, and since T_m variations are typically small, plots of ΔH versus $T\Delta S$ ($T \approx 300$ K) are approximately linear. The overall result of these opposing enthalpic and entropic effects is that as cooperative interactions are added to an interface, positive cooperativity is exercised by an overwhelming increased exothermicity of binding and a partially compensating more adverse entropy of binding (Figure 7).[24]

The observed thermodynamic effects of cooperativity appear to be a necessary consequence of the binding event itself, in that motional restriction due to the addition of positively cooperative interactions *necessarily* leads to an increased exothermicity and more adverse entropy of binding through a pseudo-heat capacity effect. In the present work, cooperativity is exercised at a *single interface*. In terms of Figure 1, the thermodynamic changes observed in passing from Figure 1a to 1b may stem from several contributions: the enthalpic chelate effect developed here and previously,[15,16] the classical chelate

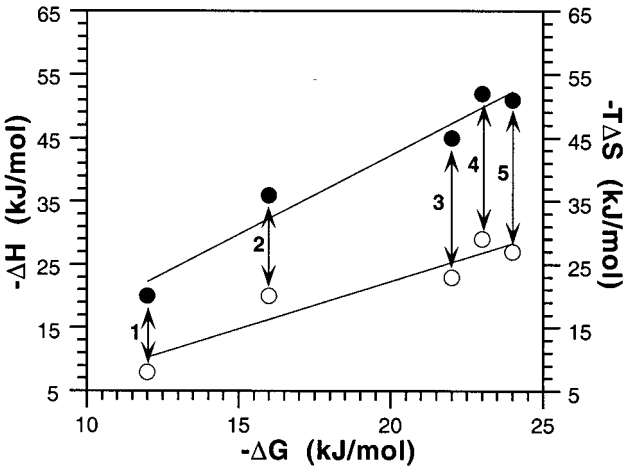

Figure 7. Plot of the free energy of dimerization against enthalpy of dimerization and entropy of dimerization at 300 K for vancomycin group antibiotics. Filled circles represent enthalpies and open circles represent entropies (in terms of TΔS). Note that the rate of increase of enthalpy of dimerization with respect to the free energy of dimerization is greater than that of TΔS; this reflects that the increase in free energy is composed of an entropic penalty and an overwhelming benefit in enthalpy.

effect,[28,29] and contributions due to the **Y**/receptor interaction itself. Thus, for example, if **Y** in Figure 1b corresponds to a large hydrocarbon moiety that is buried upon complex formation, the favorable entropy due to the hydrophobic effect may overwhelm the unfavorable entropy of motional restriction, resulting in a more favorable entropy overall. However, if the motional restriction can be achieved by the addition of an interaction remote from the affected interface, then we can directly measure the effects of this motional restriction in the absence of these complicating effects. Vancomycin group antibiotics bind peptides terminating in D-alanine with high affinity in 1:1 stoichiometry (in the case of di-*N*-acetyl-L-lysyl-D-alanyl-D-alanine, K_{lig} = ca. 10^6 M^{-1}), thus potentially forming a quaternary ligand−antibiotic−antibiotic−ligand complex. Previous work has shown that in general, ligand binding is cooperative with dimerization (that is, dimeric antibiotic binds ligand more strongly than monomeric antibiotic, and ligand-bound antibiotic dimerizes more strongly than unbound antibiotic).[30]

Of relevance to the present work, it has been shown that the intermonomeric distances in several vancomycin group dimers

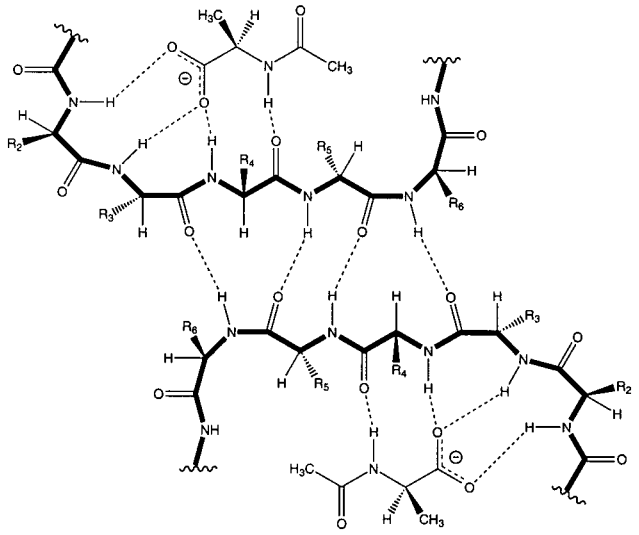

Figure 8. Structure of the quaternary ADA−vancomycin−vancomycin−ADA complex. The vancomycin backbone is represented by thick lines. Hydrogen bonds are represented by dashed lines.

decrease upon ligand binding, in accord with a model in which ligand binding restricts motion at the dimer interface.[14] More importantly, in the case of the vancomycin/acetyl-D-alanine (ADA) complex (Figure 8), it has been shown by van't Hoff analysis of x_4 chemical shift data that in the presence of ADA, dimerization is more exothermic (−62 kJ/mol vs −36 kJ/mol for vancomycin alone) and more entropically adverse (−44 kJ/mol vs −20 kJ/mol for vancomycin alone at 300 K) than in the absence of ligand.[30] Since the van't Hoff technique used to measure these data directly assays the populations of monomer and dimer present independently of other binding events, *these thermodynamic changes are exclusively due to cooperativity*. Through its restriction of motion at the dimer interface, binding of ADA by vancomycin results in the sequence of events detailed above. These data show unequivocally that in the case of the vancomycin/ADA system at least, the motional restriction results in structural tightening, an increase in dimerization enthalpy, and a more adverse dimerization entropy.

Extension to Unrelated Systems. This model provides a plausible rationale for properties observed in unrelated systems. Crystal structures exist for several polycyclic aromatic hydrocarbons. Molecules within such crystals interact mainly through van der Waals and π-stacking interactions, so that increasing the number of carbon atoms increases the extent of potentially cooperative interactions within the crystal. Our model predicts that as the number of carbon atoms increases and the network of cooperative interactions expands, the crystal should suffer a structural tightening. Packing fractions have been calculated for several of the polycyclic aromatic hydrocarbons, and a correlation has been observed between tighter packing in a crystal and the number of carbon atoms in the molecule.[31] As the extent of positively cooperative interactions in the crystal increases, the structure of the crystal tightens, in accord with the model of enthalpic cooperativity.

Conclusions

The above considerations, taken together, support a model of noncovalent binding in which positive cooperativity is expressed enthalpically. In part, cooperativity is related to the classical chelate effect,[28,29] but these results suggest a further enthalpic basis for cooperativity. In terms of the abstract model

(24) We note that in the case of more strongly dimerizing antibiotics (e.g., eremomycin, K_{dim} > 105 M^{-1}),[14] isothermal titration calorimetric measurements indicate that enthalpies of dimerization are smaller that this model would predict.[25,26] However, ITC methods are limited to reporting the sum of thermodynamic contributions from the entire system. Indeed, ITC data for the dimerization of chloroeremomycin could not be fit to a simple monomer−dimer equilibrium, an observation consistent with the presence of other, simultaneous thermodynamic processes complicating the analysis.[27] In contrast, the NMR methods utilized in this work directly assay the relative populations of monomer and dimer. We can therefore have confidence that the NMR method truly reflects the temperature variation of these populations (from which ΔH is determined). For a general discussion of the variations between van't Hoff enthalpies and calorimetric enthalpies, see: Naghibi, H.; Tamura, A.; Sturtevant, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5597.

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given in Figure 1, the information that is transferred by a linker that allows positive cooperativity between binding interactions is structural tightening. Increasing cooperativity at a binding interface tightens the interface, resulting in a more enthalpically favored, but more entropically adverse, binding event.

The correlations reported here should be applicable to other noncovalent complexes. These results shed light on the interplay between enthalpy, entropy, and noncovalent structure, and give insights into how natural systems are able to exploit the balance between these three factors.

Experimental Section

Materials. Eremomycin, chloroeremomycin, and monodechlorovancomycin were donated by Eli Lilly and Co. (Indianapolis, IN). Vancomycin (hydrochloride salt) was donated by Abbott Laboratories (Chicago, IL). Analytical reverse phase HPLC was performed on a Hewlett-Packard series 1050 HPLC instrument with a Phenomenex Jupiter column (15 μm, C-18; 300 Å; 250 $\times$ 4.6 mm). Preparative reverse-phase HPLC was performed on a Shimadzu SPD-10A HPLC instrument with a Phenomenex Luna column (5 μm, C-18; 300 Å; 150 $\times$ 21.2 mm). Mass spectrometry (ESI) was performed on a Micromass Quattro instrument.

NMR Studies. Antibiotics were lyophilized twice from D_2O before use. Samples were dissolved in 50 mM KD_2PO_4 buffer, pH 3.7. Sample pD readings were measured with a Corning pH meter 125 equipped with a combination glass electrode. No corrections were made for an isotope effect. NMR spectra were acquired on a Bruker DRX500 spectrometer over the temperature range 300−335 K. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-*2,2,3,3-d*$_4$-propionate, sodium salt (TSP, $\delta = 0.00$ ppm). Where necessary, proton x_4 was assigned by NOESY spectra.

General Procedure for the Synthesis of Pseudoaglycones. The fully glycosylated antibiotic was dissolved in a minimum of 1 M HCl by sonication (final concentration of antibiotic ca. 100 mM). The reaction mixture was heated in a boiling water bath for 5 min, at which point a precipitate formed. The reaction mixture was cooled to room temperature and lyophilized. The pseudoaglycone was isolated by reverse-phase HPLC as a white fluffy solid. The purity of the isolates was confirmed by analytical HPLC. Eremomycin pseudoaglycone: isolated in 30% yield ($C_{60}H_{67}O_{19}N_9Cl$, $[M + H]^+$ found 1252.4, calcd 1252.4). Chloroeremomycin pseudoaglycone: isolated in 59% yield ($C_{60}H_{66}O_{19}N_9Cl_2$, $[M + H]^+$ found 1286.4, calcd 1286.4).

Acknowledgment. We thank the Winston Churchill Foundation of the U.S. (C.T.C.) and B.B.S.R.C. for financial support. We also thank F. W. Goldberg for the synthesis of chloroeremomycin pseudoaglycone and eremomycin pseudoaglycone.

JA003016Y