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Importance of Structural Tightening, as Opposed to Partially Bound States, in the Determination of Chemical Shift Changes at Noncovalently Bonded Interfaces

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Abstract: Two models (**A** and **B**) have been proposed to account for decreased downfield chemical shifts of a proton bound by noncovalent interactions at a ligand/antibiotic interface as the number of ligand/antibiotic interactions is decreased. In model **A**, the proton involved in the noncovalent bond suffers a smaller downfield shift because the bond is, with a relatively large probability, broken, and not because it is longer. In model **B**, the proton involved in the noncovalent bond suffers a smaller downfield shift because the bond is longer, and not because it is, with a relatively large probability, broken. We show that model **A** cannot account for the chemical shift changes. Model **B** accounts for the process of positively cooperative binding, in which noncovalent bonds are reduced in length and thereby increase the stability of the organized state.

Introduction

The strengths of covalent bonds are relatively well-defined, but this claim cannot be made with regard to the strengths of noncovalent bonds. The factors that define the strengths of the latter are crucial to the understanding not only of binding constants but also to the stability of proteins, DNA duplexes, etc., indeed to the whole field of biological function. In this paper, we address the question of noncovalent bond strengths as they are affected by the important phenomenon of cooperativity. Noncovalent interactions are here considered as positively cooperative when they are mutually reinforcing,[1] as often found within proteins.[2,3]

We have previously[4] bound carboxylate-containing ligands into the binding pocket of glycopeptide antibiotics (Figure 1). This binding pocket consists of three amide NH groups, identified as w_2, w_3, and w_4; these are labeled in Figure 1. The pocket can be saturated with various carboxylate-containing ligands, of widely varying affinities. Where the antibiotic is ristocetin A, and the ligands that provide the carboxylate group are, respectively, acetate, *N*-Ac-D-Ala, *N*-Ac-D-Ala-D-Ala, and *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala, the ligand binding constants are close to 10, 10^3, 10^5, and 10^6 M^{-1}.[4] In saturating the binding sites with these four ligands, the downfield shift suffered by w_2 is close to 1.4, 2.5, 3.4, and 3.6 ppm, respectively, and it is the binding of the last of these that is illustrated in Figure 1. Two models, called here Model A and Model B, have been proposed to account for these findings. We first describe these models and then experiments that differentiate between them.

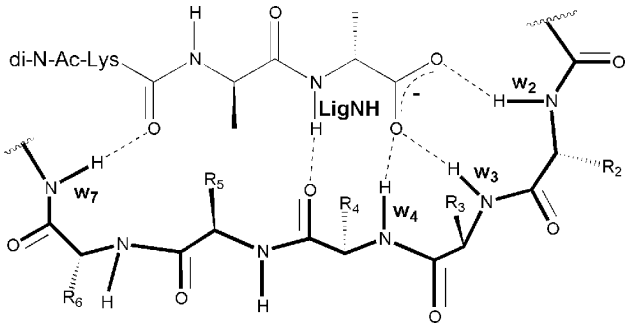

Figure 1. Hydrogen bonds formed from *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala to the antibiotic ristocetin A.

Model A

This model (Figure 2) is proposed by Hunter and Tomas[5] to account for the data. The chemical shift data are rationalized in terms of the probability, in the bound state, that a noncovalent bond to the carboxylate group (w_2, w_3, or w_4) is made or is broken. It is w_4 that is specified in the model proposed,[5] but the published chemical shifts that are required to fit the model are in fact those of w_2 (to which we refer below). The model does not consider that noncovalent bonds may, in different circumstances, have different lengths; a bond is either broken or made. To estimate the probability that a noncovalent bond is broken in the bound state of any ligand, a pathway is proposed for dissociation of each of the ligands. Thus, a pathway for dissociation of *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala occurs via several steps. These are the successive breaking of the bonds to w_7, to

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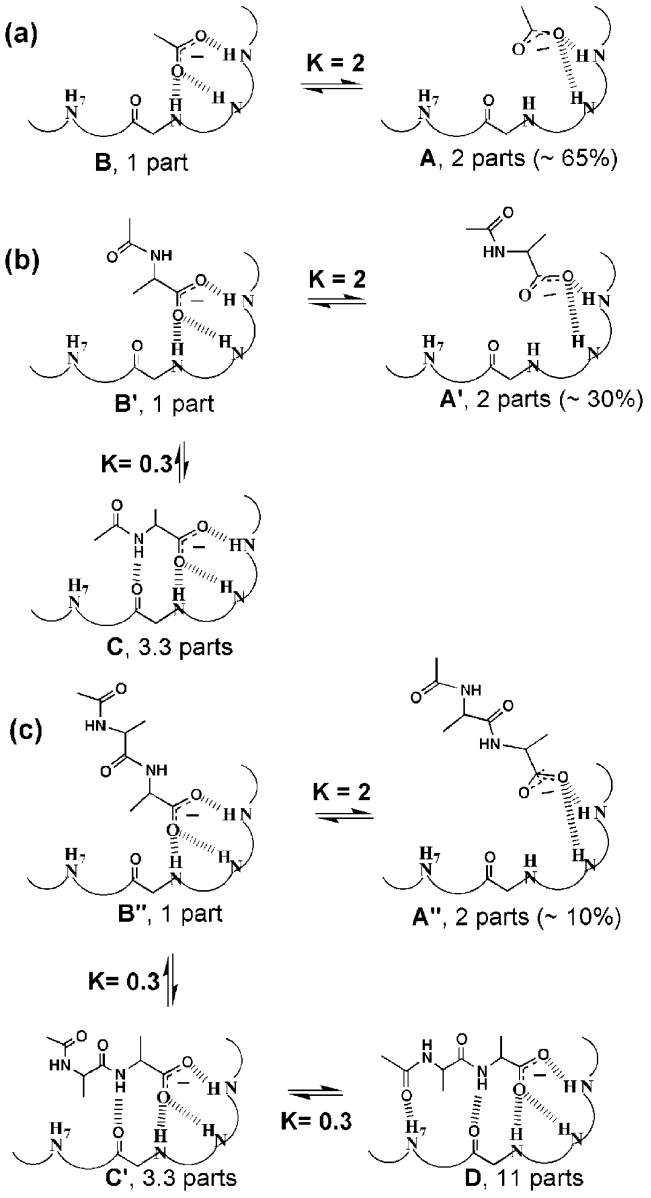

Figure 2. Model in which partially bound states are used to rationalize the occurrence of smaller limiting chemical shifts for more weakly binding ligands.[5] We present the model of Hunter and Tomas in Figure 2 as in ref 5. However, although they rationalize the NMR data in terms of w_4 in the model, they in fact use the NMR data for w_2 (see Figure 1 for the labeling of these protons).

the NH of the ligand, to one of the NHs in the pocket that binds the carboxylate, and finally to the remaining NHs in the carboxylate pocket [Figure 2c, where the last step is not shown]. The later steps in this "unzippering" process are required to be followed in the dissociation of the smaller ligands [Figure 2a and b].[5]

Since variable hydrogen bond lengths are not permitted in the model, the very low-field w_2 chemical shift of ca. 11.6 ppm for the binding of di-*N*,*N*′-di-Ac-Lys-D-Ala-D-Ala (**D**) must also be that found in **B**, **B**′, **B**″, **C**, and **C**′ (Figure 2). The observed chemical shift changes of w_2 for the various ligands require specific relative concentrations (see Figure 2) of the intermediates that are proposed for the dissociations. Thus, the pathway for the unzippering is assumed, and the *K* values are chosen to fit the chemical shift data.

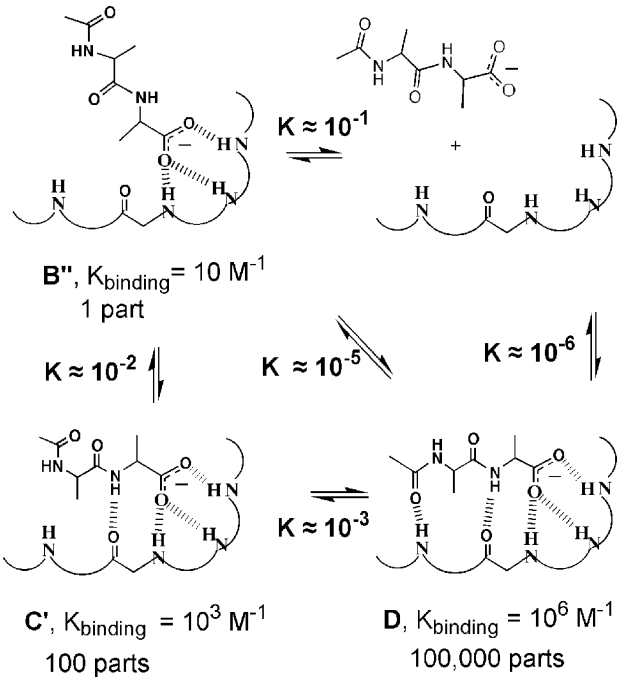

Figure 3. Model in which longer bonds are used to rationalize the occurrence of smaller limiting chemical shifts for more weakly binding ligands.

$K = 2$ is required for the bond-breaking involved in the binding of acetate. This value is dictated by the observation that the limiting chemical shift of acetate is about ca. 33% of that for *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala. It is therefore proposed that, in the bound state for the antibiotic/acetate complex, the hydrogen bond to acetate is broken ca. 65% of the time [Figure 2a]. This is required not only for **A** relative to **B** but also for **A**′ relative to **B**′ [Figure 2b] and **A**″ relative to **B**″ [Figure 2(c)]. $K = 0.3$ for the bond-breaking involving the NH of the C-terminal D-Ala (hereafter referred to as "ligand NH") is required, since the limiting chemical shift for the binding of *N*-Ac-D-Ala is ca. 67% of that for *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala. Therefore, the population of species with the w_2 hydrogen bond broken (**A**′) is required to be ca. 30% [Figure 2b]. $K = 0.3$ for the species involving breaking the hydrogen bond between the acetyl carbonyl group and 7-NH requires that the w_2 hydrogen bond is, in the *N*-Ac-D-Ala-D-Ala case,[5] broken only ca. 10% of the time (more precisely, 2 parts in a total of 17.3 parts).

We emphasize that, irrespective of whether the strongest binding ligand is taken as *N*-Ac-D-Ala-D-Ala[5] or *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala (as in the present work), model **A** requires similar, and important, contributions from partially bound states to rationalize the chemical shift data.

Model B

In model **B**, for the bound state of *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala, the hydrogen bond to w_2 is shorter than that for the binding of acetate because the additional interactions help to anchor the carboxylate group in its binding site.[4] This model may be understood by reference to Figure 3. It is proposed that the differences in the chemical shift data for the NHs that bind the carboxylate arise because these hydrogen bond lengths are shorter in **D** than in **C**′ and in **C**′ than in **B**″.[4] In this model, the populations of partially bound states **C**′ and **B**″, relative to **D**,

are assumed to be negligible (in the sense that they would not significantly influence the observed chemical shifts).

Differentiating Between the Models

We have previously determined the binding constants of truncated ligands that make the same interactions to the antibiotic as the intermediates that are proposed for dissociation in Figure 2. When the antibiotic is ristocetin A and the truncated ligands are acetate and *N*-Ac-D-Ala, the observed binding constants are, respectively, 10^1 and 10^3 M^{-1} (which should be compared with 10^6 M^{-1}, for *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala).[4] We note that the adverse entropy of an association is only a logarithmic function of the mass of the smaller of the two entities that are involved. Therefore, the interactions that are common between the binding of the truncated ligands and the intermediates proposed for dissociation should be of similar strength. Thus, if the dissociation pathway follows the "unzippering" pathway assumed in Figures 2c and 3, the equilibrium constants given in Figure 3, and not those given in Figure 2, must, to a good approximation, apply. The values of the equilibrium constants that determine the populations of the intermediate states are grossly different between Figures 2 and 3. Using the equilibrium constants of Figure 2, in the dissociation of *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala, the population of species that make the same binding interactions as acetate are ca. 25% of those of fully bound *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala. On this basis, acetate should bind $\geq 10^5$ M^{-1}. Experimentally it binds 10^1 M^{-1}, and therefore the population of species that are partially bound states must be very much less than those in model **A**. Model **A** is therefore unable to account for the chemical shift differences that are found for a variety of bound ligands.

However, model **A** has the possible merit of proposing a dissociation pathway for a peptide ligand. If such pathways can be tested, they would represent a useful advance in studies of bimolecular binding phenomena. The concept of such partially bound states in dissociation pathways is analogous to the stepwise opening of "breathing channels" in proteins where the process is unimolecular.[6−8] However, so far as we are aware, such processes have not been tested where they are bimolecular, as in the present case. We have therefore carried out experiments to test whether the pathway finds support when combined with equilibrium values that are consistent with all the binding data (Figure 3).

Determination of the Population of Dissociation Intermediates through the Measurement of NH Protection Factors

There are five amide NH groups involved in the ligand binding site for *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala (**D**, Figure 3). Three of these (w_2, w_3, and w_4) lie in the binding pocket that binds the carboxylate anion of the ligand, one is provided by the ligand (ligand NH), and the other is an antibiotic NH (w_7) (Figure 1). When these NH groups are in the free state (i.e., not involved in complex formation), each one of these has an intrinsic exchange rate (k_{int}) for exchange with water (or D_2O) in an aqueous environment. In a peptide consisting of the standard amino acids, for an amide backbone NH that is remote from a formally charged site, this intrinsic exchange rate is ca. 5 s^{-1} at room temperature and pH 7.[9] Since the process of exchange is catalyzed significantly only by OH^- at pH's $\geq$ 4, this value decreases by a factor of 10 for every reduction of the pH by 1 unit in the range 7−4. Therefore, at pH 4.5 (at which pH we have made our measurements) the rate of exchange of a solvent-exposed peptide amide NH that is not subject to atypical effects due to adjacent positive or negative charges should be close to the range 10^{-1}−10^{-2} s^{-1}. Because, at pH 4.5, these intrinsic exchange rates are many orders of magnitude less than the on-rate for the ligand ($k_{on} \approx 10^5$ s^{-1}), we can be confident that in exchanging the amide NHs in the complexes we are dealing with EX2 exchange. Thus, the data (see below) give us reliable protection factors.

The corresponding exchange rates in a complex (k_{ex}), or folded protein, can also be determined.[10,11] The factor (k_{int}/k_{ex}) by which the exchange rate is reduced gives "the protection factor" of the NH. This protection factor is the equilibrium constant *K* which measures the ratio of the population with the NH "hidden" from solvent (in the present work, through hydrogen bond formation in the complex) to the population with the NH exposed to solvent. Thus, the equilibrium constants for the breaking of the various hydrogen bonds proposed to occur in the dissociation of *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala (**D**, Figure 3) can be determined. If the pathway is correct, the protection factors should be smallest at the beginning, and greatest at the end, of the zipper. For an NH involved in the hydrogen bond that is the last to break in the dissociation of the ligand interface, the protection factor should have a value close to,[12] or equal to,[9,13] the equilibrium constant for overall binding.

Chloroeremomycin (CE, also known as A82846B) is an antibiotic of the vancomycin group that binds *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala by the same set of hydrogen bonds as do both vancomycin and ristocetin A.[14] We have used chloroeremomycin in the present study because four (w_2, w_4, the ligand NH, and w_7) of the five hydrogen bonds (Figure 1) that are involved in forming the complex with *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala can be monitored in all the required experiments. It binds acetate, *N*-Ac-D-Ala, and *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala with respective binding constants of 10^1, 300, and 10^6 M^{-1} and in so doing induces respective limiting downfield shifts of w_2 of 1.8, 2.4, and 2.85 ppm.[15] Therefore, the three equilibrium constants successively required in the "unzippering process" that is shown in Figure 2c are, for the case of CE, $K = 1$, $K = 1$, and $K = 0.5$. Thus, as in the case of vancomycin and ristocetin A, rationalization of the limiting chemical shifts in terms of Model **A** requires important contributions from partially bound states.

The required k_{int} and k_{ex} values were determined at pH 4.5. Since the k_{int} values required for the free CE and ligand components correspond to relatively fast rates of exchange (with one exception; see below), these were determined using the

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Table 1. Values of k_{int}, k_{ex}, and Protection Factors for the Binding of *N,N′*-di-Ac-Lys-D-Ala-D-Ala to Chloroeremomycin[a]

NH	k_{int}	k_{ex}	protection factor	req. model A[b]	req. model B[c]
w_7	1.0×10^{-3}	1.0×10^{-6}	10^3	~0.4 (1.7)	10^3
lig	2×10^{-3}	2×10^{-7}	10^4	~1.3 (4.8)	10^5 (10^4)
w_4	0.35	1.6×10^{-6}	2×10^5	~6 (8)	10^6 (10^4)
w_2	10	9×10^{-4}	1.1×10^4	~6 (8)	10^6 (10^4)

[a] Rate constants are in units of s^{-1}. Data were obtained at 285 ± 7 K. For the free components, 20 mM chloroeremomycin, or ligand, pH 4.5 was used (except as recorded in the text). Measurement of the exchange rates of the antibiotic NHs in the complex employed 20 mM chloroeremomycin and 30 mM *N,N′*-di-Ac-Lys-D-Ala-D-Ala, pD 4.5, whereas, for measurement of the exchange rate of the bound ligand NH, 30 mM chloroeremomycin and 20 mM *N,N′*-di-Ac-Lys-D-Ala-D-Ala was used ("ligand NH" refers to the NH of the C-terminal Ala of *N,N′*-di-Ac-Lys-D-Ala-D-Ala). [b] The protection factors are calculated by taking the ratio of the populations of states in which the hydrogen bond is proposed to be made vs broken in model A for either chloroeremomycin (first given values in the column) or ristocetin A (values in parentheses, using the populations given in Figure 2). [c] The values given in parentheses correspond to the maximum protection factors expected on the basis of the concentrations of ligand and antibiotic used in the experiments (see text for details).

method of transfer of saturation from water.[16,17] In contrast, k_{int} for the w_7 resonance of CE and all the k_{ex} values of the complex correspond to half-lives for exchange greater than 5 min. These values were therefore conveniently determined by examination of the complex in D_2O solution, and measuring directly the rate at which the four NHs that were in involved in hydrogen bonds in the complex, and could be monitored, were removed from the spectrum.

The value of k_{int} determined for the ligand NH requires comment. To allow the desired comparison with its k_{ex} value, it should correspond to the exchange rate occurring in the ligand carboxylate anion (since it is this species that binds to the antibiotic). The anion is of course highly stabilized when it is bound to the antibiotic, but at pH 4.5 in the free ligand the anion is probably significantly protonated. The k_{int} value was therefore determined at pH 7.5, 6.5, 5.5, and 4.5 as 2.5, 0.24, 0.06, and 0.02 s^{-1}, respectively. Since a pH vs log k_{int} graph is required to have a slope of 1 for a defined species,[12] the true value for the anion at pH 4.5 (2×10^{-3} s^{-1}) can be obtained by extrapolating the slope (+1) available from the pH 7.5 and 6.5 measurements to pH 4.5. The value used in Table 1 is therefore 2×10^{-3} s^{-1}. As could be anticipated, the value of 0.02 s^{-1} measured directly at pH 4.5 is greater (by a factor of 10) because partial protonation of the anion at this pH produces a neutral species that is able to undergo faster base-catalyzed exchange than does the anion.

The k_{int}, k_{ex}, and protection factor ($K = k_{int}/k_{ex}$,) values that are relevant to analysis of the binding of *N,N′*-di-Ac-Lys-D-Ala-D-Ala to chloroeremomycin are given in Table 1.

Intrinsic Rate Constants for Exchange of the Amide NHs involved in Binding

In the free states of the two species that give rise to the complex, the intrinsic rate constants (k_{int}) lie in the order $w_2 > w_4 >$ ligand NH ≈ w_7. It is in accord with expectations that the exchange rates of w_2 and w_4 are unusually fast, with the rate of exchange of the former being even greater than that of the latter. Since the exchange is catalyzed by a negatively charged entity (HO^-), it is accelerated by the proximity of positive charge to the NH undergoing exchange. In the carboxylate binding pocket, the w_2 and w_4 NHs each experience a positive charge associated with the adjacent NH(w_3)−CO dipole (Figure 4). Additionally, the positively charged N-terminus of the antibiotic is at the residue immediately adjacent to w_2 and will increase the intrinsic exchange rate in the order $w_2 > w_4$ (Figure 4). Since exchange is catalyzed by HO^-, an enhanced rate of exchange is of course anticipated in a pocket that binds the carboxylate anion.

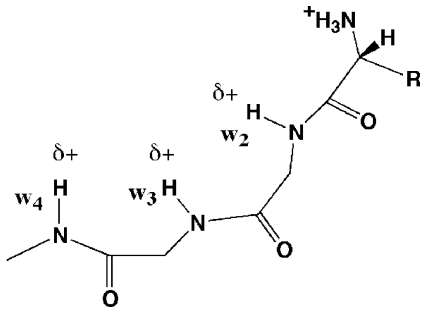

Figure 4. Illustration of the proximate positive charges that are anticipated to increase the rate of base-catalyzed exchange of the amide NHs w_2, w_3, and w_4. Since the positively charged N-terminus of the antibiotic is the residue adjacent to w_2, these exchange rates lie in the order $w_2 > w_4$.

Protection Factors of the Amide NHs involved in Binding

The binding constant of *N,N′*-di-Ac-Lys-D-Ala-D-Ala to chloroeremomycin has previously been reported as 1.0×10^6 in water.[18] Since we have worked in D_2O solution at pH 4.5 in measuring protection factors in the present work, it was necessary to determine the binding constant under these conditions. We found the binding constant to be 1×10^5 in H_2O at pH 4.5 but to be 1×10^6 in D_2O at the same pH.

However, in the case of a bimolecular association, it is not valid to assume that the maximum possible protection factor will therefore be 10^6. In the case of proteins, the dissociation of specific amide hydrogen bonds is unimolecular, and protection factors (for nonaggregating proteins) are independent of the protein concentration. In the case of bimolecular associations (as here), the fraction in the unprotected (dissociated) form depends on the concentrations of the associating species. For example, at a concentration of 0.5 mM, for a complex with $K = 10^6$ M^{-1} (the approximate value of the binding constant of *N,N′*-di-Ac-Lys-D-Ala-D-Ala to chloroeremomycin), ca. 5% of the complex will be in the dissociated state. So the apparent protection factor would be only ca. 20. The protection factor would only be 10^6 at molar concentration. Since concentrations for proton NMR experiments are effectively limited to ≤20mM for molecules of the molecular weights used here, we have worked at these upper limits. Additionally, the component of the complex whose protection factor is not being measured should be in as large an excess as practically possible. This precaution not only ensures efficient complexation of the component under investigation but also avoids the danger of a slight excess of that component if an ostensibly 1:1 ratio was examined. Such small excess would necessarily reduce the

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measured degree of protection (through exchange with the unbound form) and give rise to a misleading result.

In view of the above points, we determined the protection factors of the hydrogen bonded antibiotic amide NHs at concentrations of 20 mM chloroeremomycin and 30 mM *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala. Similarly, we determined the protection factors of the ligand amide NHs that are involved in a hydrogen bond that hold the complex together (NH of the C-terminal D-Ala) at concentrations of 20 mM *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala and 30 mM chloroeremomycin. At these concentrations, the maximum protection factor expected for any of the four NHs stipulated in Table 1 (due to unavoidable dissociation effects) is readily calculated from eq 1.

$$K = 1 \times 10^6 = [\mathrm{A/B}]/[\mathrm{A}][\mathrm{B}] \quad (1)$$

where [A/B] is the concentration of complex, [A] is taken as the concentration of the component that is in excess, and [B] is the concentration of the component that is not in excess. Since we are working in circumstances under which [A/B]/[B] must be extremely large, and the initial concentrations of A and B are 30 mM and 20 mM, we can write

$$10^6 = [20 \times 10^{-3}]/[10 \times 10^{-3}][\mathrm{B}] \text{ and therefore } [\mathrm{B}] = 2 \times 10^{-6}\ \mathrm{M}^{-1} \quad (2)$$

Thus, for the component that is not in excess, the fraction that occurs in its dissociated state is 1 part in 10^4 (i.e., $2 \times 10^{-6}/2 \times 10^{-2}$). Thus, 10^4 should be the maximum protection factor for any exchange process that is under thermodynamic control (i.e., is measured under equilibrium conditions) in our experiments.

The data establish the following:

(i) The largest protection factor (2×10^5) is found for w_4. Therefore, partially bound states generated by breaking this hydrogen bond in a possible dissociation pathway for the peptide are populated to an extent of only 0.0005% of the fully bound state. The very large protection factor clearly excludes model **A**. In this model, a high population of a partially bound state in which the hydrogen bond involving this NH is broken is required.

The observed protection of w_4 is greater than the maximum expected protection by a factor of 20. The maximum protection factor is calculated on the basis that the binding constant of the ligand is $1 \times 10^6\ \mathrm{M}^{-1}$, and this is measured by UV spectroscopy; this binding constant could be in error by a factor of 2. Errors in k_{int} could be a factor of 5, although those in k_{ex} are thought to be reliable within a factor of 2. Thus, the possible errors are comparable to the degree by which the measured factor exceeds the expected maximum. However, the key conclusion is that the observed protection is very large.

(ii) Smaller protection factors, but still very large in absolute terms (10^3-10^4), are found for the ligand NH, w_7 and w_2. The protection factor of 10^3 for w_7 excludes model **A**. In this model, the hydrogen bond involving this NH is broken in a relatively large fraction of the states proposed to make up the assembly of partially bound states.

Implications of the Work

In considering further the implications of the data (Table 1), let us start with the (physically implausible) assumption that the pathway for ligand dissociation involves no fraying but simply that the ligand dissociates by breaking all the hydrogen bonds at the same time. The hydrogen bonds would therefore all be protected to the same extent, and if we were able to work at molar concentrations, each hydrogen bond would have a protection factor equal to the equilibrium constant for binding (10^6). Since we must work at less than molar concentrations, this model would require in our experiments (within experimental error) a protection factor of 10^4 (see earlier) for every NH involved in the binding site. For the four probed hydrogen bonds involved in ligand binding, the experimental protection factors are essentially within the range $10^{4\pm1}$ (Table 1). Thus, although this "all-or-nothing" binding scenario is physically implausible, it provides a much better approximation than a model involving high populations of partially bound states. What is this result telling us about positively cooperative binding?

The rather similar NH protection factors found for different parts of the binding site emphasize the importance of positive cooperativity. When formed together, the probability of breaking *any* of the hydrogen bonds (at the NMR concentrations at which we have worked) is low ($10^{-4\pm1}$). Even the one (to w_7) separated by two internal rotors of the ligand from the next (to lig-NH) is broken with a probability of only 10^{-3} (Table 1). Although this probability of "fraying" is low, it is physically plausible that ligand dissociation commences by breaking this hydrogen bond. The alternative that dissociation commences at the other end (first breaking the bond to w_2) seems less likely. The carboxylate binding pocket is so highly organized that simultaneous breaking, or weakening, of several hydrogen bonds would then seem necessary.

Therefore, partially bound states cannot account for increases in limiting chemical shifts of the amide NHs as the numbers of adjacent binding interactions is increased. The original conclusion that *adjacent binding interactions serve to strengthen existing hydrogen bonds* and to shorten these hydrogen bond lengths is reinforced. In fact, it can be concluded from the data that such effects are large. Thus, when acetate is bound to the antibiotic, the probability of breaking all its interactions to the carboxylate binding pocket of the antibiotic is 10^{-1} at a molar concentration of acetate ($K = 10\ \mathrm{M}^{-1}$). Therefore, the binding energy provided by the interactions to the carboxylate is, at room temperature, ca. 6 kJ mol^{-1} in the case of acetate. The probability of breaking all of the same interactions when the carboxylate is part of *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala is, within a factor of about 10, 0.5×10^{-5} (from the protection factor of w_4, Table 1). Thus, when the carboxylate group is part of *N*,*N*′-di-Ac-Lys-D-Ala-D-Ala, the cost in free energy to break these interactions *and* the associated weakening of the adjacent noncovalent bonds is ca. 30 kJ mol^{-1}. Therefore, the cooperativity gives rise to a large benefit (mutual reinforcement) in the bonding interactions, in the manner also indicated by the chemical shift data.

The concept that hydrogen bonds may be shortened when a single noncovalently bonded interface is extended is supported by crystal structure data. In dimers of glycopeptide antibiotics, nonbonded distances at the dimer interface are reduced as the interface is extended and as the dimerization constant is increased.[19] Additionally, the expectation that shorter hydrogen

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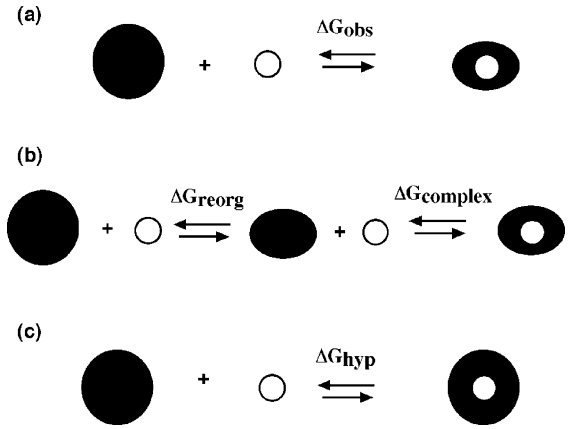

Figure 5. Binding of a ligand (small open circle) to a receptor (large black circle). (a) With contraction of the receptor structure, (b) analysis of the binding when a free energy cost is incurred through contraction of the free receptor structure, (c) representation of binding occurring without contraction of the receptor structure in the bound state. (b) Is irrelevant to the free energy of binding, since it is only the bound structure of the receptor that contracts. The contraction of the structure of the bound receptor is favorable in free energy because, in positively cooperative binding, the binding of the ligand reduces the dynamic behavior of the receptor.

bonds provide greater binding energy is supported. Thus, as anharmonic potential energy wells become deeper, the cost in entropy that occurs with a benefit in enthalpy[20] is concluded[21] to lead to an increase in the free energy of binding even for relatively weak bonds (bond dissociation energies of ca. 7 kJ mol^{-1} or greater). However such favorable local interactions can develop into repulsions if long-range attractions compress the local interaction onto the repulsive portion of the its potential energy curve.[22]

The above positive cooperativity, occurring within a single bonding interface (Figure 1), is also found when ligand binding results in reductions in noncovalent bond lengths within the receptor (cooperativity over two, or more, interfaces).[23,24]

In proposing model **A**, it was concluded[5] that although cooperativity is a general property of intermolecular interactions, the origins of the effect remain obscure. Additionally, it was concluded that enthalpy/entropy compensation does not relate to changes in free energy. We have provided contrary evidence that positively cooperativity binding occurs where sets of noncovalent interactions are mutually enhancing in free energy due to reductions in bond lengths of the interactions.[1,23,24] These reductions in bond length are accompanied by a benefit in enthalpy and a cost in entropy[20,25,26] and do give a benefit in free energy[23] to the binding of ligands to organized states. Negatively cooperative binding has the converse properties.[24] Where there is no cooperativity, structural tightening will not occur and such circumstances seem appropriate to describe the system studied by Hunter and Tomas.[5] A further example where cooperativity is not displayed is found in the binding of polyamines to DNA, where the affinity can be described simply as a function of the number of possible contributions of each ammonium center.[27]

Despite the available evidence,[23] it is sometimes perceived that a receptor system cannot *contract* its structure upon binding a ligand with a consequent *stabilization* of the receptor system (and therefore with a favorable contribution to ligand binding). The reason that it may seem improbable is evident from a fact recently emphasized by Wildes and Marqusee [Figure 5a and b].[12] Their considerations show that if the structure of a receptor is modified from that existing in the native state of the isolated receptor, then the observed ligand binding energy (ΔG_{obs}) must be less favorable than the binding energy ($\Delta G_{complex}$) of the ligand to the modified form of the receptor. This is true because ΔG_{reorg} must always be positive [Figure 5a and b]. However, a free energy change of reorganization is only required to be positive to reorganize the *free* receptor. Since ligands that bind to their receptors with positively cooperative binding reduce the dynamic behavior of the receptor,[1,24] the more compact state of the receptor is more stable than its less compact (free) state once the ligand is bound (i.e., has become part of the receptor system). Adding the ligand to the receptor is analogous to increasing the number of layers of N_2 atoms on a cooled surface from n to $n + 1$; the dynamic behavior of the system is thereby reduced, and it thereby moves toward a more stable organized state.[28]

Strictly speaking, the receptor should not be considered to exist as a definable thermodynamic entity once the ligand is bound; it has become a new thermodynamic entity (receptor/ligand). The receptor portion of this system adopts a more compact state because of the motional restraints imposed by ligand binding. ΔG_{reorg} [Figure 5b] is irrelevant to the analysis because it is a price that is never paid. The crucial point is that ΔG_{obs} is more favorable than the hypothetical free energy change (ΔG_{hyp}) of Figure 5c, and contraction of the receptor portion is, in the bound state, a spontaneous process that makes a favorable contribution to the overall change in free energy.

Conclusion

That bonds stretch before they fray is unambiguous from the anharmonicity of potential wells. In the limit of weak bonding, they will stretch and *then* fray. We have shown that a model (Model **A**) in which chemical shifts for binding vary because bonds are frayed, rather than stretched, is, in the case specified here, incompatible with the low concentration of frayed species deduced from experiments on equilibrium binding and studies of NH protection. The available evidence is consistent with the conclusion that reductions in the lengths of noncovalent interactions are associated with positively cooperative binding.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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