Toward the Semiquantitative Estimation of Binding Constants. Guides for Peptide–Peptide Binding in Aqueous Solution

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Abstract: An expression is presented for the estimation of approximate binding constants for bimolecular associations in solution. The consequences of the approach have been examined for the bimolecular association of two peptide components in aqueous solution: specifically for the binding of two vancomycin group antibiotics, vancomycin itself and ristocetin A, to the peptide cell wall analogue *N*-Ac-D-Ala-D-Ala and related ligands. Uncertainties in the treatment are relatively large, but the physical insights gained into the binding process (in part with the aid of calorimetric data obtained by others) are enlightening. We conclude that for amide–amide hydrogen bond formation in aqueous solution at room temperature, the intrinsic binding energy is ca. 24 kJ mol^{-1} (an intrinsic binding constant of ca. 10^4); this process is almost completely driven by a favorable entropy change associated with the release of water molecules from the amide NH and CO groups involved in hydrogen bond formation. The bimolecular association of *N*-Ac-D-Ala-D-Ala with ristocetin A has a remarkably small entropy change at 298 K ($T\Delta S$ = 3 ± 1.5 kJ mol^{-1}). We conclude that the release of water from polar and hydrocarbon groups involved in the binding almost exactly compensates for (i) the unfavorable entropy change due to the freezing out of four rotors of *N*-Ac-D-Ala-D-Ala upon binding and (ii) the unfavorable entropy change of a bimolecular association. A crude quantitation of these effects is presented. We also present an estimate of the increase in translational plus rotational free energy, as a function of the ligand mass, occurring when a ligand binds to a larger receptor. This quantity, fundamental to all binding processes, is relatively insensitive to the shape of the ligand. Extension of the approach will allow, in those cases where there is good complementarity between ligand and receptor, the prediction of approximate peptide–peptide binding constants in aqueous solution.

Introduction

Much research is currently being carried out to increase our understanding of the processes by which one molecule, **A**, specifically binds reversibly to another, **B**, in solution to give a complex **A·B**. However, an approach which, given the solution structures of **A**, **B**, and **A·B**, would allow an estimate (even if crude) of the binding constant for the association in the appropriate medium is not, so far as we are aware, available. In earlier work, fundamental contributions toward solving this problem have been made by Jencks and colleagues,[1-3] and it is on these contributions which we build.

In the following, we propose and apply an expression (eq 2) for estimating the free energy change (ΔG) upon association of **A** and **B**, where **A** and **B** exhibit good complementarity for binding. The general application of eq 2 will require the compilation of an extensive set of intrinsic binding constants for functional group interactions which are important in biology. Such intrinsic binding constants (which it is hoped will, to a useful approximation, be transferrable from one binding site to another) can be determined by experiment. As such, the approach will make a useful alternative approach to the elegant computational methods which are being developed.[4-7] Such computational methods are already able to lead us to an understanding of the difference in free energies of binding of closely related ligands and may in the future lead to the successful prediction of binding constants from theoretical principles.

The bimolecular association of **A** and **B** has an unfavorable entropy term since each of these components has three degrees of translational freedom and three degrees of rotational freedom prior to association, and these 12 degrees of freedom are reduced to only six (three of translation and three of rotation) in **A·B**. This reduction in entropy has been considered by Page and Jencks,[1] and they conclude that for reactions in solution, the unfavorable contribution to $T\Delta S$ at room temperature is ordinarily in the region of 50–70 kJ mol^{-1} (where one of the components in the association typically has a relative molecular mass in the range 100 to a few hundred mass units). This represents an adverse effect on binding of 10^x where x = 50–70/5.7 ≈ 9–12 (all ΔG, ΔH, and $T\Delta S$ values in the present paper can be converted to effects on binding constants in this manner). This conclusion is in good accord with our estimate for the specific system we discuss (see later) and for the generalized case (Appendix). Additionally when **A** and **B** associate, there is an overall loss of three degrees of rotational freedom, and the enthalpy loss from this rotation leads to an exothermicity of ca. 4 kJ mol^{-1} (see later for details). Together, these entropy and enthalpy terms give $\Delta G_{(trans+rot)}$, the change in translational and rotational free energy upon association; this is the first term in eqs 1 and 2.

$$\Delta G = \Delta G_{(trans+rot)} + \Delta G_{rotors} + \Delta H_{conform} + \sum \Delta G_i + \Delta G_{vdW} + \Delta G_H \quad (1)$$

$$\Delta G = \Delta G_{(trans+rot)} + \Delta G_{rotors} + \sum \Delta G_i + \Delta G_{vdW} + \Delta G_H \quad (2)$$

In the association of **A** and **B** to give **A·B**, the complex is frequently stabilized by several spatially separated polar interactions. The formation of these interactions commonly requires the freezing out of internal rotations of **A** and **B**. This gives rise to a second unfavorable free energy term, ΔG_{rotors} (largely associated with an adverse entropy change, though enthalpy does make a small contribution). Page and Jencks[1] conclude that it is reasonable to take a value in the range 5–6 kJ mol^{-1} per rotor frozen

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out upon complexation. This value is also in accord with our own estimate of the unfavorable free energy change for each rotor frozen out in a peptide backbone upon association, after allowance is made for residual torsional vibrations of these "frozen" rotors. It accounts for the second term in eqs 1 and 2.

In addition, new motions (e.g., soft vibrations) are generated in the **A·B** complex. In the present approach, the favorable effects upon binding of new soft vibrations in the complex (e.g., associated with hydrogen bond formation) are automatically included in the experimentally determined intrinsic binding constants (ΔG_i, see later).

In the general case for a bimolecular association, the conformational enthalpy of **A** and **B** will often be lower than the conformational enthalpy in the **A·B** complex. This is because the minimum energy conformations of **A** and **B**, available when each is free in solution, may have to be distorted in the **A·B** complex (e.g., if the **A** and **B** minimum energy conformations are not complementary). This possible difference (which is well-suited to assessment from molecular modeling studies) accounts for the third term ($\Delta H_{conform}$) in eq 1. If, however, **A** and **B** are able to associate with each in its conformational enthalpy minimum, then this term is zero (eq 2). $\Delta H_{conform}$ is the strain energy in the complex associated with the introduction of unfavorable bond lengths, bond angles, dihedral angles, etc. Essentially no strain is present in unbound **A** and **B**.

The $\Delta G_{(trans+rot)}$, ΔG_{rotors}, and $\Delta H_{conform}$ terms discussed above are all unfavorable for association. If association nevertheless occurs with a negative value of ΔG ($K_{association} > 1\ M^{-1}$), they must be outweighed by factors favorable for association. To a good approximation, just two (normally) favorable factors may be considered for the case of a nonaqueous solvent. First, we consider the free energies of interactions between polar functional groups in the complex. The challenge to measure experimentally such changes in free energies for functional group interactions involved in a binding site has been addressed by Jencks.[3] Briefly, if a receptor has a binding site for **X**, then simply to measure the association constant of **X** in this binding site does not measure its intrinsic affinity for the receptor, since for example the rotational and translational entropy of **X** (see above) must be lost before it can bind. The solution to this problem lies in choosing a receptor which will bind **XY** and also **Y** (Figure 1). If the free energies of binding of both these entities are measured, then the difference between them gives the intrinsic free energy of binding of **X** to the receptor (if a correction is made for the small difference in translational plus rotational entropy of **Y** and **XY** and for any rotational motions in the connectivity of **X** and **Y**). The approach does of course further require that **X** and **Y** bind into their binding sites in the same way both when separately bound and jointly bound as **X·Y**. This is an exacting requirement, but one which we believe is adequately met in the systems we shall discuss, and indeed may be met in many instances where **X·Y** is a natural substrate (or closely related to a natural substrate) of the receptor. The important point about this experiment is that since the binding of **X** was assessed when it was attached to **Y**, the large effect of its rotational and translational entropy is removed. Thus, if this intrinsic binding energy can be determined for every portion of a component involved in a binding interaction or if the appropriate intrinsic binding energies are known from prior work, then the sum of these terms can be used in eqs 1 or 2. This sum ($\sum \Delta G_i$) is the fourth term in eq 1 and the third term in eq 2. $\sum \Delta G_i$ contains all the interactions between polar (non-hydrocarbon) groups. It is emphasized that intrinsic binding energies have the property of being parameters of essentially the same value from system to system (for a similar environment and the same solvent). For example, we derive later, from calorimetric data, the binding constant for amide–amide hydrogen bond formation in aqueous solution.

The second (normally) favorable term for binding arises because the molecular packing in a complex (formed either intramolecularly or intermolecularly) may be more efficient than that of the dissociated form with the solvent. That is, the van der Waals interactions are better in the complex. We designate this change ΔG_{vdW}, and it is the fifth term in eq 1 and the fourth in eq 2. This term will, for example, normally be favorable in protein folding, since the hydrophobic interior of a globular protein frequently has a packing efficiency which makes this interior almost akin to a hydrocarbon solid. It need be considered only for the hydrocarbon-to-hydrocarbon interactions in the complex, since improved packing efficiency of polar–polar interactions will automatically be taken care of in the experimentally measured ΔG_i values. If complementarity and good packing between nonpolar surfaces is not achieved, ΔG_{vdW} will be positive and will oppose binding.

The last term to be considered is relevant only to binding in aqueous solutions. It is due to the hydrophobic effect. From our considerations of thermodynamic data on the solubilities of hydrocarbons in water,[8-10] and guided by the earlier work of others,[11,12] we have concluded[13] that the hydrophobic effect stabilizes the **A·B** complex by 0.125 kJ mol^{-1} for every square Angstrom (Å^2) of hydrocarbon removed from water accessibility upon complex formation (where surface areas are calculated with a low data point density on MACROMODEL[4]). This quantity ($0.125A_H$) is, where the structures of free and bound components are known, also well-suited to determination from molecular modeling experiments. Alternatively, if calorimetric experiments over a range of temperatures have allowed the determination of the change in heat capacity (ΔC_p) associated with the binding process, then ΔC_p (J K^{-1} mol^{-1})/1.6 gives the area of hydrocarbon removed from water accessibility upon binding.[13] Since the hydrophobic effect (ΔG_H) is entropy driven at room temperature, this final term ($0.125A_H$, term 6 in eq 1, and term 5 in eq 2) favors binding entropically. It is physically associated with the release of ordered water from the surface of hydrocarbon which is accessible to water before binding but not accessible to water after binding. There are thus two terms for the contribution of nonpolar groups to binding. ΔG_H gives the transfer free energy of a nonpolar group from water to a nonpolar solvent, when can be readily measured and calculated. ΔG_{vdW} gives the free energy of transfer from a nonpolar solvent to the binding site. In nonaqueous solvent, therefore, only ΔG_{vdW} applies.

The molecular description of binding processes demands an understanding of that portion of the binding process which is driven by changes in electrostatic energies (enthalpy changes, whereby heat may be released to randomize the surroundings of the system under study) and that portion of the binding process which is driven by entropy changes. To summarize the relative enthalpy and entropy contributions where ligand and receptor are not fully complementary (eq 1): terms 1 and 2 are largely based on entropy and unfavorable for association, term 3 is exclusively based on enthalpy and also unfavorable for association, and terms 4, 5, and 6 are favorable for association—term 4 entropically and/or enthalpically, term 5 enthalpically, and term 6 entropically at room temperature.

The simpler equation (eq 2) is applicable in those cases where ligand and receptor show good complementarity and no strain in the bound complex. It should be appropriate for the analysis of binding sites which show good complementarity and no strain by virtue of having evolved under the pressure of natural selection to maximize the binding strength. The partitioning of the free energy into five terms in the equation is justified in terms of an analytical breakdown of binding into five features: (i) the probability of bringing ligand and receptor together, (ii) the probability of orienting the ligand appropriately once it is together with the receptor, (iii) the free energy of electrostatic interactions in the complex relative to the prior ones with solvent (and including the favorable release of solvent molecules), (iv) the change in hydrocarbon-to-hydrocarbon packing efficiency in the complex

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(relative to hydrocarbon-to-solvent packing efficiency in unassociated states), and (v) the favorable release of water from hydrocarbon surfaces buried in the binding process. It is the only equation applied in the present work.

In the following sections, we use calorimetric data and the temperature variation of binding constants to separate entropy and enthalpy contributions and so derive a relatively detailed molecular description of a peptide–peptide association in aqueous solution. All the relevant generalizations do not appear to have been tested together previously, although Andrews et al.[14] have enunicated the principles involved for a large number of drugs in the absence of detailed knowledge (or, due to necessity in most cases, any knowledge) of the receptors involved. For interactions occurring at a well-defined receptor, eq 2 therefore appears to give for the first time a basis for making comparisons between theory and experiment by this approach.

The System Used for Analysis of a Peptide/Peptide Association in Aqueous Solution. We now turn to a system suitable for the application of eq 2: the interaction of the antibiotics vancomycin and ristocetin A with cell wall mucopeptide precursors terminating in D-Ala-D-Ala, and related analogues, in aqueous solution. Equation 2 (rather than eq 1) is applied for the following reasons. First, vancomycin and ristocetin A undergo only restricted conformational change upon binding to the cell wall analogues. Yet it is true that the N-terminal regions of both antibiotics do change their conformations upon association with the cell wall analogues[15-17] and that the sugars of the antibiotics can subtly influence the binding[16,18] and may thereby modify their rotational motion upon ligand binding. Thus, this is a simplifying but not totally realistic assumption. However, it is an assumption which should not compromise conclusions based upon comparisons between the binding of two related ligands to the same antibiotic. Second, *N*-Ac-D-Ala-D-Ala appears to bind to the antibiotics in a conformation which is close to the energy minimum found for the free peptide (cf. the X-ray structure of *N*-Ac-L-Ala-L-Ala[19]). Third, space-filling models indicate a close complementarity between those portions of the peptides and antibiotics which come together in forming a 1:1 complex between them. The simplification of being able to approximate $\Delta H_{conform}$ to zero is an important one, since the separation of the binding energy into the components of eqs 1 and 2 may well be less reliable in the absence of good ligand/receptor complementarity. Last, in applying eq 2 in the analysis of this particular receptor–ligand interaction, we assume $\Delta G_{vdW} = 0$, since we are dealing with a small ligand that is seen to have good complementarity of hydrocarbon–hydrocarbon interactions with the receptor, but these are not so extensive as to give the kind of cooperativity found in solids.

It is well-established that, in the complex formed by ristocetin A and *N*-Ac-D-Ala-D-Ala,[20] the cell wall analogue is oriented relative to the antibiotic as shown in Figure 2. In the figure, dotted lines indicate hydrogen bond formation between the carbonyl group of one component and an NH group of the other component. It can be seen that in the proposed complex, the carboxyl group of the C-terminal alanine of the cell wall analogue forms no less than three hydrogen bonds to three NH groups which lie in a pocket, bounded by substituted benzene rings, at one end of the antibiotic structure. As these interactions occur, additional hydrogen bonds can be formed between the carbonyl oxygen of the acetyl group of the cell wall analogue and an NH which is seen at the left-hand part of the antibiotic structure and between

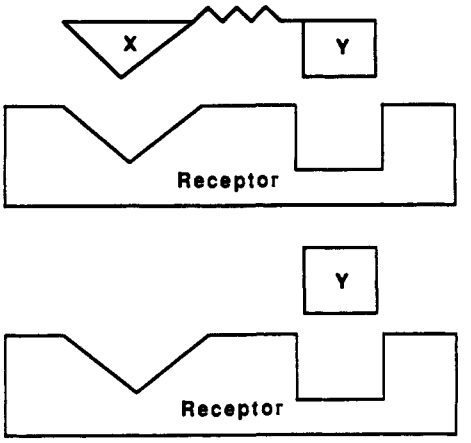

Figure 1. Schematic representation of a receptor which will separately associate with Y and XY, and where X can thereby be presented into its binding site without a correction for rotational and translational entropy. If the binding constant for Y is K_Y, and for XY is K_{XY}, then the intrinsic binding constant of X is K_{XY}/K_Y (after correction for any internal rotors in XY which may have to be frozen out in order to present X appropriately to the receptor).

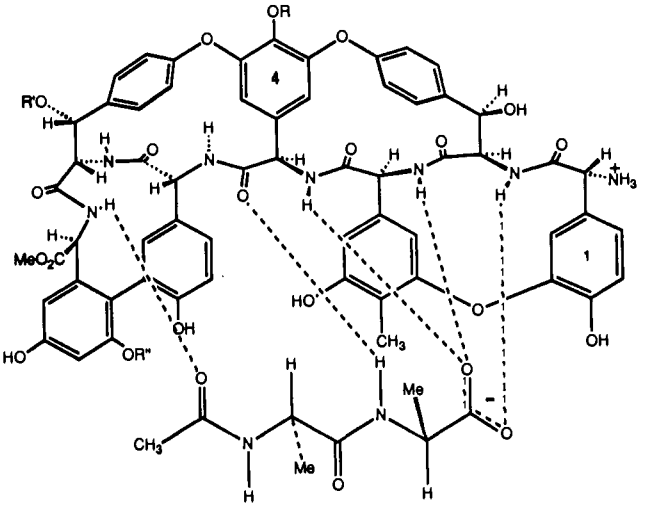

Figure 2. A model of the interaction between ristocetin A and the bacterial cell wall analogue *N*-acetyl-D-Ala-D-Ala. The broken lines indicate intermolecular hydrogen bonds formed on complexation.

the NH of the C-terminal alanine and a carbonyl group of the antibiotic. The geometry of these hydrogen-bonded interactions allows simultaneously favorable hydrophobic interactions to occur between the two alanine methyl groups of the cell wall analogue and parts of the benzene rings of the antibiotic. The above model for the binding interaction has been checked by use of the powerful technique of intermolecular nuclear Overhauser effects (NOEs).[20] The NOE data for the ristocetin–dipeptide complex showed that the binding model represented in Figure 2 is indeed correct in its essential details. Those protons of the antibiotic and cell wall analogue which are demanded to be near in space by the binding model do give mutual NOEs. The complex between vancomycin and the dipeptide forms all the analogous hydrogen bonds, the major difference between the two complexes being that the hydrophobic wall of the carboxylate anion pocket formed by the substituted benzene ring side chain of residue 1 in Figure 2 is replaced by the side chain of *N*-methylleucine in the case of the vancomycin complex. Thus, the molecular basis of action of the antibiotics is well-founded. Additionally, by utilizing, and extending, earlier work by Nieto and Perkins,[21-23] it has been possible

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to measure the binding constant between the two components by the use of UV spectroscopy. In the following work, we also use circular dichroism (CD) to measure binding constants.

The Intrinsic Binding Constant (ΔG_i) for Amide–Amide Hydrogen Bond Formation. In the antibiotic/peptide complexes, there are two amide–amide hydrogen bonds (see the first two dotted line from the left in Figure 2), reminiscent of the hydrogen bonds found in β-sheets of proteins. In addressing the estimation of an intrinsic binding constant (ΔG_i) for such amide–amide hydrogen bonds, we discuss first the interaction as it occurs unimolecularly in proteins, and second as it occurs bimolecularly in a peptide–peptide interaction. Since intrinsic binding constants have the property of measuring binding after overcoming the adverse free energy of bringing the interacting groups into the appropriate position, they will be the same for unimolecular and bimolecular association.

A. The Amide–Amide Bond Formed Unimolecularly. Comments on the β-Sheet Structure of Proteins. There is experimental evidence that isolated α-helix and β-sheet structures are, prior to tertiary interactions, populated only to a similar extent as the unfolded structures.[24] Thus, the free energies of such subunits do not differ greatly from those of their unfolded counterparts (i.e., $\Delta G \approx 0$ kJ mol^{-1}). We make the simplifying assumptions that the structures of β-sheets do not involve significant van der Waals repulsions and that they can be nucleated without a critical reliance on the amino acid side chains. Given these assumptions, then the main adverse free energy change is that of organizing the peptide backbone into a β-sheet structure by freezing out two rotors for each amino acid to be incorporated in the organized structure. The rotors which must be frozen out are the N–C_α and C_α–CO–bonds. For the freezing out of a free rotor, the unfavorable free energy change has been estimated (see earlier), and in the case of a short peptide backbone calculated[25] to be ca. 5 kJ mol^{-1} at room temperature (taken as 300 K). According to our calculations, this change can be taken to a good approximation to correspond almost completely to an unfavorable entropy change ($T\Delta S$ ca. 5 kJ mol^{-1} at 300 K). Although there is formally a favorable exothermicity (due to the passing of the kinetic energy of the rotor to its surroundings) of ca. 2.5 kJ mol^{-1},[25] we take this as being offset by a roughly equal and opposite retention of heat in the residual torsional vibration of the rotor.

Since we argue that ΔG for formation of a short isolated β-sheet is near zero per residue, then ca. 10 kJ mol^{-1} must be almost exactly counterbalanced by favorable changes. These are (i) the enthalpy term [ΔH(H)] for the formation of an amide–amide hydrogen bond and (ii) the entropy term [$T\Delta S$(W)] for the water released by the formation of this hydrogen bond. Since in the formation of an isolated β-sheet, one hydrogen bond is formed per two residues, then

$$\Delta H(\mathrm{H}) - T\Delta S(\mathrm{W}) + 20 = 0 \text{ kJ mol}^{-1} \qquad \text{at 300 K} \quad (3)$$

Thus, the intrinsic binding energy associated with amide–amide hydrogen bond formation is estimated to be ca. 20 kJ mol^{-1}. (We recognize that eq 3 and all our following relationships are approximations, but it will be seen later that these approximations lead to predictions which are in good accord with experimental measurements.)

B. The Amide–Amide Bond Formed Bimolecularly. The intrinsic binding constant for the amide–amide bond formed bimolecularly can be measured in principle through comparison of the binding free energies of two ligands which differ in their binding to a common receptor by deletion of such a bond. To do this, we first describe [sections (i) and (ii)] the general strategy for comparing the binding parameters for differing ligands to a given receptor. In section (iii), we apply this strategy to deduce the intrinsic binding constant.

Table I. Calculated Gas-Phase Rotational and Translational Free Energies (kJ mol^{-1}, at 300 K) of the Receptor and Selected Ligands

compound	no. waters "bound"	G_{trans}	G_{rot}	$G_{trans+rot}$
acetate	5	−44.5	−34.3	−78.8
alanine	8	−46.5	−37.6	−84.1
glycolate	6	−45.6	−35.7	−81.3
lactate	6	−45.8	−36.4	−82.2
N-Ac-D-Ala	8	−47.1	−39.2	−86.3
N-Ac-D-Ala-D-Ala	11	−48.5	−42.1	−90.6
ristocetin A	33	−55.6	−54.5	−110.3

Table II. Semiempirical Values of $G_{(trans+rot)}$, $TS_{(trans+rot)}$ and $H_{(trans+rot)}$ (kJ mol^{-1}, at 300 K) of Ristocetin A and Selected Ligands

compound	$TS_{(trans+rot)}$	$H_{(trans+rot)}$	$G_{(trans+rot)}$
acetate	61	7	−54
alanine	65	7	−58
glycolate	62	7	−55
lactate	63	7	−56
N-Ac-D-Ala	67	7	−60
N-Ac-D-Ala-D-Ala	70	7	−63
ristocetin A	85	7	−78

(i) Corrections for Differences in the Translational and Rotational Free Energies of the Ligands. In comparing the difference in binding parameters for two ligands to a receptor, allowance must be made for their different rotational and translational free energies, due to their different moments of inertia and mass. We do this on a semiempirical basis. Prior work,[26] and data presented subsequently in this paper, suggests that in water the following polar functional groups behave as though they carry the following numbers of water molecules: NHCO (3), CO_2^- (5), NH_3^+ (3), and OH (1). This is of course an average behavior, representing a dynamic attachment to a larger set of transiently attached water molecules. However, the above numbers seem appropriate to reflect the fact that peptide molecules in aqueous solution will have larger effective masses and moments of inertia than their unsolvated counterparts. With these descriptions, the gas-phase rotational and translational free energies of relevant ligands which bind to the antibiotics can be calculated (Table I). The rotational free energies were calculated with MACROMODEL;[4] the gas-phase translational free energies were calculated from the Sackür–Tetrode equation.[27] However, experimental determinations of both liquid- and gas-phase entropies show that liquid states commonly have lower entropies than gases by $T\Delta S$ about 25 kJ mol^{-1} at 300 K (Trouton's rule); the unusual order existing in liquid H_2O relative to gaseous H_2O is reflected by a larger $T\Delta S$ of 33 kJ mol^{-1}.[28] These differences arise in part because of the volume decrease occurring upon condensation (1 atm to neat liquid) and in part because of the greater order existing in liquids. Since the ligands and antibiotics considered in this paper are in aqueous medium and will partly appear to be analogous to water due to their extensive solvation, we first reduce the calculated gas-phase entropies of the solvated species by $T\Delta S = 30$ kJ mol^{-1} (a value bounded by the two above values). Since the semiempirical values thus obtained correspond to hypothetical neat liquids, we finally add to the derived values $2.3RT \log_{10} 1000/\mathrm{RMM}$ to allow for the entropy change at a molar concentration. The results, separated into enthalpy ($3RT$) and entropy contributions ($T\Delta S$), are presented in Table II. The $3RT$ term finds its origins in (i) $RT/2$ for the kinetic energy in each rotational degree of freedom and (ii) $RT/2$ for the kinetic energy in each translational degree of freedom.

The data (Tables I and II) illustrate the (relatively small) effect of increasing mass and increasing moments of inertia. Because of these relatively small effects, when small ligands bind to much

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(25) Doig, A.; Williams, D. H. Unpublished work; the unfavorable free energy change for freezing out a rotor of a long peptide backbone (such as found in a protein) rises to ca. 10 kJ mol^{-1}, due to the larger associated momenta of inertia.

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(27) See, for example: Atkins, P. W. *Physical Chemistry*; Oxford University Press: 1978; Chapters 20 and 21.

(28) Atkins, P. W. *Physical Chemistry*; Oxford University Press: 1978; p 134.

larger receptors, as in the present work, the rotational and translational entropy of the resulting complex is not significantly different from that of the receptor itself. Thus, on complexation of the ligand, the loss in rotational and translational entropy is well approximated by the value for the ligand alone. Indeed, in the present case, when allowance is made for the loss of water molecules from the ligand and receptor upon complex formation, the value of $T\Delta S_{(trans+rot)}$ is the same for the receptor and the complex within 1 kJ mol^{-1}. On the other hand, in any process in which two molecules combine to give one complex, the overall loss of three degrees of rotational freedom implies an intrinsic exothermicity (due to loss of rotational enthalpy) of $3RT/2$. However, perhaps surprisingly, when two molecules associate to give a complex through the action of weak intermolecular forces, there is no decrease in the average translational kinetic energy. It has been pointed out[29] that the average translational kinetic energy of a molecule is $3kT/2$ irrespective of whether it is in the gas, liquid, or solid state; the molecule's motion is merely restricted to a narrower region of space around a potential energy minimum. Thus, the resulting exothermicity is due to the loss of three degrees of rotational freedom only ($3RT/2$, 4 kJ mol^{-1}).

In conclusion, the unfavorable rotational and translational free energy change for all the bimolecular associations we consider is obtained simply by taking the free energy column for the ligands in Table II, inverting the sign, and increasing the resulting value by 4 kJ mol^{-1}, i.e., for *N*-Ac-D-Ala and *N*-Ac-D-Ala-D-Ala, the values are 64 kJ mol^{-1} and 67 kJ mol^{-1}. We emphasize that although the approach is a simple approximation, the entropy changes depend on the logarithms of the masses and moments of inertia involved in such a way that they are relatively insensitive to the details of the model assumed. This is particularly true where comparisons are being made between the binding of ligands to the same receptor. In these cases, we find that the change in the loss of rotational and translational free energy on binding different ligands is the same within 2 kJ mol^{-1}, independent of the precise details of the model.

The adverse change in translational and rotational free energy ($\Delta G_{trans+rot}$) when a ligand of a given molecular mass binds to a large receptor is a quantity of such fundamental importance that we give our semiempirical estimates for this quantity (Appendix, Figure 7). Fortunately, to a useful approximation, this quantity is independent of the shape of the ligand (Appendix).

(ii) Corrections for the Change in Numbers of Free Rotors and Hydrophobic Interactions between the Ligands. In general, the binding of ligands of differing size to a given receptor will involve the freezing out of different numbers of ligand rotors and occasionally will involve different hydrophobic interactions. Correction must be made for these differences, with the appropriate values given in the Introduction.

(iii) The Intrinsic Binding Free Energy, Enthalpy, and Entropy for the Bimolecular Amide–Amide Interaction from the Acetyl Carbonyl Group of *N*-Ac-D-Ala-D-Ala to the Antibiotics (Figure 2). For a molecular description of this process, analogous to the same hydrogen bond formed in β-sheets, we need to know whether this process is enthalpy or entropy driven (or both). To obtain the intrinsic binding enthalpy and entropy for the hydrogen bond to the acetyl carbonyl group, we compare the thermodynamic binding parameters for *N*-Ac-Gly-D-Ala and *N*-Ac-D-Ala, since one of the changes involved in binding the latter relative to the former ligand is loss of the hydrogen bond under consideration (cf. Figure 2). In comparing the binding of the two ligands, we note that only two of the C–H bonds of the acetyl methyl group of *N*-Ac-D-Ala interact strongly with the antibiotics at any one instant and that these interactions are retained in the *N*-Ac-Gly-D-Ala/antibiotic complex. In the latter complex, the *N*-acetyl methyl group points away from the antibiotic and is therefore assumed not to perturb the binding significantly.

We need the differences in enthalpy of binding [$\Delta(\Delta H)$] and entropy of binding [$\Delta(T\Delta S)$] of the two ligands. The former quantity will give the enthalpy [$\Delta H_{H\text{-}bond}$] of the hydrogen bond. The relevant data are available from a comparison of the calorimetric data of Rodriguez-Tebar et al.[30] on the binding of *N*-Ac-D-Ala and *N*-Ac-Gly-D-Ala to vancomycin and ristocetin A. The experimental $\Delta(\Delta H)$ [or $\Delta H_{H\text{-}bond}$] values are -3.4 ± 2.8 in the case of ristocetin and $+1.4 \pm 2.3$ kJ mol^{-1} in the case of vancomycin, at 298 K. The important point about these values is that they are both very small, with a mean exothermicity of -1 kJ mol^{-1}. Thus, it is concluded that amide–amide hydrogen bond formation in water is associated with an enthalpy change close to zero.

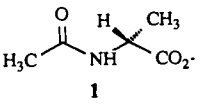
In contrast, the calorimetric data of Rodriguez-Tebar et al.[30] also show that the entropy change associated with the formation of the same hydrogen bond, and concurrently freezing out the two rotors of glycine necessary to make this hydrogen bond, is (in terms of $T\Delta S$ at 25 °C) larger: 11 ± 3 kJ mol^{-1} in the case of vancomycin and 8 ± 4 kJ mol^{-1} in the case of ristocetin. Thus, the mean entropy change is ca. 10 kJ mol^{-1}. This change in the entropy of binding will originate from three sources. First, *N*-Ac-D-Ala-D-Ala will be captured with greater difficulty than *N*-Ac-D-Ala by the receptor site of the antibiotic due to the larger rotational and translational free energy of the former; we estimate this difference to be 3 kJ mol^{-1} (Table II). Second, we have calculated that the freezing out of the two rotors of glycine is entropically unfavorable by ca. 10 kJ mol^{-1} at room temperature (see above). Third, there will be the entropy change (y) associated with hydrogen bond formation. Thus

$$10 = y - 3 - 10; \qquad \text{therefore } y = 23 \text{ kJ mol}^{-1}$$

We conclude that in these systems (and by extrapolation in isolated β-sheets in general), amide–amide hydrogen bond formation in aqueous solution is essentially an entropy driven process, the favorable entropy change being associated with water release from the C=O and NH groups which associate. The reasonable consistency between the intrinsic binding energy ($\Delta G = \Delta H - T\Delta S = -1 - 23 = -24$ kJ mol^{-1}) for amide–amide hydrogen bond formation based on the mean data for the two experiments (and coupled to the freezing out of two rotors), with that estimated earlier on the basis of freezing out four rotors alone (−20 kJ mol^{-1}), supports our estimate for the free energy cost of freezing out the backbone rotors of a short peptide. In the light of these values, we take 24 kJ mol^{-1} for the intrinsic binding energy of this hydrogen bond. This corresponds to an intrinsic binding constant of ca. 10^4 M^{-1} at room temperature.

Approximate Binding Constants Estimated for Acetate Anion

A. Application of Eq 2 To Calculate the Free Energy of Acetate Binding to Vancomycin. We now use the approximate intrinsic binding parameters for amide–amide hydrogen bond formation ($\Delta H = -1$ kJ mol^{-1}, $T\Delta S = 23$ kJ mol^{-1} at 25 °C) to estimate the approximate binding constant for acetate anion to ristocetin A, based on the terms listed in eq 2. The thermodynamic data, obtained by the relatively precise method of calorimetry,[30] for the binding of *N*-Ac-D-Ala (**1**) are $\Delta H = -28.3 \pm 1.4$ kJ mol^{-1}, $T\Delta S = -11 \pm 2$, and $\Delta G = -17.4 \pm 0.5$ kJ mol^{-1} at 25 °C. The amide



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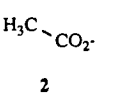

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NH of **1** is assumed, by analogy to the known mode of binding of *N*-Ac-D-Ala-D-Ala, to be bound by hydrogen bond formation to an amide carbonyl of the antibiotic (Figure 2). We estimate first the enthalpy of binding (x) of acetate (**2**). Since **2** should tend to bind less exothermically than **1** because of the small exothermicity (1 kJ mol^{-1}) of hydrogen bond formation, then

$$x = -28.3 \pm 1 \approx -27 \text{ kJ mol}^{-1}$$

(29) Israelachvili, J. N. *Intermolecular and Surface Forces*; Academic Press: London, 1985; p 21.

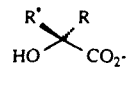
(30) Rodriguez-Tebar, A.; Vazquez, D.; Perez Velazquez, J. L.; Laynez, J.; Wadso, I. *J. Antibiot.* **1986**, *39*, 1578–1583.

If $T\Delta S$ for the binding of acetate is y, then this must be increased by the intrinsic binding entropy for hydrogen bond formation (23 kJ mol^{-1}) but decreased by 10 kJ mol^{-1} to allow for the freezing out of two rotors of **1**. Additionally, the rotational and translational entropy of **1** is larger than that of acetate by 6 kJ mol^{-1} (Table II). Finally, the methyl of the acetyl group of **1** and the methyl group of the alanine residue make hydrophobic interactions with the antibiotic. From molecular modeling studies, we estimate that the partial removal of the acetyl methyl group from water will increase binding by 2 kJ mol^{-1}. The increase in binding energy due to the methyl group of the C-terminal alanine is 9 kJ mol^{-1}. This value is reliably based on the average increase in binding energy of 9 kJ mol^{-1} found for *N*-Ac-D-Ala over *N*-Ac-Gly, *N*-Ac-D-Ala-D-Ala over *N*-Ac-D-Ala-Gly, or di-*N*-Ac-Lys-D-Ala-D-Ala over di-*N*-Ac-Lys-D-Ala-Gly, by various methods in numerous laboratories (Table III). The calorimetric data of Rodriguez-Tebar et al.[30] show that the decrease in binding energy caused by substitution of C-terminal Gly for D-Ala is very largely (83%) entropy driven, as required for a hydrophobic effect at room temperature. In summary, we conclude that removal of the acetyl methyl and alanine methyl groups decreases the binding energy of **2** relative to **1** by 11 kJ mol^{-1} and that to a good approximation this is an entropy effect. Thus

$$y + 23 - 10 - 6 + 11 = -11, \text{ i.e., } y = -29 \text{ kJ mol}^{-1}$$

This estimated unfavorable entropy of binding of acetate is essentially equal and opposite in influence to the estimated favorable enthalpy of binding given above. Thus, the estimated free energy of binding of acetate anion is approximately zero (K = 1 M^{-1}), and the loss in binding energy on passing from **1** to **2** is ca. 19 kJ mol^{-1}. Considering the simplicity and approximations involved, the approach could be considered successful if the experimentally determined binding constant for acetate lay in the range 0.1–10 M^{-1} (ΔG = +6 to −6 kJ mol^{-1}). Moreover, we predict that this ΔG value should be constituted from a relatively large negative enthalpy of association (ca. 27 kJ mol^{-1}), offset by an almost equal $T\Delta S$ term at 298 K. This follows since the differences in binding affinity between *N*-Ac-D-Ala and acetate are due to changes in amide–amide hydrogen bonding, hydrophobic interactions, the freezing out of rotors, and the change in translational plus rotational free energy—all terms which have previously been ascribed as arising almost exclusively from entropy effects. This prediction of the magnitudes of ΔH and $T\Delta S$ for acetate binding is a test of our approach in addition to the actual value of the binding constant. An analogous approach to the binding of **2** to vancomycin is summarized in Table IV (with data also presented in Table V), giving rise to the prediction of a binding constant in the range 0.1–10 for acetate anion in this case also. Once more it is predicted that the binding will be largely enthalpy driven but opposed by a $T\Delta S$ term of similar magnitude. In the following section, we show experimentally that these order of magnitude predictions are correct, and thereby are consistent with (but do not prove) the validity of the approach.

B. Experimental Determination of Acetate Binding Parameters. (a) Free Energy of Binding. It is first necessary to search for evidence that simple carboxylate anions do indeed bind, albeit weakly, at the expected site in the antibiotics. Acetate anion does not contain a convenient proton probe for binding, but the simple lactate enantiomers **3a** and **3b** offered promise in this respect.



3a: R = H, R' = CH_3

3b: R = CH_3, R' = H

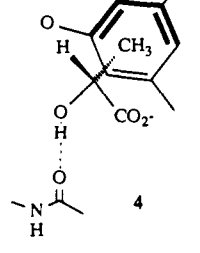


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Table III. Differences in Free Energies of Binding (kJ mol^{-1}) of Ligands to Ristocetin in Which Glycine is Substituted for Alanine[a]

technique	$\Delta\Delta G$ for Ala → Gly at C-terminus	$\Delta\Delta G$ for Ala → Gly at non-C-terminal residue	comments
UV[23]	8.9	0.8	*b*
UV[31]	10.0	4.6	*c*
solid phase assay[31]	8.4	2.9	*c*
calorimetry[30]	11.4	0.4	
UV[23]	8.2	3.3	*b, d*
UV	7		*e*

[a] The comparison ligand is *N*-Ac-D-Ala-D-Ala, and the receptor is ristocetin A, unless otherwise stated. [b] Receptor ristocetin B. [c] Ligand *N*-benzoylated rather than N-acetylated. [d] Ligand di-*N*-Ac-Lys-D-Ala-D-Ala. [e] Present work, from comparison of binding of *N*-Ac-D-Ala and *N*-Ac-Gly.

D-Lactate (**3b**) should bind more strongly than acetate (experimentally ΔG = −5 kJ mol^{-1}, see later) to ristocetin A by (i) the magnitude (x) of the intrinsic binding constant (see **4**) of a hydroxyl group to an amide carbonyl (now known to be associated with a negative ΔG[34]) and (ii) the magnitude of the hydrophobic effect between the lactate methyl group and the benzene ring of residue 4 of ristocetin [9 kJ mol^{-1} by analogy to *N*-Ac-D-Ala-D-Ala (Table III)]. Against these advantages for the binding of lactate must be set (i) the entropic disadvantage of freezing out in **3b** the CH–CO_2^- rotor (ca. 5 kJ mol^{-1}) and the CH–OH rotor (only ca. 3 kJ mol^{-1}, due to the smaller associated moments of inertia), and (ii) the disadvantage of binding a larger ligand whose translational and rotational entropy is greater by 2 kJ mol^{-1} (Table II). The minimum energy mode of binding (see **4**) will place the methyl group of D-lactate in the shielding region of the benzene ring of residue 4, and the resulting degree of shielding will be a measure of the binding constant. The relevant data for **3** and related simple bifunctional carboxylates are summarized in Table VI; several points arise out of the data. First, the chemical shift changes of the methyl groups of the optically active carboxylates upon association with the antibiotic are in all cases more to higher field for the D-isomers than for the L-isomers. These data not only demand that the carboxylates are associating into an asymmetric environment but also are in accord with the anticipated geometry of binding (**4**). Second, if it is assumed that the limiting upfield shift of the methyl group of D-lactate would be the same as is observed for the methyl group of the C-terminal D-alanine of di-*N*-Ac-L-Lys-D-Ala-D-Ala (0.8 ppm,[33]), then the derived binding constant of D-lactate is 70 M^{-1} (this assumption could in principle be a poor approximation, but a much more reliable binding constant (100 M^{-1}), determined by UV spectroscopy,[34] in fact agrees with it quite closely). Third, the relatively small upfield shift of the methyl group of D-alanine suggests that this ligand binds more poorly into the binding site (see **4**, with OH replaced by NH_3^+) than both D-lactate and D-thiolactate (Table V). This finding initially surprised us, since it was felt that the NH_3^+—O=C—NH— interaction would have a large intrinsic binding constant. However, this assumption turns out to be false in the present binding pocket, as has been confirmed by the low binding constant for D-alanine (<10 M^{-1}) determined by UV spectroscopy.[34] The large upfield shift of one of the methyl groups of prochiral α-hydroxyisobutyrate but only small shifts of the methyl groups of α-aminoisobutyrate (Table VI) are also in accord with a weaker intrinsic binding constant for an ammonium ion to the residue 4 amide CO than for a hydroxyl group (see Figure 2).

(31) Smith, P. W.; Chang, Y.; Still, W. C. *J. Org. Chem.* **1988**, *53*, 1587–1590.
(32) Brown, J. P.; Ternius, Y.; Feeney, J.; Burgen, A. S. V. *Mol. Pharmacol.* **1975**, *11*, 126–132.
(33) Williamson, M. P.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 1* **1985**, 949–956.
(34) Williams, D. H.; Cox, J. P. L.; Nicholls, I. A. Unpublished work.

Table IV. Prediction of the Thermodynamic Parameters of Binding of Acetate Anion to Vancomycin from the Free Energy of Binding of *N*-Ac-D-Ala

	differences				
	rotors	H[a]	(T + R)[b]	HB[c]	
ΔH(*N*-Ac-D-Ala)					predicted ΔH(acetate)
−30.6 ± 1.2[d]				+1	−29.6
$T\Delta S$(*N*-Ac-D-Ala)					predicted $T\Delta S$(acetate)
−17 ± 2[d]	+10	−7	+6	−23	−31

[a] H = hydrophobic effect; the value of 5 kJ mol^{-1} is taken for the alanine methyl group, representing the average of six experimental determinations (Table V), and, as in the case of ristocetin, 2 kJ mol^{-1} is allowed for the interaction of the acetyl methyl group with CH groups of residues 5 and 6 of the antibiotic which underlie it. [b] The difference in rotational and translational entropy of the two substrates (Table II). [c] The intrinsic enthalpy or entropy for the hydrogen bond formation. [d] Data from ref 30.

Table V. Differences in Free Energy of Binding (kJ mol^{-1}) of Ligands to Vancomycin in Which Glycine is Substituted for Alanine[a]

technique	$\Delta\Delta G$ for Ala → Gly at C-terminus	$\Delta\Delta G$ for Ala → Gly at non-C-terminal residue	comments
UV[23]	3.2	1.4	
calorimetry[30]	6.1	2.0	
UV[31]	4.6	2.1	*b*
solid phase assay[31]	5.0	2.5	*b*
NMR[32]	6.3	2.1	
UV[23]	6.1	6.1[d]	*c*

[a] The comparison ligand is *N*-Ac-D-Ala-D-Ala unless otherwise stated. [b] Ligand N-benzoylated rather than N-acetylated. [c] Ligand di-*N*-Ac-Lys-D-Ala-D-Ala. [d] This value, considerably larger than the others, should not be regarded as anomalous; in the case of this tripeptide cell wall analogue binding to ristocetin, it has been shown[33] that the hydrocarbon side chains of lysine and the non-C-terminal alanine interact. This interaction probably does not give the optimum hydrophobic stabilization in this case due to the proximity of mannose. In the case of vancomycin, the same interaction can occur in the absence of any steric encumbrance by mannose.

Table VI. Proton Chemical Shift Changes (ppm) for Methyl Group Probes in Carboxylate Anions of the General Formula RR′CHCO$_2^-$ due to Association with Ristocetin A[a]

compound	R	R′	δ	compound	R	R′	δ
D-lactate	CH_3	H	−0.33	L-lactate	H	CH_3	−0.17
D-alanine	CH_3	H	−0.11	L-alanine	H	CH_3	−0.05
D-thiolactate	CH_3	H	−0.25	L-thiolactate	H	CH_3	−0.18
α-hydroxy isobutyrate	CH_3	H	−0.26 −0.10	α-amino- isobutyrate	CH_3	CH_3	−0.07 −0.06

[a] Chemical shift changes were measured for 20 mM solutions of ligand in the presence and absence of an equimolar quantity of ristocetin. All chemical shifts were measured relative to external dioxan at 295 K.

In the light of the above NMR evidence that simple carboxylates bind in the same manner as acetylated mono- and dipeptides, we next determined the binding constant for acetate anion. In using UV difference spectroscopy to measure these binding constants, it was encouragingly observed that the binding of *N*-Ac-D-Ala and acetate gave similar UV difference spectra: for the two ligands to ristocetin A, λ_{max} = 250 and 290 (*N*-Ac-D-Ala) and λ_{max} = 250 and 287 nm (acetate) and to vancomycin, λ_{max} = 246, 273, 282 (*N*-Ac-D-Ala) and λ_{max} = 247, 273, 282 nm (acetate). These observations constitute further evidence that the ligands are binding in the same binding site. The binding constant to ristocetin A, as determined by UV spectroscopy, was 5 ± 4 M^{-1} and, as determined CD, was 13 ± 5 M^{-1}. The binding constant to vancomycin, as determined by UV spectroscopy, was 12 ± 6 M^{-1} and, as determined by CD, was 16 ± 6 M^{-1}. Relevant experimental data are given in Figures 3 and 4. These values agree with the binding constants predicted from our semiempirical analysis to within 6 kJ mol^{-1}. This remarkably good agreement supports the validity and utility of the approach, which is further demonstrated from the temperature variation of these binding constants presented in the following section.

(b) Determination of ΔH and ΔS of Acetate Binding from the Temperature Variation of ΔG. The enthalpies and entropies (the latter in terms of $T\Delta S$ at room temperature) of association for acetate anion have been determined from the temperature variation of UV difference spectra. It was not anticipated that these data would give perfectly linear classical van't Hoff plots of $\log_{10} K$ vs $1/T$; this would occur only if ΔH and ΔS were temperature invariant. However, in all the associations under investigation, hydrophobic interactions occur to some extent. These interactions are entropy driven at room temperature (see Introduction) but are increasingly enthalpy driven at higher temperatures.[11,12,35] As a consequence, $\log_{10} K$ could show a somewhat greater dependence on $1/T$ at higher temperatures, i.e., at lower values of $1/T$. Nevertheless, should significant curvature of the plots occur, ΔH can be obtained at any temperature by drawing a tangent to the curve obtained by plotting $\log_{10} K$ against $1/T$. A plot of ln K against $1/T$ (K) for the binding of acetate to ristocetin A is given in Figure 5; the plot does in fact show slight curvature. Other determinations suggest that this curvature is systematic, and it is indeed in the direction consistent with the expression of some hydrophobic effect in the binding. The derived value of ΔH is −24 kJ mol^{-1} and of $T\Delta S$ is −19 kJ mol^{-1} at 298 K [from the straight line (least-squares fit) shown in the figure]; from the best straight line drawn through the four points at highest and lowest temperatures, the derived values are ΔH = −29 kJ mol^{-1} (307 K) and −19 kJ mol^{-1} (290 K). This temperature variation corresponds to ΔC_p = −500 J K M^{-1}, implying the burial of ca. 300 Å^2 of hydrocarbon from water upon the binding of acetate into the carboxylate binding pocket. This value is of course subject to gross uncertainty (80 Å^2 is estimated from molecular modeling—see later), and we draw attention only to the fact that it is of the anticipated sign. The plot is typical of the quality of the data obtained in five independent determinations of the temperature variation of the binding constant. In the other four determinations, the values of (ΔH, $T\Delta S$) obtained were (−13, −8), (−21, −16), (−48, −43), and (−26, −21) (the first three in the temperature range 279–314 K, and the last in the temperature range 303–333 K). Although the binding constants in one set of data could be measured with a relatively high precision (±1 M^{-1}), this uncertainty is unfortunately not small compared to the variation in the binding constant (i) from determination to determination and (ii) in the temperature range available for study. Thus, in the temperature range given for the first three determinations, K varied in the range 10.4–5.8, 8.0–2.5, 7.0–1.1 and for the last determination, 4.4–1.4 M^{-1}, hence the large variation in the derived values of ΔH and $T\Delta S$. We conclude that the enthalpy of association of acetate anion with ristocetin A is exothermic with a most probable value in the region of −26 kJ mol^{-1} and an unfavorable entropy change with a most probable value of $T\Delta S$ = −21 kJ mol^{-1} at room temperature (mean value of five determinations) but that this value is subject to a relatively large uncertainty. Despite this relatively large uncertainty, the most probable thermodynamic parameters are in reasonable agreement (considering the experimental uncertainties and the theoretical approximations) with those (ΔH = −27 and $T\Delta S$ = −29 kJ mol^{-1}) estimated on the basis of the semiempirical analysis. Determinations of the temperature variation of the binding constant of acetate to vancomycin gave values of (ΔH, $T\Delta S$) (room tem-

(35) Tanford, C. *The Hydrophobic Effect*, 2nd ed.; Wiley: New York, 1980.

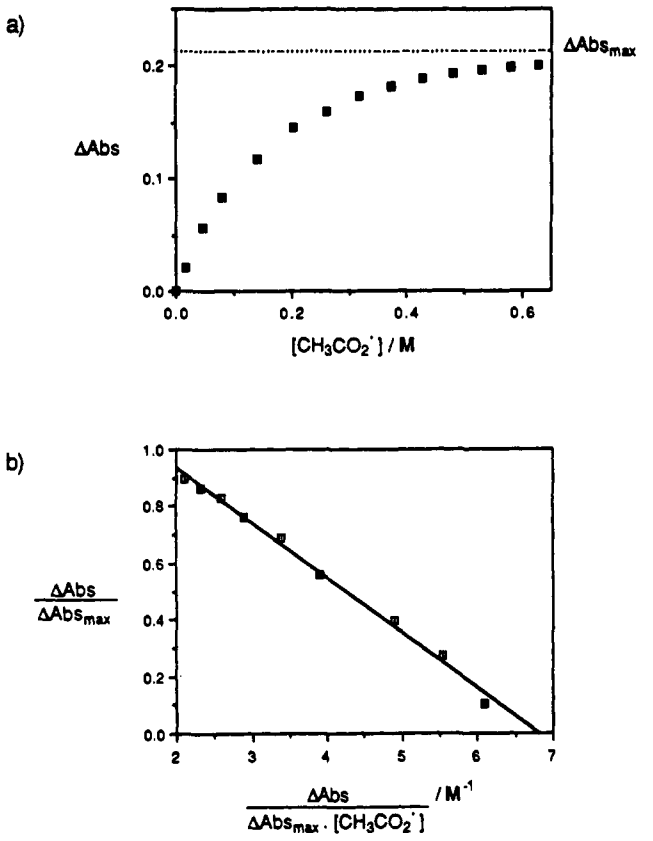

Figure 3. (a) Dependence of the UV absorption of ristocetin A upon the concentration of added acetate anion and (b) Scatchard plot for the data given in (a).

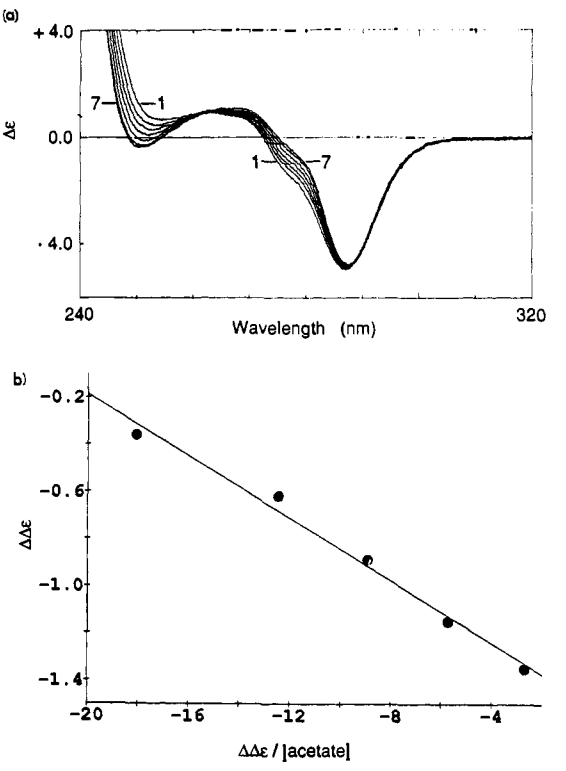

Figure 4. (a) CD spectra of vancomycin (0.1 mM) in H_2O at pH 5.0–5.1, titrated with acetate at 298 K. Spectrum 1 is in the absence of acetate; the other spectra correspond to 0.02, 0.05, 0.10, 0.20, 0.50, and 1.00 M (spectrum 7) acetate. $\Delta\epsilon$ is in units of mol^{-1} cm^{-1}. (b) Scatchard plot of CD data at 251 nm from the titration of vancomycin with acetate; the least-squares line is also shown. $\Delta\Delta\epsilon$ and acetate are in units of mol^{-1} cm^{-1} and mol, respectively.

perature) of greater precision; thus, two determinations in the temperature range 282–328 K gave values of (−28, −22) and (−27, −21) kJ mol^{-1} for these thermodynamic parameters. Once more, these mean values are in reasonable agreement with the values [ΔH = −29.6, $T\Delta S$ = −31, Table IV] predicted from the semiempirical analysis. For each antibiotic it appears likely that the entropy of binding of acetate is experimentally less adverse than that estimated from the semiempirical approach to an extent that allows acetate to bind more strongly than estimated by just over one order of magnitude in the binding constant. Both sets of data support the earlier conclusion that the amide/amide hydrogen bond in water predominantly supports binding entropically rather than enthalpically. In view of this success, we are currently using the method to determine intrinsic binding constants for some fundamental functional group interactions which are important in molecular recognition in aqueous solution. For cases of good ligand/receptor complementarity, these values will then permit the rough estimation of binding constants in aqueous solution, through the application of eq 2. Such work will not of course necessitate the breakdown of ΔG into ΔH and ΔS contributions.

A Molecular Interpretation of the Binding Process. Following the considerations of Page and Jencks[1] and those in this paper (Table II), we can take the unfavorable entropy term for association of acetate anion, *N*-Ac-D-Ala and *N*-Ac-D-Ala-D-Ala, due to their loss of rotational and translational entropy upon association with ristocetin A as 58, 64, and 67 kJ mol^{-1}, respectively (free energy values in Table II, converted to $T\Delta S$ values by correction for the rotational exothermicity of the 2 → 1 association of 4 kJ mol^{-1}—see earlier). Given these estimates, the earlier analysis of the thermodynamics of binding, and noting that the binding of *N*-Ac-D-Ala-D-Ala to ristocetin has ΔH = −25.4 ± 1.6 kJ mol^{-1} and $T\Delta S$ = 3 ± 1.8 kJ mol^{-1} at 298 K,[30] then the following molecular picture of the binding process emerges.: The unfavorable entropy change due to a bimolecular association (ca. 70 kJ mol^{-1}) and the freezing out of four rotors of the dipeptide (20 kJ mol^{-1}) is almost exactly balanced by a favorable entropy change. This total unfavorable entropy change at room temperature (90 kJ mol^{-1}) is countered by the following favorable entropy changes.

(i) The hydrophobic interactions involve mainly the interactions of the two alanine methyl groups and the α-CH of the non-C-terminal alanine with the various substituted benzene rings of ristocetin. In addition, it is known[17] that the benzene ring of residue 1 of ristocetin (Figure 2) folds over the carboxylate anion of the bound dipeptide. The total area of hydrocarbon buried in the binding process has been calculated by molecular graphics to be ca. 200 Å^2, and therefore the total favorable entropy-based contribution to binding is calculated to be ca. 25 kJ mol^{-1} (see Introduction). The above estimate for the area of hydrocarbon buried in the binding process is in reasonable agreement with that calculated from the relationship $\Delta C_p/\Delta A_H$ = 1.6 J K^{-1} mol^{-1} Å^{-2}, where ΔC_p is the change in heat capacity associated with the binding process (see Introduction). The experimental value of ΔC_p for the association in question is −385 ± 52 JK^{-1} mol^{-1},[30] giving 240 ± 32 Å^2 as the area of hydrocarbon buried in the binding process.

(ii) With the release of water molecules from polar groups involved in the binding there is a favorable $T\Delta S$ term of approximately 46 kJ mol^{-1} for water released in the formation of the two amide–amide hydrogen bonds. In order to obtain the experimental value of $T\Delta S$ (+3 kJ mol^{-1}) for the overall association (see above), the water released upon binding of the carboxylate anion into its receptor pocket of three amide NHs must therefore give a favorable $T\Delta S$ term z such that

$$3 = -90 + 25 + 46 + z$$

i.e., z should be ca. 22 kJ mol^{-1}. It is gratifying that this term is large and positive, since any physically reasonable model would seem to demand release of water from these polar groups upon their association. It is perhaps only surprising that the estimated value is similar to the value for formation of a single amide–amide hydrogen bond (corresponding to the release of water from one

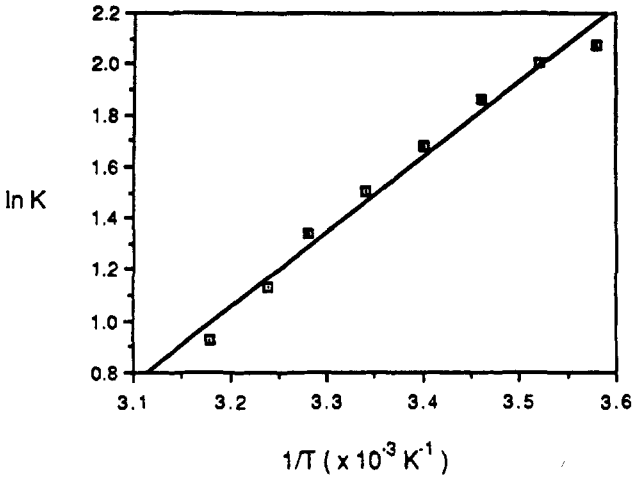

Figure 5. Plot of ln K vs $1/T$ for the binding of acetate anion to ristocetin A.

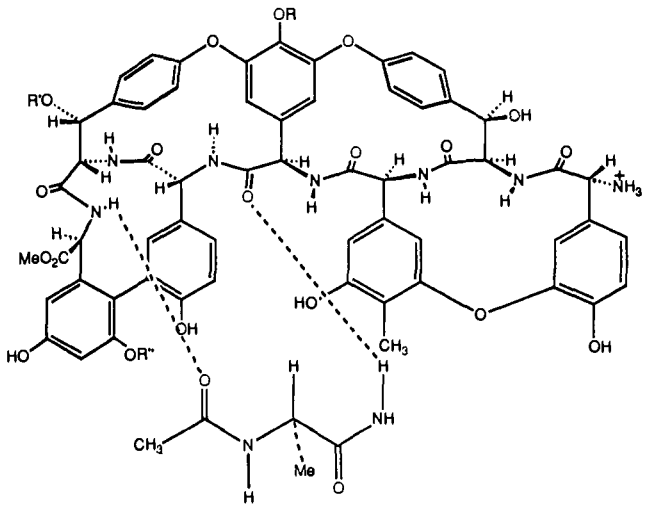

Figure 6. Mode of binding of *N*-Ac-D-Ala-amide relevant to the binding constant predicted in the text.

amide NH and one amide carbonyl group). This may reflect steric hindrance to the binding of water into the anion pocket prior to association of the peptide and/or inadequate approximations made in the analysis.

The water released in the binding process is of course an assembly of water molecules "attached" (but in a dynamic sense) to each polar group eventually involved in the binding. From $T\Delta S$ for the melting of 1 mol of ice (adjusted to 298 K), the release of 1 mol of water from an almost fully ordered state (ice) to the disordered state existing in bulk water corresponds to $T\Delta S = +6.5$ kJ mol^{-1} (see also ref 2). On this basis, amide–amide hydrogen bond formation would be associated with the release of 23/6.5 (i.e., between 3 and 4) fully ordered water molecules or, more realistically, with the release of a number of partially ordered water molecules which interact directly with the polar functionalities and of further less ordered water molecules from more distant coordination spheres. It is this water release from all the polar functional groups involved in the binding process, aided by the release of ordered water from hydrocarbon (the hydrophobic effect, $T\Delta S = 25$ kJ mol^{-1}), that is able to completely offset the otherwise unfavorable entropy of a bimolecular association which also requires the freezing out of four rotors in the dipeptide component. Additionally, we note that were it not that the peptide backbones of vancomycin group antibiotics were largely rigidified through cross-linking of the amino acid side chains, they would be unable to act as antibiotics. Strong bimolecular association of two small peptides (lacking cross-links) in aqueous solution is not sufficiently favorable in free energy to be possible (without the intervention of metal ions or other strong ionic interactions).

Thermodynamic parameters for the intrinsic binding constant for acetate into the pocket of three amide NHs can be estimated. The estimate is subject to relatively large uncertainties due to the uncertainties in separating free energies into their enthalpic and entropic parts and in the absolute values of rotational and translational entropies. From the binding of acetate anion into the pocket, we know an approximate, but most probable, value of $\Delta H = -26$ kJ mol^{-1}. Since any process in which two molecules combine to give one should release ca. 4 kJ mol^{-1} due to loss of rotational enthalpy (see earlier), then the estimated intrinsic binding enthalpy of acetate anion into the pocket is -22 kJ mol^{-1}. It is striking that three appropriately orientated amide NHs can electrostatically bind a carboxylate anion more strongly than bulk water. This is an experimental manifestation of calculations made by Warshel.[36,37] He concluded that two or three fixed dipoles in an enzyme (for example, NHs of backbone amide groups) can stabilize a charge at least as effectively as bulk water. The intrinsic binding entropy of acetate anion is then given by the extent to which its loss of rotational and translational entropy upon binding (−61 kJ mol^{-1}, Table II) differs from the experimental value of $T\Delta S$ (−21 kJ mol^{-1}). The large intrinsic binding entropy obtained (40 kJ mol^{-1}) can, on the basis of the surface area of hydrocarbon removed from water on the binding of acetate (ca. 80 Å^2 due to partial protection of the H_3C–C of acetate and its two surrounding benzene rings in the complex), be subdivided into 10 kJ mol^{-1} for the hydrophobic effect and 30 kJ mol^{-1} for release of water from the three amide NHs in the pocket and the carboxylate anion. This last value is in satisfactory agreement with the corresponding value (22 kJ mol^{-1}) derived earlier from an analysis of the binding of *N*-Ac-D-Ala-D-Ala.

The intrinsic binding free energy of the acetate anion into the pocket of three NHs is impressively large (22 + 40 = 62 kJ mol^{-1}); this corresponds to an intrinsic binding constant of ca. 10^{11}. This also includes an electrostatic attraction between the N-terminal NH_3^+ group and the carboxylate anion. In vancomycin this is worth 6 kJ mol^{-1}, shown by a change of the pK_a of the NH_3^+ group upon binding *N*-Ac-D-Ala-D-Ala;[38] in ristocetin this is worth 4 kJ mol^{-1}, shown by chemically changing the NH_3^+ group to OH.[39] An experimental binding constant for acetate near to 10 M^{-1} results because the rotational and translational free energy increase on binding is ca. 58 kJ mol^{-1} (adverse to binding by 10^{10}). This value emphasizes the difficulty of binding any peptide in a manner which does not use the carboxylate anion binding pocket. For example, the futility of attempting to bind *N*-Ac-D-Ala-amide to the antibiotics in the manner depicted in Figure 6 is clear.[40] The intrinsic binding constant (ca. 10^{11}) of the carboxylate has been lost, as has that ($10^{1.5}$) of the methyl group of the C-terminal alanine. Thus, the binding constant relative to *N*-Ac-D-Ala-D-Ala (10^6) is reduced by $10^{12.5}$. All that is gained in the binding of the smaller ligand is the freezing out of two fewer rotors (10^2), and the greater ease of "catching" the smaller ligand (ca. $10^{0.5}$, cf. Table II). Thus, the predicted binding constant for the interaction shown in Figure 6 is ca. 10^{-4} M^{-1}. A similar value can be derived by an alternative approach. The rotational and translational free energy gain in binding *N*-Ac-D-Ala-amide to the antibiotic receptor is ca. 64 kJ mol^{-1} (cf. Table II), and two rotors are frozen out when it binds as in Figure 6, giving total adverse effects on binding of ca. 10^{13}. Binding is promoted by the formation of two amide–amide hydrogen bonds (10^8) and

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(40) We are concerned here only with this hypothetical binding mode and do not preclude the possibility that *N*-Ac-D-Ala-amide could bind with the C-terminal functionality occupying the pocket normally occupied by the carboxylate anion [see data on the binding of di-*N*-Ac-Lys-D-Ala-D-Ala-amide to vancomycin (Popieniek, P. H.; Pratt, R. F. *Anal. Biochem.* **1987**, *165*, 108–113)].

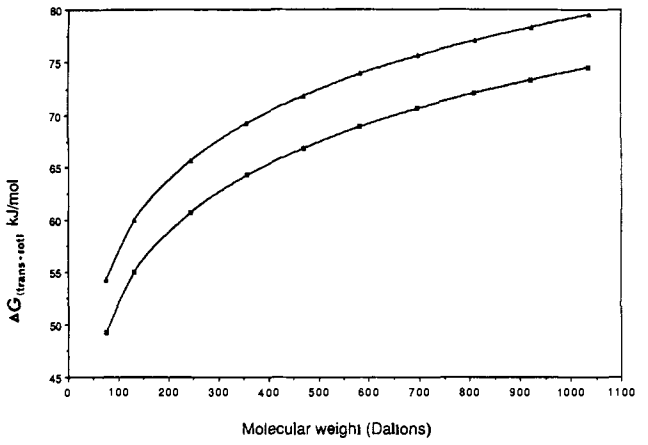

Figure 7. Plots of the approximate unfavorable translational plus rotational free energy of binding [$\Delta G_{(trans+rot)}$] for binding to a receptor of mass >2000 Daltons as a function of the mass of the ligand (■ ■ ■, aqueous medium; ▲ ▲ ▲, nonpolar medium).

hydrophobic effects (10^1), again giving a predicted binding constant of 10^{-4} M^{-1}. These examples serve to illustrate how even a very approximate approach to the analysis of binding can give a clear indication, in both pharmaceutical and academic research, as to whether productive binding is feasible.

Conclusion

We note that although it is the sign and order of magnitude of the thermodynamic parameters which we have discussed which are important, the specific values are reasonably self-consistent where comparisons are possible, and the resulting model is physically reasonable. The approach assumes approximate additivity of binding contributions, and the successful prediction of the binding constant for acetate in the two antibiotics within one order of magnitude supports this assumption. Indeed, the fact that acetate binding is slightly stronger than predicted suggests that any cooperativity between binding interactions may have been outweighed by an opposing effect. If such an opposing effect exists, then it is plausible that it may arise because a small ligand with a weak binding constant retains more residual motion in the binding site than a larger ligand with a stronger binding constant. This explanation is consistent with our observation that the experimental entropy change upon binding acetate is slightly less negative than predicted. If such an explanation is supported by subsequent research in which, as in the present work, ligand binding constants are studied as the ligand is "cut" into smaller components, then allowance could be made for such effects.

For ligand/receptor interactions of good complementarity, the application of eq 2 will allow the estimation of rough binding constants. $\Delta G_{trans+rot}$ is taken from Figure 7, ΔG_{rotors} is $5n$ kJ mol^{-1} where n is the number of rotors frozen out upon binding, ΔG_i is the intrinsic free binding energy for each interaction of polar functional groups, and ΔG_H is available from the area of hydrocarbon removed from exposure to water. The immediate task is the determination of additional intrinsic free energies of binding for functionalities which are important in biological interactions. Such work is in hand. These intrinsic free energies of binding should be applicable to binding processes in general, providing the geometry of the functional group interactions is sensibly similar. In subsequent work, the collection of additional experimental data and refinement of the theoretical approach may allow the thermodynamic parameters derived in the present work to be either confirmed or refined.

Experimental Section

UV Measurements. UV spectra were recorded on UVIKON 860 and 940 dual beam spectrophotometers at 303 ± 3 K. Antibiotic (A) and ligand (L) solutions were buffered with KH_2PO_4 (0.05 M)/NaOH (0.029 M), pH 7.0. Initial concentrations of vancomycin and ristocetin A were 0.25 and 0.15 mM, respectively. The λ_{max} for a particular complex was determined from a four-cell tandem arrangement in which the two cells in the reference beam contained the antibiotic and ligand solutions, respectively, while the two cells in the sample beam contained a mixture of the antibiotic and ligand solutions and buffer solution, respectively. Antibiotic concentrations in different cells were the same, as were ligand concentrations. Typically the max to be monitored fell in the region 245–255 or 275–290 nm.

Titration was carried out at the previously determined wavelength in a two-cell arrangement, where buffer and antibiotic solutions were placed in the reference and sample beams, respectively. Equal aliquots of the ligand solution were added successively to the two cells, followed by stirring (2 min) and measurement of the absorbances. Absorbance changes (ΔAbs) were initially plotted against the total ligand (L_T) concentrations (Figure 3a) to obtain an estimate of ΔAbs_{max}, the absorbance change corresponding to complete complexation of antibiotic. The binding constant for the ligand–antibiotic interaction was derived from the gradient of a Scatchard plot of $\Delta Abs/\Delta Abs_{max}$ (v) against $v/[L]_F$ (Figure 3b), where $[L]_F = [L]_T - v([A]_T)$.

Variable-temperature experiments were carried out in a 6 × 6 cell compartment, with the two sets of cells containing equal concentrations of buffer and antibiotic solutions, respectively. Successively increasing aliquots of the ligand solution were added to five of the six pairs of cells so as to emulate the titration above. Absorbance measurements were made between 278 and 343 K at 5° intervals, and the data at each temperature were treated as above to yield a set of K values as a function of temperature. The enthalpy of binding was determined from the gradient of a van't Hoff plot of ln K versus $1/T$.

NMR Measurements. ^1H NMR measurements were made on Bruker WM400 and AM400 instruments at 295 K. All samples were made up in a pH 7.0 D_2O buffer containing KD_2PO_4 (0.05 M) and NaOD (0.029 M). The deuterated inorganic salts were prepared by successive dissolution and lyophilization with D_2O. The pD was measured with a Corning pH meter 125 equipped with a combination glass electrode. Chemical shifts were measured with respect to external dioxan contained within a sealed glass capillary inside the NMR tube. Sample concentrations were 20 mM with respect to the test ligand. Binding samples employed a 1:1 mixture of ligand/antibiotic.

CD Measurements. A solution of the antibiotic (0.10 mM) in water was adjusted to pH 5.0 and then titrated with an aqueous solution of acetic acid (6.5 M) containing sufficient sodium hydroxide to maintain the pH at 5.0–5.1. Circular dichroism (CD) spectra (Figure 4a) were recorded at each point in the titration, with the solution in a 1.0-cm pathlength cylindrical cell (Hellma, England) thermostated at 298 K. The spectra were recorded on a JASCO J-600 spectropolarimeter with a 2.0-nm band width, 4-s time constant, and 10 nm/min scan speed; each spectrum is the average of two scans. Binding constants were obtained by plotting the CD data in the form of a Scatchard plot based on eq 4, where $\Delta\Delta\epsilon$ is the change in $\Delta\epsilon$ of the antibiotic caused by the corresponding concentration of acetate, and K is the binding constant (Figure 4b). The least-squares line for each plot was calculated with the FIT line function in the RS1 software package.

$$\Delta\Delta\epsilon = -\Delta\Delta\epsilon/K[\text{acetate}] + \Delta\Delta\epsilon_{max} \quad (4)$$

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Appendix

The increase in rotational and translational free energy (at 300 K) for binding a molecule of mass M (<1100 Daltons) to a receptor (mass ≥2000 Daltons) is such an important quantity for studies in molecular recognition that we give a plot (Figure 7) of this free energy against the molecular mass of the ligand (M). The two sets of data (Figure 7) are for a cylindrical molecule [(leucine)$_n$] binding to a larger receptor in an aqueous medium (■ ■ ■) or a nonaqueous medium (Δ Δ Δ). In each plot, the point at lowest mass M is calculated for glycine. These lines differ by 5 kJ mol^{-1} simply to allow for the slightly greater degree of order that will exist in an aqueous environment prior to binding (see

text for details of calculations). When the calculations are repeated for disc-shaped molecules (modeled by fused benzenoid aromatics) or globular molecules (modeled by a diamond lattice), the values of $\Delta G_{(trans+rot)}$ are smaller at all ligand masses by approximately 2 and 4 kJ mol^{-1}, respectively. Since these differences are smaller than the uncertainties in the semiquantitative values of $\Delta G_{(trans+rot)}$, we produce only the data for the molecular cylinder, which should be of general applicability. Uncertainties in $\Delta G_{(trans+rot)}$ arise not only from the semiempirical correction for gas-phase to solution entropies but also from the lack of detailed knowledge of residual molecular motions in the lig- and/receptor complexes. Nevertheless, the plot serves as a useful guide, for use in conjunction with eqs 1 and 2 (see text), as to whether a hypothetical association is likely to be productive. The adverse effect on binding constant (10^x) is given by $x = \Delta G_{(trans+rot)}/5.7$.

Registry No. 1, 19436-52-3; 2, 64-19-7; D-Ala, 338-69-2; L-Ala, 56-41-7; *N*-Ac-D-Ala-D-Ala, 19993-26-1; vancomycin, 1404-90-6; ristocetin A, 11021-66-2; D-lactic acid, 10326-41-7; L-lactic acid, 79-33-4; D-thiolactic acid, 33178-96-0; L-thiolactic acid, 57965-30-7; α-hydroxyisobutyric acid, 594-61-6; α-aminoisobutyric acid, 62-57-7.

Communications to the Editor

Efficient Electrocatalytic and Stoichiometric Oxidative Cleavage of DNA by Oxoruthenium(IV)

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The oxidative cleavage of DNA by metal complexes is important in drug applications,[1] the development of synthetic restriction enzymes,[2] and studies of tertiary DNA structure.[3] Polypyridyl complexes are extraordinarily attractive in studies of both DNA binding and oxidative cleavage.[4] The complex $Ru^{IV}(tpy)(bipy)O^{2+}$ (bipy = 2,2′-bipyridine, tpy = 2,2′2″-terpyridine) oxidizes organic hydrocarbons and alcohols via hydride transfer (eq 1).[5,6] We have found that this complex also oxidizes

(1)

DNA efficiently under anaerobic conditions. This oxidation can be performed either chemically by addition of $Ru^{IV}(tpy)(bipy)O^{2+}$ or electrocatalytically by controlled potential electrolysis of $Ru^{II}(tpy)(bipy)OH_2^{2+}$ at 0.8 V (Ag/AgCl).

The changes in the optical absorption spectrum of $Ru^{IV}(tpy)(bipy)O^{2+}$ that occur during the oxidation of isopropyl alcohol to acetone have been characterized in detail,[6,7] and we observe analogous changes during the oxidation of DNA.[8] Oxidation by the $Ru^{IV}O^{2+}$ form dominates the early stage of the reaction, and spectra taken during this stage are characterized by an isosbestic point at 363 nm (Figure 1A). The later stage is dominated by oxidation of the DNA by $Ru^{III}(tpy)(bipy)OH^{2+}$, which is generated by comproportionation of the Ru(IV) and Ru(II) species. Accordingly, a new isosbestic point at 406 nm is observed, as is an increase in absorption at 477 nm due to the quantitative formation of the $Ru^{II}(tpy)(bipy)OH_2^{2+}$ (Figure 1B). In solutions containing a 1:1 molar ratio of calf thymus DNA (nucleotide phosphate) to $Ru^{IV}(tpy)(bipy)O^{2+}$, the isosbestic behavior at 406 nm is reached in approximately 1 h. Under the conditions shown in Figure 1B ([DNA-nucleotide phosphate] = 2 mM, $[Ru^{IV}(tpy)(bipy)O^{2+}]$ = 0.1 mM), the later stage of the reaction is reached before a spectrum can be acquired; *i.e., all of the $Ru^{IV}(tpy)(bipy)O^{2+}$ is consumed immediately upon mixing with the DNA.* The kinetics are strongly dependent on the extent of binding of the Ru complex to the DNA; a detailed kinetic study is underway.

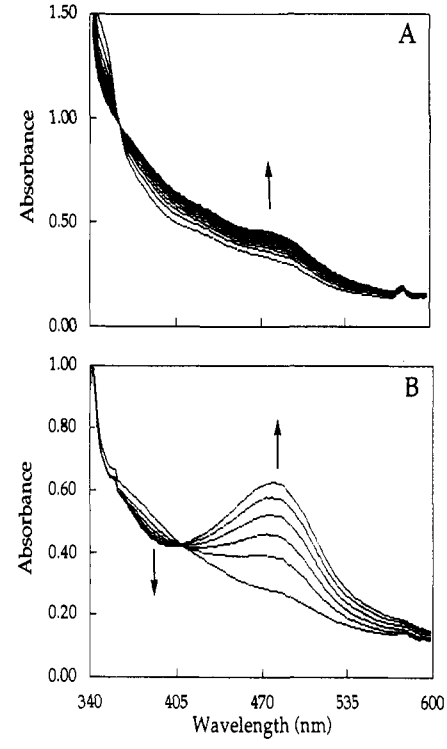

Figure 1. (A) UV–vis spectra taken at 2-min intervals during the oxidation of calf thymus DNA (0.5 mM nucleotide phosphate) by $[Ru^{IV}(tpy)(bipy)O](ClO_4)_2$ (0.5 mM) under N_2. (B) UV–vis spectra taken at 5-min intervals during the oxidation of calf thymus DNA (2 mM) by $[Ru^{IV}(tpy)(bipy)O](ClO_4)_2$ (0.1 mM) under N_2.

The cyclic voltammogram of $Ru^{II}(tpy)(bipy)OH_2^{2+}$ shows two waves corresponding to the $Ru^{III}(tpy)(bipy)OH^{2+}/Ru^{II}(tpy)(bipy)OH_2^{2+}$ ($E_{1/2}$ = 0.49 V) and $Ru^{IV}(tpy)(bipy)O^{2+}/Ru^{III}(tpy)(bipy)OH^{2+}$ ($E_{1/2}$ = 0.62 V) redox couples (cyclic voltam-

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