et al.[36] The experimental data indicate that deoxygenation (dehydroxylation) of tetrasaccharides results in both a less negative enthalpy and a less negative entropy of binding. In line with the hypothesis presented earlier, we propose that the entropy of binding becomes more negative (unfavorable) as the extent of the oligosaccharide interaction with the protein interface increases (more negative enthalpy of interaction). Lemieux et al.[13a] propose that deoxygenation results in a less negative enthalpy of binding because a substrate hydroxyl group in the "free" ligand is surrounded by "high-energy" water which contributes a favorable enthalpy of binding when such water molecules are released to bind more strongly to the bulk solvent. This proposal is inconsistent with the conclusion that neutral-neutral hydrogen bonds in aqueous solution have only small or negligible exothermicities.[29a]

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Conclusions

The thermodynamics of phase transitions (melting and sublimation) undergone by organic crystals have been used to suggest the approximate entropic cost of rotor restrictions, and the loss in translational and rotational motions, appropriate to the formation of weakly bound complexes in solution. The data indicate that the loss in translational and rotational entropy increases gradually from very small to limiting values as the endothermicity of the dissociation increases. Some β-adrenergic agonists bind with relatively large exothermicities; this presumably permits the formation of a relatively high ordered agonist/receptor complex, suitable for precise conformational change and hence for signalling at a distance.

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Partitioning of Free Energy Contributions in the Estimation of Binding Constants: Residual Motions and Consequences for Amide-Amide Hydrogen Bond Strengths

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Abstract: Any bimolecular association is entropically unfavorable because of degrees of freedom of translation and rotation lost when two molecules come together to form a complex. For a ligand of molecular weight 200, the formation of a "rigid" dimer (one in which there is no residual relative motion of the associating components **A** and **B** in the complex **A·B**) opposes binding by ca. 10^{-9} to 10^{-10} M^{-1} in binding constant. If relative motions (including new soft vibrations) in the complex are then credited to the functional group interactions then the amide-amide hydrogen bonds, for example, those involved in the reported formation of lactam dimers in solution, are concluded to promote dimerization by ca. 10^4 per hydrogen bond (Doig, A. J.; Williams, D. H. *J. Am. Chem. Soc.* **1992**, *114*, 338). An alternative approach is to regard residual relative motions remaining in the complex as constituting translational and rotational entropy of **A** and **B** that was not lost. In this paper and in the preceding paper we have attempted to quantitate the contribution of residual motions in weakly bound complexes from literature data on the fusion, sublimation, and dissolution of model compounds. If the entropic advantage of the residual motions is removed as entropy that is not lost in the bimolecular association, then free energies for amide-amide hydrogen bond formation are obtained that are not significantly different from the conventional view of these bonds of between −2 and −8 kJ mol^{-1}. The same conclusion is reached in ligand extension studies for the binding of peptide cell wall analogues to the antibiotics vancomycin and ristocetin A if credit for residual motions is removed, and allowance is made for a larger hydrophobic effect than originally envisioned (Williams, D. H., et al. *J. Am. Chem. Soc.* **1991**, *113*, 7020).

Introduction

In several recent publications,[2-4] we have considered an approach to the factorization of the free energy of binding for molecular associations in aqueous solution, by partitioning free energy contributions into four principal terms. Our analysis is based upon the pioneering work of Jencks[5] and Page and Jencks.[6] A similar factorization has previously been used by Andrews et al.,[7] and the relevance and physical basis of the factors involved have been summarized by Fersht.[8] The consideration of only four terms is justified only if the ligand and receptor show good van der Waals complementarity, and if the conformations of the bound components correspond closely to conformational energy minima in the separated states.[2,3] These terms are considered as

(1) Abbreviations: ΔG, ΔH, and ΔS, change in Gibb's free energy, enthalpy and entropy; K, binding constant; T, temperature (K); T_m, melting temperature (K); ΔG_{t+r} and ΔS_{t+r}, change in free energy and entropy of translation and rotation; ΔG_r and ΔS_r, change in free energy and entropy of internal rotations; ΔG_p, change in free energy for polar group interactions; ΔG_h, change in free energy due to the hydrophobic effect; ΔG_{conf}, change in free energy due to conformational strain; ΔG_{vdW}, change in free energy due to van der Waals interactions; ΔA_{np}, change in nonpolar surface area (Å^2).

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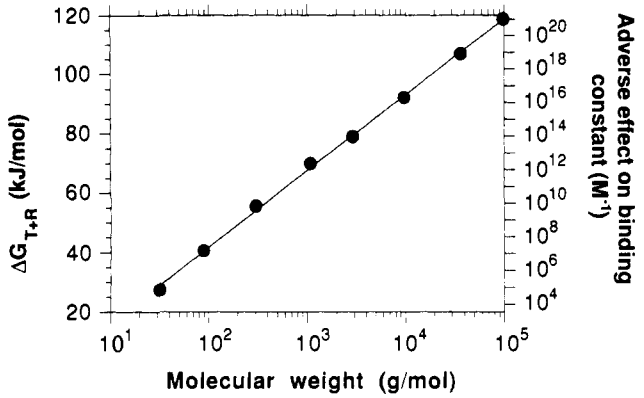

Figure 1. An estimate of the adverse effect on binding constant in the hypothetical case where all the entropy of translation and overall rotation of the components A and B is lost upon association to form the complex A·B.

follows: (i) the low probability of "catching" the ligand on the receptor in the absence of intermolecular forces; (ii) the adverse free energy change (largely entropic) associated with the restriction of any internal rotations of either component upon complex formation; (iii) the promotion of binding if hydrocarbon is removed from exposure to water upon complex formation; and (iv) the promotion of binding due to the favorable interactions between polar functional groups in the complex. These four parameters are now enumerated, and the consequences of residual motions in complexes formed from weak associations (as outlined in the preceding paper[9]) are elaborated. Problems associated with the designation of particular values to the parameters are discussed, together with a reassessment of the strength of the amide–amide hydrogen bond in lactam dimers and peptide–antibiotic complexes.[2-4]

(i) Bimolecular Association. Any bimolecular binding process is entropically unfavorable due to the formation of a single molecule of complex, which occurs with loss of translational and rotational entropy. The unfavorable free energy of the association (ΔG_{t+r} (kJ mol^{-1}) equal to $-T\Delta S_{t+r}$ at 298 K) as a function of the molecular weight of a ligand binding to a larger receptor with complete loss of the translational and rotational entropy of the smaller component can be estimated[2] and is given in Figure 1.[10] As with all other free energy changes given in this account, it can be converted to an effect on log K by division by 5.7 (for room temperature binding); this scale is given on the right-hand side of the figure. We find that within 4 kJ mol^{-1}, the same values apply for any molecular shape (rod, disc, or sphere) of molecular weight m binding to any receptor of molecular mass 1200 or greater.[1] Thus, for example, ΔG_{t+r} is adverse to binding by a factor of ca. 10^{-9} M^{-1} for a ligand of molecular weight 200 (where the "molecular weight" includes bound solvent molecules, which can be regarded as translating and rotating with the ligand). The figure of 10^{-9} M^{-1} is the entropic price to be paid when there is no residual overall relative translation and rotation of the associating components **A** and **B** in the complex **A·B**. We note later that this situation is unlikely to be achieved in practice; rather, some (variable) fraction of 10^{-9} M^{-1} is the cost to be paid, as outlined in the preceding paper,[9] and as elaborated below.

(ii) Restriction of Internal Rotations. Following Page and Jencks,[6] we note that binding is adversely affected by approximately 5 to 6 kJ mol^{-1} (ΔG_r) for each rotation removed upon association. Thus, if four relatively free rotations are lost in an association, the binding is hypothesized to be adversely affected by a factor of ca. 10^4. These values correspond to severe restriction, or complete loss of a rotation; we have argued[9] that smaller values may be appropriate for associations involving non-covalent bonds.

(iii) The Hydrophobic Effect. The magnitude of the hydrophobic effect can be estimated from solvent partitioning experiments.[11] For every square angstrom (Å^2) of hydrocarbon removed from exposure to water by the binding process, we conclude (see later) the binding energy to be increased by 0.2 kJ mol^{-1}. This value is in accord with a recent theoretical reassessment,[12] and in good agreement with recent experimental data by Serrano et al.[13] on the change in solvent-accessible hydrophobic surface area on mutation of Gly to Ala on the exposed surface of α-helices in barnase.

(iv) Polar Interactions of Functional Groups. The bringing together of the two binding entities with the appropriate internal geometry is accounted for in factors (i) and (ii). However, if the free energy of binding which results from the interaction of any pair of functional groups (ΔG_p) is to be the same if the process occurs either intramolecularly or bimolecularly (which is obviously desirable if ΔG_p values are to be of general utility), then ΔG_{t+r} values cannot be taken from Figure 1. We elaborate on this point subsequently. When polar interactions occur with optimum geometry for binding, they are known as intrinsic binding energies.[5]

In summary, the free energy (ΔG, kJ mol^{-1}) of a bimolecular association following the above specifications has been approximated by

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

where $\sum \Delta G_p$ represents the free energies of binding for each set of interacting functional groups, summed over all such sets of interactions.

For the more general case where ΔG_{conf} represents the total conformational strain energy produced upon binding and ΔG_{vdW} represents the change in van der Waals energy between free and bound states (due, for example, to the existence of van der Waals repulsions or cavities in the complex), then eq 2 results:[2]

$$\Delta G = \Delta G_{t+r} + \Delta G_r + \Delta G_h + \sum \Delta G_p + \Delta G_{conf} + \Delta G_{vdW} \qquad (2)$$

In applying eqs 1 and 2, we recognize that the derived parameters will be approximate only and that the analysis may be additionally complicated by cooperativity. It nevertheless seems worthwhile to attempt a semiquantitative approach even if only to give rough estimates of the parameters and to expose any problems that the analysis may uncover.

Uncertainties in the Application of Equation 1

The Entropic Cost of Bimolecular Associations Involving Weak Interactions. The values of ΔG_{t+r} which can be read from Figure

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(10) We have made three modifications in the estimation of ΔG_{t+r} relative to the original estimate (refs 2 and 3). First, the Trouton's rule correction refers to the entropy decrease of liquids at the boiling point relative to gases. To allow for the additional order existing at temperatures below the phase transition, we have added $T\Delta S$ to the Trouton's rule correction in water, where T is 298 °C, and $\Delta S = C_p$ ln (373/298), where C_p is the heat capacity of water at constant pressure. This correction is empirical, but it seems better than ignoring an effect which is physically required in some form or another; it acts to reduce ΔG_{t+r} by 5 kJ mol^{-1}. Second, since the Trouton's rule correction is applicable to neat liquids, we had earlier (refs. 2 and 3) added $2.3RT$ log 1000/RMM to allow for the entropy increase at a molar concentration. Such a correction is appropriate for a concentration decrease in the gas phase of a single component but not for a dilution in a liquid phase, where an entropy of mixing (assuming ideal behavior) is appropriate. An entropy of mixing is small compared to the entropy of dilution, and for a ligand of effective molecular weight 200, this change decreases ΔG_{t+r} by 4 kJ mol^{-1}. Third, we originally followed the approach of Israelachvili that translational kinetic energy is not lost in an association to give a complex held together by weak intermolecular forces (Israelachvili, J. N. *Intermolecular and Surface Forces*; Academic Press: London, 1985; p 21). In view of the case made later in this manuscript that considerable translational and rotational motions remain in complexes where the components are held together by non-covalent interactions, we now carry out the accounting by regarding kinetic energy of both translation and rotation ($RT/2$ per degree of freedom) to be retained in the complex; this modification increases ΔG_{t+r} by 4 kJ mol^{-1} ($3RT/2$). The overall effect of all these changes for a ligand of molecular weight 200 is to decrease ΔG_{t+r} by only 5 kJ mol^{-1} relative to the original estimate[2,3] and, in view of the large uncertainties in this parameter, these changes are cosmetic rather than of great consequence.

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1 are not experimental and solution entropies of complex molecules, upon which they depend, are uncertain. However, the values do accord well with the adverse entropy changes (on which they largely depend) for reactions in which two small molecules react to one *covalently* bound entity.[5] A much more difficult problem lies in searching for the most acceptable method to do the "accounting" for the free energy change, ΔG_{t+r}, appropriate for *weak* associations. The preceding paper, dealing with the use of entropies of fusion and sublimation of crystals as models for entropy changes in weak associations, highlights the problem. Not only may the value of ΔG_{t+r} read from Figure 1 be much larger than the loss in translational and rotational free energy for a weak association, but the degree by which it is too large seems likely to be dependent on the exothermicity of the association.[9] Weakly exothermic associations are expected to have relatively small adverse entropies, and strongly exothermic associations to have much larger adverse entropies. In some cases, the benefit of translational and rotational entropy which remains in the complex plus the entropic advantage of new low-frequency vibrations may almost balance the formal loss of $T\Delta S_{t+r}$ (close in magnitude to the ΔG_{t+r} values which are given in Figure 1) associated with the formation of a rigid complex.[9]

The values for ΔG_{t+r} in Figure 1 correspond to making a complex in which all the relative overall translation and rotation of the associating entities has been lost. Thus, if the values in Figure 1 are used directly in the application of eq 1 to an association **A** + **B** → **A·B**, then entropically advantageous relative translation and rotation of **A** and **B** which remain in **A·B** are credited to the functional group interactions in $\sum\Delta G_p$. Using this approach and the data of Susi et al.[14] for valerolactam dimerization in aqueous solution, ΔG_p for each amide–amide hydrogen bond in the dimer (**1**) is −27 kJ mol^{-1}.[4] In broad terms, this is because the reported association constant for dimerization is ca. 10^{-1} M^{-1}, and the loss of the translational and rotational entropy of one component of the dimer is adverse to binding by ca. 10^{-9} M^{-1} (cf. Figure 1). Thus, the amide–amide hydrogen bonds are concluded to promote dimerization by ca. 10^{8} M^{-1}, or ca. 10^{4} M^{-1} per hydrogen bond. The entropically favorable low-frequency vibrations which permit relative translation of the two parts of the dimer and other entropically favorable motions in the dimer have been credited to the ΔG_p values of the hydrogen bonds.

An alternative approach is not to use ΔG_{t+r} values taken from Figure 1, but smaller values which therefore treat motions remaining in the complex as constituting translational and rotational entropy that was not lost. A good case can be made that the loss in translational and rotational entropy in the formation of the dimer **1** is in reasonable accord with the expectations from the fusion and sublimation models presented in the preceding paper.[9] Further, Murphy and Gill[15] have shown that the entropy change (in terms of $T\Delta S$ at 298 K) upon dissolving crystals of diketopiperazine in aqueous solution is +11.5 kJ mol^{-1}. If allowance is made for the fact that the two methylene groups of this molecule will order water (through the hydrophobic effect) in aqueous solution, causing an adverse $T\Delta S$ on dissolution of 5–10 kJ mol^{-1}, then freeing the molecule from the crystal is seen to be favorable entropically ($T\Delta S$) by 16.5–21.5 kJ mol^{-1}. In other words, in passing from free diketopiperazine in aqueous solution to its crystal lattice (a model for a bimolecular association involving similar weak interactions), the molecule loses only about 33–43% of its translational and rotational free energy (i.e., about these percentages of the value read from Figure 1). Since diketopiperazine is held in its crystal structure by 4 amide–amide hydrogen bonds, there can be little doubt that the proposed solution dimer **1** would lose an even smaller fraction of ΔG_{t+r}. Plausibly, the adverse change in ΔG_{t+r} ($-T\Delta S_{t+r}$) in the formation of dimer **1** would lie in the range 11–17 kJ mol^{-1}, opposing dimer formation by a factor of only 10^{-2} to 10^{-3} M^{-1}. Thus, if ΔG_p = −(5 to 6) kJ mol^{-1} (promotion of dimerization by a factor of 10^{1} M^{-1}) for the amide–amide hydrogen bond, two of these would be not quite adequate to overcome the adverse entropy of dimerization, to give an observed dimerization constant in the region of 10^{-1} M^{-1}. In summary, large negative ΔG_p values are obtained for the hydrogen bond if it is credited with the favorable entropy of residual motions and small negative ΔG_p values if it is not credited with this favorable entropy. We recommend that ΔG_p values be obtained by the "anchor principle"[5] (or "ligand extension"—see following section), since the entropic costs of weakly exothermic bimolecular associations are difficult to determine. Additionally it seems unsatisfactory to credit associations which have no enthalpic barrier to the reverse (dissociation) step with the entropic advantage of 10^{10} M^{-1} which such a process would permit, i.e. no loss in translational and rotational entropy.[9]

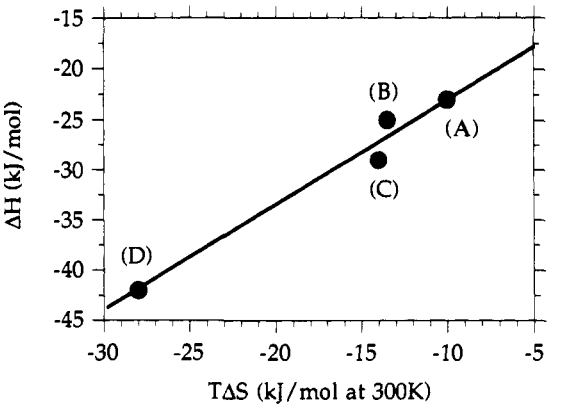
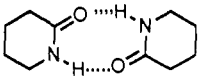


Figure 2. Plot of enthalpy versus entropy ($T\Delta S$ at 298 K) of dimerization of (A) ϵ-caprolactam (in CCl_4), (B) 2-pyridone ($CDCl_3$), (C) γ-butyrolactam (CCl_4), and (D) δ-valerolactam (CCl_4).

In relation to the above discussion, it is noteworthy that the evidence for the formation of dimer **1** in aqueous solution, although



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long accepted, is not particularly strong. Against this point may be set the similar dimerization constants (ca. 10^{-1} M^{-1}) proposed for assumed analogous dimers of urea[16] and diketopiperazine[17] in aqueous solution and the fact that dimerization constants in this region are consistent with the ΔG_p values (−5 to −6 kJ mol^{-1}) assumed for amide–amide hydrogen bonds on the basis of protein engineering experiments (which do not credit this hydrogen bond with the advantageous entropy of the large residual motions which its formation allows; see below). Evidence for the formation of hydrogen-bonded dimers of γ-butyrolactam, δ-valerolactam, and ϵ-caprolactam in carbon tetrachloride is much stronger. The respective values of K, ΔH, and $T\Delta S$ at 298 K are the following: 460 M^{-1} γ-butyrolactam, −29 and −14 kJ mol^{-1}; 432 M^{-1} δ-valerolactam, −43 and −28 kJ mol^{-1}; 168 M^{-1} ϵ-caprolactam, −23 and −10 kJ mol^{-1}.[18] These data give support to the model which uses crystal formation as a guide to the entropic costs of complex formation. From the dimerization constants, it can be seen that the ΔG values for dimer formation are all similar (−15, −15, and −13 kJ mol^{-1}, respectively). Thus, where dimer formation is most exothermic (δ-valerolactam), the extent of dimer formation is similar to the other cases. This is because the most exothermic dimerization pays the largest cost in entropy. Here again we see the entropy/enthalpy compensation alluded to in the preceding paper[9] and illustrated graphically in Figure 2 (the data for the dimerization of 2-pyridone in $CDCl_3$[19] are also included in the

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Table I. Change in Solvent Accessible Surface Area ($\Delta\Delta A_{np}$), Binding Free Energy ($\Delta\Delta G$), and Enthalpy ($\Delta\Delta H$) for Deletion of a Methyl Group (Ala → Gly) from *N*-Acetyl/*N*-Benzoyl Peptides Binding to Ristocetin A[a]

ligands	$\Delta\Delta G$	$\Delta\Delta H$	$\Delta\Delta A_{np}$ (complex)	$\Delta\Delta G/\Delta\Delta A_{np}$
N-X-Ala-Ala/N-X-Ala-Gly	11.4,[b] 8.4,[c] 10.1[c]	1.8 ± 2.7[b]	47	−(0.24–0.18)
N-X-Ala-Ala/*N*-X-Gly-Ala	0.4,[b] 2.9,[c] 4.6[c]	6.3 ± 3.0[b]	53	−(0.007–0.09) −(0.12–0.21)[e]
N-X-Ala-Ala/*N*-X-Gly-Gly	13.0,[c] 15.2[c]	nd	76	−(0.17–0.20)
N-X-Ala-Gly/*N*-X-Gly-Gly	4.6,[c] 5.0[c]	nd	30	−(0.15–0.17)
N-X-Gly-Ala/*N*-X-Gly-Gly	10.1,[c] 10.5[c]	nd	30	−(0.34–0.35)
N-X-Ala/*N*-X-Gly	7.0[d]	nd	36	−0.19

[a] Where X represents acetyl or benzoyl, see footnotes *b–d* following; $\Delta\Delta G$ and $\Delta\Delta H$ in kJ mol^{-1}; $\Delta\Delta A_{np}$ (change in nonpolar surface area) in Å^2; $\Delta\Delta G/\Delta\Delta A_{np}$ in kJ mol^{-1} Å^{-2}. [b] Data of Rodriguez-Tebar et al.[21] for *N*-acetyl-substituted ligands. [c] Data of Smith et al.[22] for *N*-benzoyl ligands. [d] Data of Williams et al.[2] for *N*-acetyl ligands. [e] Value obtained when exothermicity ($\Delta\Delta H$) is subtracted from $\Delta\Delta G$.

plot). Covering all three lactams, the entropic costs of dimerization, in terms of the adverse cost of $T\Delta S$ at 298 K on the dimerization constant, lie in the range $10^{-1.7}$ to $10^{-4.7}$ M^{-1}. The smallest entropic cost is for the least exothermic association, and the largest cost is for the most exothermic. This observation and the range of the entropic cost support the guides given by the crystal model. We incidentally note that the variation in the exothermicities for the dimerization of the three saturated lactams could reflect, at least in part, variation in the dryness of the carbon tetrachloride solvent (with the expectation that the driest solvent would result in the most exothermic dimerization; see below).

Studies Involving the Formation of the Amide–Amide Hydrogen Bond in a Ligand Extension; Proportioning Free Energy Changes between ΔG_{t+r}, ΔG_r, and ΔG_h. An analysis of the increased binding constant of *N*-Ac-Gly-D-Ala (*2*) over *N*-Ac-D-Ala (**3**) to the antibiotics ristocetin A and vancomycin gave an average binding energy (ΔG_p) of −24 kJ mol^{-1} for the amide–amide hydrogen bond found between the NH of glycine and an amide CO of the antibiotics (Figure 3).[2] This value was obtained by taking the mean increase in binding energy of **2** over **3** (11 kJ mol^{-1} at 25 °C), adding 10 kJ mol^{-1} to this value (for the free energy cost of restricting two rotors at 5 kJ mol^{-1}), and also allowing for the estimated extra entropic cost (3 kJ mol^{-1}) for catching a ligand of larger mass.

$$\sum\Delta G_p = -11 - 10 - 3 = -24 \text{ kJ mol}^{-1} \qquad (3)$$

In the method of accounting used to derive this value, the value for rotor restriction (5 kJ mol^{-1}) may give too little credit for the favorable free energy of residual torsions in the restricted rotors of weak complexes. If the values for rotor restriction in the formation of crystals from melts of C_nH_{2n+2} hydrocarbons (where n is even) at T_m are used (3.5 kJ mol^{-1}, see preceding paper[9]) as a lower limit but 5 kJ mol^{-1} is retained as an upper limit [since rotor restrictions are more costly in free energy when at sp^2–sp^3 bonds (peptides) than at sp^3–sp^3 bonds (hydrocarbons)],[6] then −10 is replaced by −(7 to 10) kJ mol^{-1}. Second, the factor of −3 kJ mol^{-1} (eq 1) is based upon the fact that the entropy of a ligand is dependent upon the logarithm of its mass.[2] In the method of accounting adopted, the amide–amide hydrogen bond is credited with the larger adverse entropy that has to be overcome in catching a ligand of larger mass. No such credit may accrue if it is considered that the ligands **2** and **3** retain some fraction of their free ΔG_{t+r} when associated (rather than losing all their free ΔG_{t+r} and then crediting residual motions in the complex to ΔG_p values). The reason for this (as discussed in the preceding paper[9]) is that the ΔG_{t+r} value of the free ligand is dependent upon a term log (am) and the ΔG_{t+r} value of the bound ligand upon a term log (bm) (where a and b are constants and m is the mass of the ligand). Therefore, the difference between the ΔG_{t+r} values for the free and bound states is dependent upon a term log (am) − log (bm) [i.e., log (a/b)], and it becomes independent of mass. In summary, if the ΔG_p value for the amide–amide hydrogen bond is not given the credit for any internal motions remaining in the complex, then −24 kJ mol^{-1} (eq 3) becomes −(18 to 21) kJ mol^{-1}.

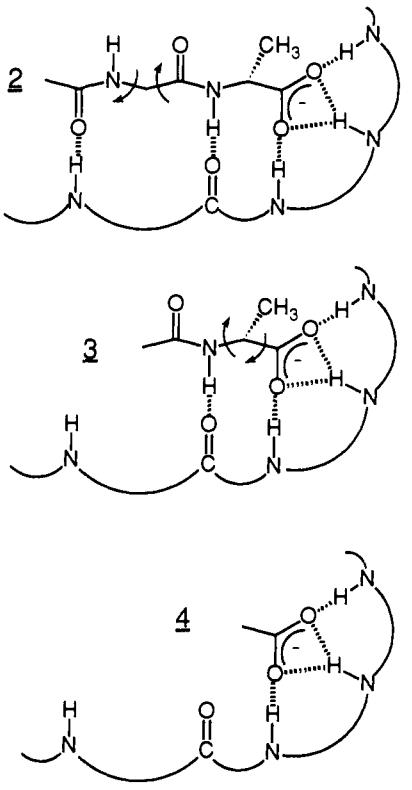

Figure 3. Application of ligand extensions to the study of amide–amide hydrogen bonds in peptide–antibiotic complexes. Schematic representation of the interaction of (**2**) *N*-Ac-Gly-D-Ala; (**3**) *N*-Ac-D-Ala; and (**4**) acetate with the binding pocket of ristocetin A.

The above changes in ΔG_p simply reflect different methods for crediting the advantages of residual motion in the complex, in proportioning the free energy changes between ΔG_{t+r}, ΔG_r, and ΔG_p. However, in addition to these "accounting method" differences, we conclude that the original analysis underestimated the role of the hydrophobic effect in increasing the binding of **2** over **3**. In the original analysis,[2] it was argued that since the *N*-acetyl methyl group of **2** points away from the antibiotic, this methyl group would not perturb the binding significantly. Therefore, no increase in the binding of **2** over **3** was attributed to the hydrophobic effect (ΔG_h, eq 1). We now believe this to be an error for two reasons. First, the aromatic ring of residue 7 of the antibiotics (Figure 4) can approach this methyl group in the complex. Second, CPK models and molecular graphics representations of the complex show that other C–H groups of the antibiotic interact with the polar parts of the CH_3CONH– extension added in passing from **2** to **3**. Using MacroModel[20] to calculate changes in hydrophobic surface areas, we have reassessed the contribution of the hydrophobic effect to ligand binding en-

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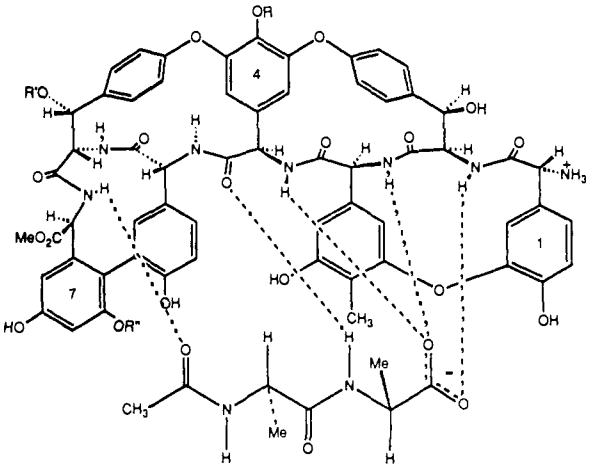


Figure 4. Expanded view of the complex formed between the antibiotic ristocetin A and the cell-wall analogue *N*-Ac-D-Ala-D-Ala (with the aromatic ring 7 of the antibiotic indicated).

ergies. In Table I we have considered the change in solvent accessible surface area that occurs on complex formation when a methyl group has been deleted for the pairs of ligands indicated. For example, in comparing the binding of *N*-acetyl-D-Ala-D-Ala and *N*-acetyl-D-Ala-Gly to ristocetin A we calculate a difference in the surface area of hydrocarbon buried ($\Delta\Delta A_{np}$; corresponding to the hydrophobic contribution from the C-terminal alanine methyl group) of 47 Å^2. This value was calculated from the surface areas of the energy minimized ligand, antibiotic, and ligand–antibiotic complex using a water molecule of radius 1.4 Å together with a high density of points on a sphere. Further, the difference in binding energy between these two ligands of −11.4, −8.4, and −10.1 kJ mol^{-1} (from several independent sources[21,22]) has been shown by calorimetry[21] to be essentially entropy driven. Therefore, we attribute the difference in binding energy to a combination of (i) the hydrophobic contribution from the alanine methyl group and (ii) any promotion of binding of Ala over Gly due to the Ala methyl group conformationally biasing the peptide backbone into the bound conformation. Fortunately, using the data of Serrano et al.,[13] these two variables can be estimated separately. The conversion of a glycine to an alanine residue, in the absence of burial of the side chain residue (other than by contact with the peptide backbone), increases the solvent accessible hydrophobic surface area by 22 Å^2 in α-helices, but by 35 Å^2 in a β-strand[23] (or "random-coil" assuming such a "random" structure reflects largely conformers with preferred (ϕ, ψ) angles in the range found for a β-strand). Thus, when a difference in solvent accessible hydrophobic surface area of 35 Å^2 is retained, the preference for Ala over Gly in a β-strand conformation is a measure of a factor, or factors, other than the hydrophobic effect. We ascribe this preference to the conformational bias induced by an Ala methyl group toward the (ϕ, ψ) angles of a β-strand. From the data of Serrano et al.,[13] this conformational bias appears to be negligible (≤1 kJ mol^{-1}). Therefore, the above differences in binding energies (−11.4, −8.4, and −10.1 kJ mol^{-1}) directly give the increase in binding to be ascribed to the hydrophobic effect. Thus, when the difference in binding energy is equated with the change in surface area of 47 Å^2, we estimate a hydrophobic contribution of −0.18 to −0.24 kJ mol^{-1} per Å^2 buried (considering the full range of experimental binding data) in this system. This range is in good accord with the recent value (of −0.23 kJ mol^{-1} Å^{-2}) found in protein engineering experiments on α-helix stability.[13,24]

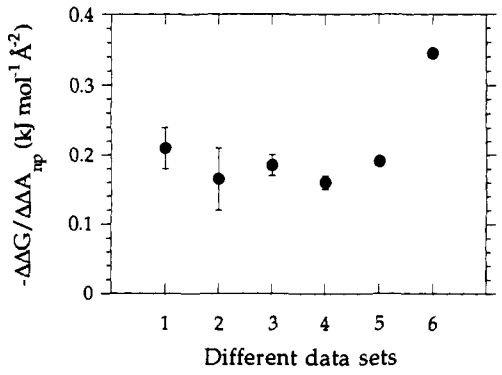
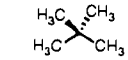
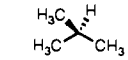
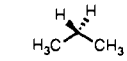
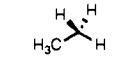


Figure 5. Surface area dependence of the hydrophobic effect ($\Delta\Delta G/\Delta\Delta A_{np}$; kJ mol^{-1} Å^{-2}) corresponding to methyl group changes (Ala → Gly) for peptide ligands binding to ristocetin A; six different ligand comparisons are considered (see Table I).

Table II. Hydrocarbon Surface Areas

hydrocarbon	solvent-accessible surface area (Å^2) total	methyl
	200	12
	188	18
	170	28
	142	

Similar analyses to those above, comparing the binding of five other pairs of ligands that correspond to methyl group substitutions (Ala → Gly), are also included in Table I. Four of the six cases considered result in similar surface area dependent changes in binding energies, giving $\Delta\Delta G/\Delta\Delta A_{np}$ values of −0.15 to −0.24 kJ mol^{-1} Å^{-2}, in general agreement with earlier work.[13] However, there are two anomalous values (one much smaller and one much larger; Table I) that warrant further consideration, both involving the binding of *N*-X-Gly-D-Ala (where X represents acetyl or benzoyl; see legend to Table I). The deletion of the methyl group of the N-terminal residue (*N*-X-D-Ala-D-Ala versus *N*-X-Gly-D-Ala) produces relatively little change in binding energy given the relatively large change in hydrophobic surface area (53 Å^2) that is expected to promote the binding of the former over the latter. Closer examination of the calorimetry data[21] for these two ligands reveals a more exothermic binding interaction ($\Delta\Delta H$) for *N*-acetyl-Gly-D-Ala of 6.3 ± 3.0 kJ mol^{-1} that appears to compensate for the loss of hydrophobic effect from methyl group deletion. However, when a similar comparison is made for the binding of these ligands to vancomycin,[21] a much smaller exothermicity difference of 1.2 ± 2.7 kJ mol^{-1} is found that points to some feature unique to the ristocetin complex. In short, the anomalous change in binding exothermicity indicates that the small difference in binding free energy observed between these two ligands (see Table 1) cannot be interpreted only in terms of the relative contribution of the hydrophobic effect; if the difference in exothermicity is subtracted from $\Delta\Delta G$, i.e. only the difference in the entropy term is considered, then numbers per unit area buried of between −0.12 and −0.21 kJ mol^{-1} Å^{-2} are consistent with the general trend of the data. In contrast, the comparison of the binding of *N*-acetyl-Gly-D-Ala and *N*-acetyl-Gly-Gly gives a much larger change in binding energy for what we calculate to be a relatively small difference in the amount of hydrophobic surface area buried (30 Å^2). An experimental exothermicity for the binding of *N*-acetyl-Gly-Gly is not available for comparison of the two ligands which, at this stage, precludes a more detailed analysis.

We conclude from this analysis (as summarized graphically in Figure 5) together with the results of others[12,13] that if the area

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Table III. Estimation of the Free Energy of Formation of the Amide–Amide Hydrogen Bond from Ligand Extension Studies of Peptide Binding to Ristocetin A

ligands	$\Delta\Delta G^a$	$\Delta\Delta H^a$	$\Delta\Delta A_{np}$	$\Delta\Delta G_h{}^b$	$\Delta\Delta G_r$	$\Delta N_{hb}{}^c$	$\Delta G_p{}^d$
N-Ac-Ala-Ala/*N*-Ac-Ala	−11.4	+2.9 ± 3.0	85	−17	7 to 10	1	−2.9 ± 1.5
N-Ac-Gly-Ala/*N*-Ac-Ala	−11.0	−3.4 ± 2.8	33	−7	7 to 10	1	−12.5 ± 1.5
N-Ac-Ala-Gly/*N*-Ac-Ala	0	+4.7 ± 2.5	39	−8	7 to 10	1	−1.0 ± 1.0
N-Ac-Gly-Gly/*N*-Ac-Ala	+5.3	nd	9	−2	7 to 10	1	−1.2 ± 1.5
N-Ac-Ala/acetate	−11.4	nd	89	−18	7 to 10	1	−1.9 ± 1.5
N-Ac-Ala-Ala/acetate	−22.8	nd	174	−35	14 to 20	2	−2.4 ± 1.5
N-Ac-Gly-Ala/acetate	−22.4	nd	122	−24	14 to 20	2	−7.7 ± 1.5
N-Ac-Ala-Gly/acetate	−11.4	nd	128	−26	14 to 20	2	−1.3 ± 1.6
N-Ac-Gly-Gly/acetate	−6.1	nd	98	−20	14 to 20	2	−4.6 ± 1.5

[a] Data from ref 21; acetate binding from ref 2; binding of *N*-Ac-Gly-Gly, K = 150 ± 30 M^{-1} at 295 K (Williams, et al., unpublished results). [b] Using $\Delta\Delta G/\Delta\Delta A_{np}$ = −0.2 kJ mol^{-1} $Å^{-2}$. [c] Difference in the number of amide–amide hydrogen bonds. [d] Amide–amide hydrogen bond energies; nd = not determined.

of hydrocarbon buried is x $Å^2$, then the free energy change (ΔG_h) due to the hydrophobic effect may be represented by ca. $-0.2x$ kJ mol^{-1}; this conversion factor is used in subsequent calculations of binding energies.

We further note that there is no fixed value for the surface area of a methyl group because of the strong dependence of solvent accessibility on neighboring groups/residues. The change in surface area on deleting a methyl group (replacing with hydrogen) is illustrated for the hydrocarbons shown in Table II. For example, the change in surface area on replacing a methyl group of 2,2-dimethylpropane is only 11.5 $Å^2$; however, a similar transformation from propane to ethane, within the same series, produces a change in area of 28 $Å^2$, more than twice the area change for the more highly branched hydrocarbon.

Thus, after reconsidering the increase in nonpolar surface area which is removed from water when **2** binds to ristocetin A (over **3** binding to ristocetin A; 33 $Å^2$), we estimate the difference in the contribution of the hydrophobic effect $\Delta\Delta G_h$ to be $-0.2 \times 38 = -8$ kJ mol^{-1}. Applying eq 1, we then obtain $\Delta G_p = -11$ to -14 kJ mol^{-1} for the amide–amide hydrogen bond of **2**. The relative contributions to the difference in binding energy between these two ligands are presented in Table III.

In the earlier work, we also used the binding of the acetate anion (**4**, Figure 3), as a fragment of **3**, to estimate ΔG_p for the amide–amide hydrogen bond between **3** and the antibiotic and derived a value of −16 kJ mol^{-1}.[2] This value credits ΔG_p with 6 kJ mol^{-1} for a greater ΔG_{t+r} of **3** over acetate anion (**4**) and for residual motion reflected by using 5 versus 3.5–5 kJ mol^{-1} per restricted rotor. The hydrophobic effect of the alanine methyl group of **3** was allowed for by an experimental value (9 kJ mol^{-1}), but the hydrophobic interaction of the acetyl methyl group of **3** was taken as 2 kJ mol^{-1} (i.e., a total hydrophobic effect of 11 kJ mol^{-1}). A more realistic value for the contribution from the hydrophobic effect to the binding of **3** over **4** has now been estimated to be ca. −18 kJ mol^{-1}, corresponding to a difference in the nonpolar surface area buried on binding of 89 $Å^2$. Thus, when the credit for residual motion in the complex and for a larger hydrophobic effect is removed from −16 kJ mol^{-1}, we obtain a value for ΔG_p of −0.4 to −3.4 kJ mol^{-1}. We have considered the stepwise removal of hydrogen bonds for the 9 pairs of ligands indicated in Table III for binding to ristocetin A, (shown graphically in Figure 6). Calculated changes in the solvent-accessible surface areas are indicated in all cases, using the procedure described above. The relative contributions to the difference in binding energies ($\Delta\Delta G$) are itemized and the estimated free energies for amide–amide hydrogen bond formation (ΔG_p) are indicated in the final column.

To summarize these results, stepwise removal of the amide–amide hydrogen bonds made by peptide ligands to vancomycin group antibiotics gives a mean binding energy for the amide–amide hydrogen bond (using all nine data sets) of −4.4 kJ mol^{-1}, with values in the range 0 to −12.5 kJ mol^{-1} (as shown graphically in Figure 6). It can be seen that seven of the derived ΔG_p values in Table III fall in the range −1.0 to −4.6 kJ mol^{-1}, with only two much larger values (−12.5 and −7.7 kJ mol^{-1}) falling outside this range. Of these last two and possibly anomalously large values, two points are noteworthy. First, both include data for the binding

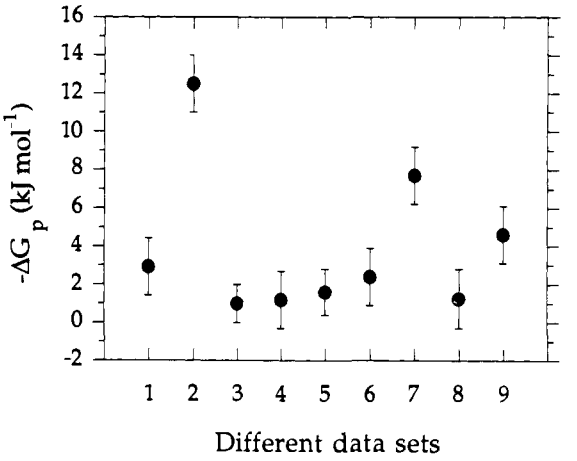

Figure 6. Summary of hydrogen bond free energies deduced from ligand extension studies of binding to ristocetin A; units along the x axis are arbitrary and correspond to ΔG_p values deduced from comparisons of binding data for nine different sets of ligand extensions (see Table III).

Table IV Estimates of Amide–Amide Hydrogen Bond Strengths (ΔG_p), Based on Differing Values of ΔG_h and ΔG_r, from a Comparison of the Binding of Peptide Ligands to Ristocetin A

assumed ΔG_h (kJ mol^{-1} $Å^{-2}$)	assumed ΔG_r (kJ mol^{-1})	derived ΔG_p (kJ mol^{-1})
−0.15	3.5	−4 ± 2
−0.15	5.0	−7 ± 2
−0.20	3.5	−1 ± 2
−0.20	5.0	−4 ± 2

of *N*-Ac-Gly-D-Ala (Table III). Second, the ΔG_p value of −7.7 kJ mol^{-1} is numerically less than the value of −12.5 kJ mol^{-1} mainly because the former is averaged over two apparent hydrogen bond strengths, whereas the latter value is the apparent hydrogen bond strength associated with only the glycine extension. Thus, both of the larger values are associated with an *apparent* hydrogen bond strength of the same glycine extension, possibly because in this case binding is promoted by a factor for which appropriate allowance has not been made. We note that if these possible anomalies are excluded from the averaging process, then an average amide–amide hydrogen bond strength of −2.2 kJ mol^{-1} is obtained.

In Table IV, we assess the effects of uncertainties in ΔG_h and ΔG_r on the estimated values of ΔG_p for the seven derived ΔG_p values that lie in the range −1.0 to −4.6 kJ mol^{-1}. We consider surface area dependences of the hydrophobic effect that encompass both the value of −0.2 kJ mol^{-1} $Å^{-2}$ used above and the smaller value of −0.15 kJ mol^{-1} $Å^{-2}$, which lies closer to the value long accepted on the basis of solvent transfer data.[11] Simultaneously, we consider the cost of rotor restriction between the limiting values of 3.5 and 5.0 kJ mol^{-1}. All estimates of ΔG_p for the extra amide–amide hydrogen bond of the dipeptide fall in the range −(1 to 7) ± 2 kJ mol^{-1}. Such are the combined uncertainties in the experimentally determined free energies of binding and the

appropriate ΔG_r and ΔG_h values used in the application of eq 1 that the derived range is in remarkably good agreement with the values of −2 to −8 kJ mol^{-1} derived for several types of neutral–neutral hydrogen bonds from protein engineering experiments.[25,26] It is encouraging that the two very different types of experiments give data which are in good accord. Note also that the ΔG_p values obtained for the binding of **2** and **3** to the antibiotics should probably bear comparison with the generalization of −2 to −8 kJ mol^{-1} from protein engineering, since in such experiments local motions in the protein are probably little changed before and after a hydrogen bond deletion resulting from a mutation.[27] Thus, the values give little or no entropic credit to the hydrogen bond for any motions it allows. We can be confident that this is the case for peptide antibiotic complexes when the difference in exothermicity of ligand binding is small or zero.[9] For the binding of **2** and **3** to ristocetin A, a small reported[21] value for $\Delta\Delta H$ of −3.4 ± 2.8 kJ mol^{-1} is consistent with this view; the difference in free energy of binding is essentially entropic in origin. $\Delta\Delta H$ values for ligand binding, where experimental data are available, are quoted in Table III.

Release of Water Molecules from Polar Groups

Previously we have concluded[2-4] that the entropically favorable release of water molecules, ordered around polar groups, provided the driving force for the formation of hydrogen bonds in aqueous solution. This may be an unnecessary hypothesis in the light of the present reconsideration of the "accounting" for residual motions and of an increased hydrophobic effect. However, the case for the adverse entropic effect of ordering of water molecules around polar groups in aqueous solution is worthy of further note. Evidence for ordering is based upon the NMR data of Kuntz,[28] while molecular dynamics simulations by Rossky and Karplus[29] of a solvated dipeptide in water indicate that polar groups have little influence on solvent mobility. More recently, elegant NMR experiments by Wuthrich and co-workers[30] seem to indicate that there is no evidence for preferentially ordered solvent molecules around charged or polar groups on the surface of BPTI. At present there is probably no good evidence for the *thermodynamic significance* of the "immobilization" of water around dipolar but uncharged groups found in peptides and proteins. However, binding studies involving hydrogen bond formation in "dry" and "wet" chloroform[31] indicate that the association of noncharged hydrated solutes in nonaqueous solvents is entropically less adverse to binding. This may suggest that substantial loss of translational and rotational freedom may be partially offset by the liberation of water molecules from the polar groups[31] in such wet nonpolar solvents. Again, enthalpy/entropy compensations work to produce similar association constants in both "dry" and "wet" environments. It is not clear that effects in "wet" chloroform can be readily extrapolated to infinitely wet solvents (aqueous solution) with similar entropic benefits. Indeed, it is a reasonable expectation that water will be more ordered by polar groups in a wet organic solvent than in aqueous solution. The small binding energies of the amide–amide hydrogen bonds that we have examined (Figure 6) may be due to a small favorable entropy change associated with water release, but this is not proven.

Conclusions

Large ΔG_p values for the amide–amide hydrogen bonds reportedly involved in dimerizations in aqueous solution are obtained if such hydrogen bonds are given credit for the residual motions which they allow. If the entropic advantage of these residual motions are estimated and removed, then the ΔG_p values obtained are not significantly different from the existing view of these bonds of −2 to −8 kJ mol^{-1}.[25,26] The same conclusion applies to ΔG_p values obtained by ligand extension (in binding to antibiotics) if credit for residual motions is removed and allowance for a larger hydrophobic effect than originally envisioned is made. Efforts to estimate solution binding constants by the application of eqs 1 and 2 will run into contradictions if ΔG_{t+r} values are taken from Figure 1 and the entropic advantages of residual motions are thereby credited to ΔG_p values.[2-4] These contradictions will arise because the a priori analysis of bimolecular associations of low exothermicity would credit much entropy of residual motions to ΔG_p values (A in Figure 7). In contrast, if ligand extensions or protein engineering experiments are used to derive ΔG_p values, then entropically favorable motions are similar in both the ligand X (B) and the extended ligand X–Y (C) (as represented schematically in Figure 7), if the extension is associated with a small or negligible increase in exothermicity of association. In these circumstances, ΔG_p values are not augmented by the favorable entropy of residual motion. Therefore, in attempts to obtain a potentially self-consistent set of ΔG_p values, the net change in ΔG_{t+r} should be employed.

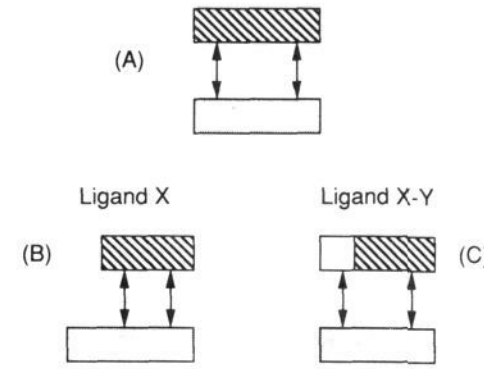

Figure 7. Residual motions in complexes of extended ligands. Entropically favorable motions in bimolecular associations (A) may be credited to functional group interactions; in contrast if residual motions are similar in both a ligand (B) and extended ligand (C), then functional group interactions are not augmented by the favorable entropy of these motions.

Our analysis of peptide binding (ligand extensions) to vancomycin group antibiotics has led us to conclude that hydrogen bonds contribute on average −4.4 kJ mol^{-1} (with estimated values in the range 0 to −12.5 kJ mol^{-1} per hydrogen bond), in general accord with previous conclusions.[25,26] Moreover, hydrogen bond free energies of ca. −4 to −5 kJ mol^{-1} are entirely consistent with observations on the stabilities of isolated α-helices in solution. It is evident from the study of model peptides[32] that many α-helices of moderate length (i.e. 15 to 50 residues) are formed with ΔG not far from zero in aqueous solution at physiological temperatures. When we consider that two rotors are restricted per residue for each hydrogen bond formed within the helix, at a cost of 3.5 to 5.0 kJ mol^{-1} per rotor (in the absence of other stabilizing interactions), we are led to the conclusion that each hydrogen bond is worth less than 7–10 kJ mol^{-1} (since polyglycine will not form a helix, and assistance from a favorable side chain is needed). Alanine-rich helices do form with ΔG not far from zero, and O'Neil and DeGrado[33] have shown that the relative stabilities of α-helices, versus their random-coil states, vary among the 20 commonly occurring amino acids by only 3 kJ mol^{-1} from the most favorable (Ala) to the least favorable (Gly). Thus, the amide–amide hydrogen bond strength can independently be estimated in the range −(7−3) to −(10−3) = −(4 to 7) kJ mol^{-1} in aqueous solution. We note that the difference between alanine and glycine

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(27) Exceptionally, we note that single amino acid substitutions of certain active site residues in phospholipase A_2 have recently been shown to convert the "rigid" enzyme structure to a highly flexible molecule with correspondingly large changes in free energies of unfolding of up to 21 kJ mol^{-1}: Dupureur, C. M.; Li, Y.; Tsai, M.-D. *J. Am. Chem. Soc.* **1992**, *114*, 2748–2749.
(28) Kuntz, I. D. *J. Am. Chem. Soc.* **1971**, *93*, 514–520.
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may, in large part, arise from the hydrophobic contribution derived from the partial burial of the hydrophobic surface of the alanine methyl rather than from any conformational bias (see above).

In this paper (and the preceding paper[9]), we have reconsidered the partitioning of free energy contributions important in bimolecular associations and in ligand extension studies and present a self-consistent approach that may prove useful in the semiquantitative estimation of binding constants. Much work remains to firmly establish the thermodynamic basis of many of the interactions found in biologically important associations. In this regard we have shown that model systems, including crystals (as analogues of tight-binding complexes), can provide useful insights that guide estimates of entropy changes in molecular recognition phenomena.

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On the Factors Controlling the Structural Specificity and Stereospecificity of the L-Lactate Dehydrogenase from *Bacillus stearothermophilus*: Effects of Gln102→Arg and Arg171→Trp/Tyr Double Mutations

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Abstract: The factors determining the L-stereospecificity of the L-lactate dehydrogenase from *Bacillus stearothermophilus* have been probed by introducing Arg171Trp/Tyr and Gln102Arg mutations. These changes preclude normal 2-keto acid substrate binding via an Arg171–COO^- electrostatic interaction and are positioned to induce a reversal of the natural substrate binding mode, thereby leading to D-2-hydroxy acid formation. However, the L-stereospecificities of the mutant enzymes remain unchanged, showing that there are important fail-safe stereospecificity determinants that take over when the key Arg171–COO^- binding interaction is removed. The effects of the mutations on structural specificity are approximately additive, resulting in the broad 2-keto acid specificity of the wild-type enzyme being changed to give catalysts highly selective for the dicarboxylic substrate oxalacetate.

Lactate dehydrogenases (LDHs)[1] catalyze C=O ⇄ CH(OH) transformations of the type shown in eq 1.[2] The natural keto

$$RCOCOOH + NADH + H^+ \underset{\longleftarrow}{\overset{BSLDH}{\longrightarrow}} RCH(OH)COOH + NAD^+ \quad (1)$$

1	2
a. R=CH_3-	h. R=$C_6H_{11}CH_2$-
b. R=CH_3CH_2-	i, R=$C_6H_5CH_2$-
c. R=$CH_3(CH_2)_2$-	j, R=HOOC-
d. R=$CH_3(CH_2)_3$-	k, R=$HOOCCH_2$-
e, R=$CH_3(CH_2)_5$-	l, R=$HOOC(CH_2)_2$-
f. R=$(CH_3)_2CH$-	m, R=$HOCH_2$-
g. R=$(CH_3)_2CHCH_2$-	n, R=$BrCH_2$-

acid substrate is pyruvic acid (**1a**), but reductions of other structurally varied 2-keto acids to the corresponding 2-hydroxy acids have been reported.[3] Because such LDH-catalyzed reductions are stereospecific, each 2-hydroxy acid product is enantiomerically pure. This is of considerable practical importance since 2-hydroxy acids are valuable chiral synthons in asymmetric syntheses of biologically important molecules.[4-15] As a consequence, organic synthetic applications of LDHs are expanding.[3,16-18]

A remarkable feature of LDHs, as well as of most other dehydrogenases, is their high fidelity with regard to the stereose-

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(1) Abbreviations used: LDH, lactate dehydrogenase; BSLDH, LDH from *Bacillus stearothermophilus*; DMLDH, LDH from spiny dogfish muscle; NAD^+ and NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide, respectively; FDP, fructose 1,6-diphosphate; MTPA, (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl; CD, circular dichroism; WT, wild-type; Q102R, glutamine 102 to arginine mutation; R171Y and R171W, arginine 171 to tyrosine or tryptophan mutation, respectively; *E. coli*, *Escherichia coli*; ES, enzyme–substrate; K_M, Michaelis constant; k_{cat}, catalytic constant.

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