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Towards a semi-quantitative description of a bimolecular association involving weak interactions in aqueous solution

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We present a description of a bimolecular association in aqueous solution by dividing the free energy of binding into a number of terms representing 'costs' and 'benefits'. Using data on the binding of cell-wall peptide analogues to vancomycin-group antibiotics, we have established an experimental basis for the adverse free energy of restricting internal rotations, the benefit in free energy from hydrophobic interactions and polar functional group interactions (amide–amide hydrogen bonds). Collating data from the literature on weak associations in non-polar solvents, a relation between the electrostatics of binding (enthalpy) and the dynamics (entropy) is presented. This provides an approximate, but useful, relation between the magnitude of the enthalpic barrier to dissociation for complexes in aqueous solution, and the cost in translational and rotational free energy of the reverse bimolecular association.

1. Introduction

We have recently taken an approach to furthering our understanding of weak interactions in aqueous solution which divides the free energy of binding (ΔG) for a bimolecular association into six terms:

$$\Delta G = \Delta G_{\mathrm{T+R}} + \Delta G_{\mathrm{r}} + \Delta G_{\mathrm{h}} + \Sigma \Delta G_{\mathrm{p}} + \Delta G_{\mathrm{conf}} + \Delta G_{\mathrm{vdW}}, \qquad (1)$$

where $\Delta G_{\mathrm{T+R}}$ is the change in translational and rotational free energy (largely entropic) associated with the low probability of 'catching' the ligand on the receptor in the absence of intermolecular forces, ΔG_{r} is the change in free energy of internal rotations, and ΔG_{h} is the change in free energy due to the hydrophobic effect (relevant when binding in aqueous solution is being considered). ΔG_{p} is the free energy contribution due to any increased electrostatic interaction occurring in the complex over those occurring in solution (summed over all such interactions), ΔG_{conf} is the change in free energy due to any conformational strain occurring in the complex, and ΔG_{vdW} is the free energy change due to a change in van der Waals interactions in the complex over those occurring in solution (e.g. due to the presence of cavities, or van der Waals repulsions in the complex). To test the utility of this approach, we have studied the binding of cell-wall analogues to vancomycin group antibiotics in aqueous solution (Williams *et al.* 1991; Searle *et al.* 1992). In such studies, the approximation $\Delta G_{\mathrm{conf}} \approx 0$ has been made since, for example, the cell wall analogue which is central to the studies (N-Ac-D-Ala-D-Ala) binds to the antibiotics

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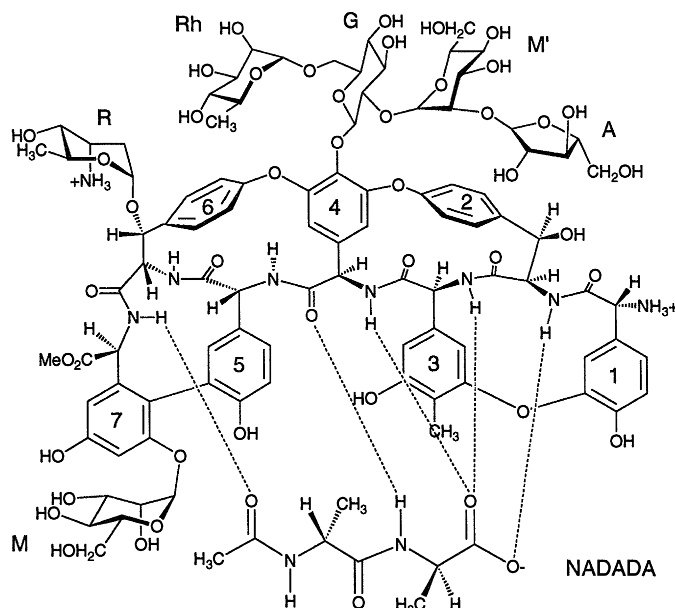

Figure 1. Structure of the N-ac-D-Ala-D-Ala–Ristocetin A complex. Antibiotic sugars are labelled R = ristosamine, Rh = rhamnose, M = M′ = mannose, and A = arabinose. Dotted lines represent intermolecular hydrogen bonds.

in a conformation which is similar to an extended β-strand, and to the conformation found in an X-ray study of N-Ac-L-Ala-L-Ala (Fawcett *et al.* 1975). Similarly, we make the assumption that van der Waals repulsions are negligible ($\Delta G_{\mathrm{vdW}} \approx 0$), since the ligands show good complementarity to the antibiotic receptors. Hence, on the basis of these approximations, equation (1) simplifies to

$$\Delta G = \Delta G_{\mathrm{T+R}} + \Delta G_{\mathrm{r}} + \Delta G_{\mathrm{h}} + \Sigma \Delta G_{\mathrm{p}}. \qquad (2)$$

Using this approach, we have derived an approximate but most probable value for the dependence of the hydrophobic effect on the solvent-accessible surface area of hydrocarbon buried on binding (-0.20 ± 0.05 kJ mol^{-1}; Searle *et al.* (1992)); this value is in reasonable accord with those based on solvent transfer experiments (adjusted for a favourable entropy of mixing; Sharp *et al.* (1991)), and protein engineering experiments (Serrano *et al.* 1992). Amide–amide hydrogen bond strengths in the range $-(0\text{–}6)$ kJ mol^{-1} were obtained (Searle *et al.* 1992) when the above value of the hydrophobic effect was used, in conjunction with free energy costs for rotor restrictions (ΔG_{r}) in the range 3–5 kJ mol^{-1} (Searle & Williams 1992; Gerhard *et al.* 1993*a*).

In this paper, we address three issues. First, we justify and estimate rough factors by which the above effects promote or oppose binding, and use these values, in conjunction with other information, to give a full (although admittedly very approximate) description of the major factors determining the binding constant of N-Ac-D-Ala-D-Ala to the vancomycin group antibiotic ristocetin A (figure 1). Secondly, we show that the dimerisation of these antibiotics, in itself a fascinating molecular recognition phenomenon, does not significantly compromise this description. Thirdly, we show that there is a relation between the exothermicity of weak associations (involving limited areas of solute–solute contact) in non-polar

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solutions and the adverse entropy ($T\Delta S$ at 300 K) of the association. This relation is used to give a rough guide to the loss in translational and rotational free energy (ΔG_{T+R}, essentially entropic in origin) in the binding of N-Ac-D-Ala-D-Ala to ristocetin from the enthalpic barrier to the reverse dissociation step.

2. Approximate factorization of binding specificities

The successful application of (2) depends upon obtaining each factor as a usefully correct approximation. In this paper we outline results obtained using the ligand extension or ligand modification approach ('anchor principle'; Jencks (1981)) in which the ΔG_{T+R} term (the cost of a bimolecular association) in (1) can usefully be regarded as common to all of the associations described and is thereby a variable which is removed from the equation when comparing the binding of ligand X-Y-Z and ligand X-Y to a common binding site. In estimating functional group contributions to binding (ΔG_p values) using this approach, it is a prerequisite that the electrostatics (enthalpies) of binding X-Y-Z and X-Y are sufficiently similar ($\Delta\Delta H \approx 0$) that any difference in binding energy ($\Delta\Delta G$) can be attributed to the functional group contribution of Z (after allowance has been made for any additional rotations restricted in the bonds connecting Y and Z) rather than to any difference in the amount of residual motion that X-Y-Z and X-Y permit (Searle *et al.* 1992; Williams *et al.* 1993). Thus, the difference in binding energy $\Delta\Delta G$ between X-Y-Z and X-Y can be usefully approximated to:

$$\Delta\Delta G = \Delta\Delta G_r + \Delta\Delta G_h + \Delta(\Sigma\,\Delta G_p). \tag{3}$$

(*a*) *Hydrophobic effect*

The ligand modification approach has enabled us to establish a correlation between free energy changes and the non-polar surface area (Å^2) buried on binding to ristocetin A in a series of dipeptide ligands with methyl group deletions corresponding to Ala→Gly 'mutations' at the C- and N-terminal positions (Searle *et al.* 1992). Comparing N-Ac-D-Ala-D-Ala and N-Ac-D-Ala-Gly realizes a difference in binding energy of $\Delta\Delta G$ of -10.0 ± 1.6 kJ mol^{-1} (average of three values from different sources), which equates with a difference in non-polar surface area buried of 47 Å^2. (Surface areas were measured from energy-minimized structures of the complexes and free ligands by rolling a sphere (corresponding to a water molecule of radius 1.4 Å) over the surface.) A surface area dependent hydrophobic effect of between -0.18 and -0.24 kJ mol^{-1} Å^{-2} is in good accord with the value (-0.23 kJ mol^{-1} Å^{-2}) recently found for Ala→Gly mutations from protein engineering experiments (Serrano *et al.* 1992). Six ligand modification comparisons have been considered which leads to a value of -0.2 ± 0.05 kJ mol^{-1} Å^{-2} at the centre of the experimental range. This value is subsequently used as the magnitude of the hydrophobic effect.

(*b*) *Internal rotations*

The cost in free energy of restricting a rotation (mainly entropy) is taken to lie in the range 3–5 kJ mol^{-1} per rotor. The lower value is that deducted from the entropic cost of crystallizing even-membered n-paraffins (C_nH_{2n+2}) (Searle & Williams 1992), where weak intermolecular (van der Waals) interactions in the crystal permit entropically significant residual torsional vibrations, while the upper limit is derived from the entropic cost in the formation of small rings from linear hydrocarbons (Page

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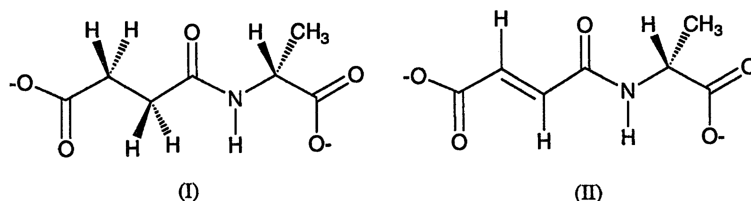
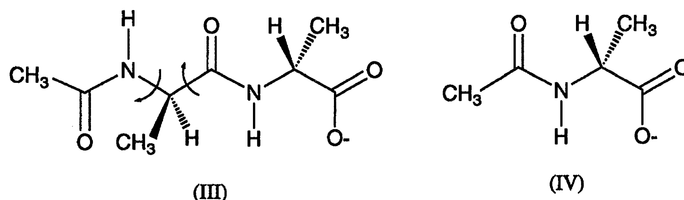
& Jencks 1971; Searle & Williams 1992), where severe restriction of rotations occurs due to covalent bond formation in the product (which permits very little residual motion). The lower value may be more appropriate for general use where rotations are restricted by weaker non-covalent interactions. This conclusion is in agreement with the value of 3.7 ± 0.9 kJ mol^{-1} estimated as the cost in free energy based on experiments using the ristocetin A system as a receptor and comparing the binding of N-succinyl-D-Ala (I) and N-fumaryl-D-Ala (II) (Gerhard *et al.* 1993*a*).



Thus, using the parameters deduced above for the contribution of the hydrophobic effect (-0.2 ± 0.05 kJ mol^{-1} Å^{-2}), and the cost of restricting a rotation (3–5 kJ mol^{-1} per rotor) it is possible to estimate ΔG_p values using the method of ligand extension and equation (3).

(*c*) *Amide–amide hydrogen bonds*

The difference in free energy of binding between N-Ac-D-Ala-D-Ala (III) and N-Ac-D-Ala (IV) to ristocetin is -11.4 kJ mol^{-1}. The results of calorimetry experiments (Rodriguez-Tebar *et al.* 1986) show that this difference is largely entropic in origin, the difference in exothermicity being small ($\Delta\Delta H = +2.9 \pm 3.0$ kJ mol^{-1}). The increase in binding energy of the dipeptide is achieved through the formation of an additional amide–amide hydrogen bond, through the burial of an extra 85 Å^2 of hydrocarbon surface, but at a cost of restricting two peptide backbone rotors (III).



The estimated difference in binding energy from the hydrophobic effect is -17 kJ mol^{-1} while the cost of two rotor restrictions is 6–10 kJ mol^{-1} (the peptide backbone is extensively cross-linked and is restricted both before and after ligand binding). Since the adverse cost of the bimolecular association in each case ($\Delta G_{\mathrm{T+R}}$) should be essentially the same for both ligands (both have similar masses and bind with similar exothermicities), then applying equation (3):

$$-11.4 = (6\text{–}10) - 17 \pm \Delta G_p \,\text{kJ mol}^{-1}.$$

Thus, the binding energy for the amide–amide hydrogen bond (ΔG_p) is deduced to lie between -0.4 and -4.4 kJ mol^{-1}. Amide–amide hydrogen bond strengths from nine such comparisons of ligand extensions have been considered (Searle *et al.* 1992), and include comparisons of the binding of X-Y-Z and X-Y and X, and of X-Y with X, where X = acetate anion, X-Y = N-Ac-D-Ala, and X-Y-Z = N-Ac-D-Ala-D-Ala, N-Ac-D-Ala-Gly, N-Ac-Gly-D-Ala and N-Ac-Gly-Gly. Seven of the nine ΔG_p values deduced fall in the range -0.5 to -4.1 (± 3) kJ mol^{-1}, with only two values falling outside this range (-12.0 and -7.2 (± 3) kJ mol^{-1}). Both larger values arise from

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the binding data for N-Ac-Gly-D-Ala and are therefore associated with a Gly extension. The first (-12.0 kJ mol^{-1}) is the *apparent* hydrogen bond strength from the Gly extension alone, whereas the second (-7.2 kJ mol^{-1}) is averaged over two apparent hydrogen bond strengths (comparison of acetate with N-Ac-Gly-D-Ala). Assuming the 'non-Gly' hydrogen bond to be 'normal', it can be seen that in both cases, the *apparent* Gly hydrogen bond strength is very large. We have recently concluded (Holroyd *et al.* 1993) that these two larger values are anomolous by virtue of being credited with the additional benefit of π-stacking interactions that the amide group of the Gly residue makes through closer approach to the π-face of the *m*-dihydroxylated benzene ring of residue 7 of the antibiotic. Such interactions are precluded in the binding of N-Ac-D-Ala-D-Ala (III) by the steric bulk of the Ala methyl group. Calorimetry results support such a model; despite the loss of the hydrophobic component to binding from the Ala methyl group (difference of *ca.* 53Å^2 of buried non-polar surface area), N-Ac-Gly-D-Ala has a similar overall binding energy to N-Ac-D-Ala-D-Ala, but binds more exothermically by 6 kJ mol^{-1} (Rodriguez-Tebar 1986; Searle *et al.* 1992). Molecular modelling studies and subsequent analysis of the binding data for (I) and (II) (and several oxamic acid analogues) (Holroyd *et al.* 1993), reveals a large binding component from the succinoyl and fumaroyl carboxylate groups of (I) and (II) which we rationalize, in large part (*ca.* 10 kJ mol^{-1}), in terms of face-to-face π-stacking interactions. Such exothermic interactions are known to occur from benzene-induced solvent shifts observed in ^{1}H NMR spectra of N,N-dimethylacetamide (Hatton & Richards 1962), and are plausible contributors to ligand-receptor binding.

Thus, when the two anomalously large values for the amide–amide hydrogen bond (-12.0 and -7.2 kJ mol^{-1}) are corrected for the additional 6 kJ mol^{-1} of exothermicity derived from π-stacking interactions, values of -6.0 and -1.2 (± 3) kJ mol^{-1} are obtained (the latter averaged over two hydrogen bonds from comparison with acetate binding). Averaging over all nine data sets (including these corrected values), gives a hydrogen bond strength of -2.1 (± 3) 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 equation (3), that the derived ΔG_p values can be considered to be consistent with hydrogen bond strengths of -2 to -8 kJ mol^{-1} found for amide–amide and other neutral–neutral hydrogen bonds from protein engineering experiments (Fersht 1986; Shirley *et al.* 1992). It is encouraging that the two very different types of experiments give data that are in such reasonable accord.

3. Dimerization of vancomycin-group antibiotics

A further feature of the vancomycin-group antibiotics, and one that has been proposed to play a possible role in antibiotic action, is their ability to form homodimers in both aqueous and mixed solvents (figure 2*a*) (Waltho & Williams 1989; Gerhard *et al.* 1993*b*). Thermodynamic characterization of the dimerization process in several related antibiotics and their derivatives (Gerhard *et al.* 1993*b*), reveals that dimer formation is enthalpy driven (*ca.* -36 to -51 kJ mol^{-1}) to an important extent. Moreover, studies of the effects of bound ligand (N-Ac-D-Ala, N-Ac-D-Ala-D-Ala, and di-N-Ac-Lys-D-Ala-D-Ala) on the dimerization constant of ristocetin Ψ (lacking the sugars on rings 4 and 7, see figure 1), shows dimerization to be enhanced by a factor of 5–10 upon ligand binding. The chemical shift of proton

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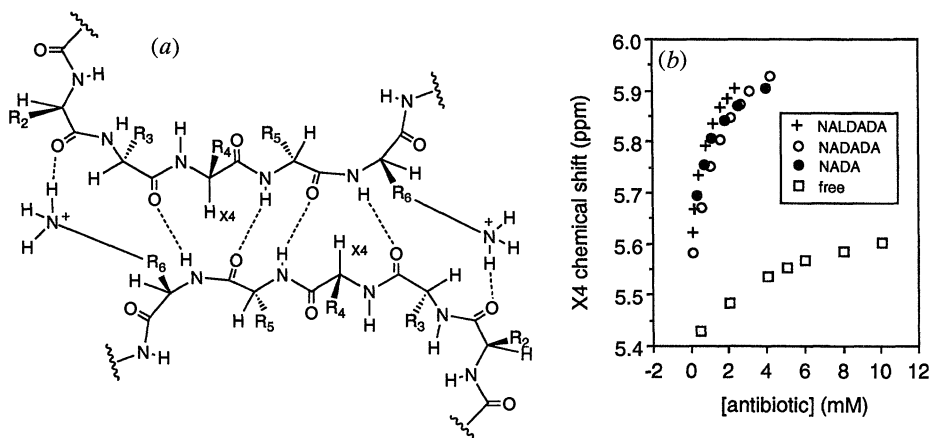

Figure 2. (*a*) Intermolecular hydrogen bonds (hashed lines) between the two halves of the ristocetin dimer. Only the antibiotic peptide backbone is illustrated, with the position of residue side chains (R_2 to R_6). The concave cavities on the outer faces of the monomer units are the binding site of the cell-wall analogues (see figure 1). In (*b*) the chemical shift of X4 is plotted against antibiotic concentration as a measure of the extent of dimerisation.

X4 (CαH proton of residue 4 in figure 2*a*) provides a convenient NMR probe of the extent of dimerization, and is plotted as a function of either free antibiotic concentration, or complex concentration, in figure 2*b*. The larger limiting shift of X4 in the dimer complex may indicate a tighter interation between the two halves of the dimer in the presence of bound ligand. The increase in the extent of dimerization upon ligand binding, calculated for the antibiotic concentrations used in calorimetry experiments (0.046 mM and 0.23 mM; Rodriguez-Tebar *et al.* (1986)), suggests that the amount of dimer increases by 2 and 8 % respectively, upon binding N-Ac-D-Ala-D-Ala. Hence, there is the potential for an additional enthalpy contribution to ligand binding that finds its origins in the large enthalpy component for dimer formation. However, as expected from such relatively small enhancements of dimerization (2–8 % in the concentration range considered), this contribution is expected to be comparable in magnitude with experimental error limits. If the effect of cell wall analogues on the dimerization of ristocetin A and vancomycin is similar to that found for ristocetin Ψ, then the analysis of the thermodynamic data for ligand binding will not be significantly compromised by the effects of dimerization.

4. Enthalpic barriers to dissociation

(*a*) *Enthalpy/entropy compensation*

The phenomenon of enthalpy/entropy compensation is of fundamental importance to molecular recognition phenomena in situations where the binding enthalpy (ΔH) is comparable with, or a few times, the thermal energy RT (R, gas constant) (Williams *et al.* 1993). The origins of the phenomenon are to be found in the depth of the electrostatic well corresponding to the associated state. In deep electrostatic wells (perhaps $> 20RT$) relatively little motion is permitted compared with the dissociated state in which the electrostatic attractions are removed. Conversely, dissociations that have small barriers ($\Delta H_{\text{barr}} < 4RT$) have much residual motion in the associated state. From these arguments, it can be seen that the adverse entropy of a weakly exothermic bimolecular association will be only a relatively small

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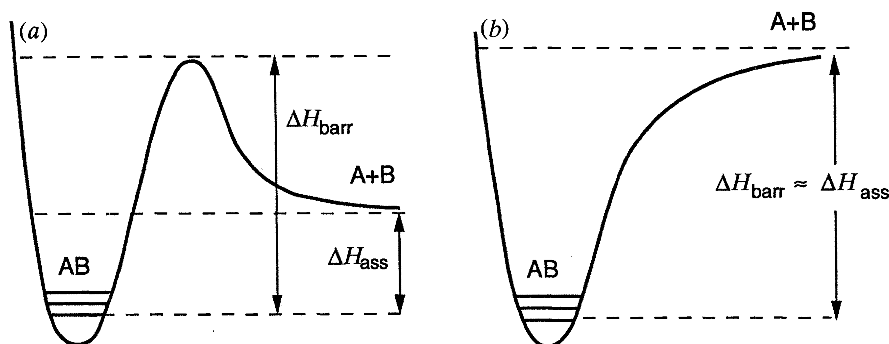

Figure 3. Energy wells for the association of A + B to give a complex AB, in which there is (*a*) an endothermic barrier to complex dissociation, and (*b*) no barrier to the association step.

fraction of the formal loss of $\Delta G_{\mathrm{T+R}}$ corresponding to the formation of a 'rigid' complex (Williams *et al.* 1993). The relation between the enthalpic barrier to dissociation (ΔH_{barr}) and the loss of $\Delta G_{\mathrm{T+R}}$ ($\approx T\Delta S_{\mathrm{T+R}}$) in a bimolecular association, appears to be of such fundamental importance to the application of (1) and (2) that we now address experimental approaches that might allow us to make an approximate correlation between ΔH_{barr} and the adverse entropy of a bimolecular association $T\Delta S_{\mathrm{T+R}}$ ($\approx \Delta G_{\mathrm{T+R}}$).

(*b*) *Problems with estimating* ΔG_{T+R}

Page & Jencks (1971) have shown (supported by much experimental data) that the loss in translational and rotational free energy (mainly entropy) of a bimolecular association costs 50–60 kJ $\mathrm{mol^{-1}}$ where covalent bond formation is involved between entities of molecular weight 100 to 300 Daltons. It has been assumed in some cases that a similar entropic price is paid in bimolecular associations involving only weak (non-covalent) intermolecular interactions (Andrews *et al.* 1984) (i.e. hydrogen bonds, van der Waals interactions). However, it appears that in general the cost will be some fraction of 50–60 kJ $\mathrm{mol^{-1}}$, and a sensitive function of the enthalpic barrier to dissociation. In support of this conclusion are the small favourable entropies of sublimation of organic compounds (such as hydrocarbons) which involve relatively weak interactions in the crystal, while larger entropy changes are associated with the sublimation of polar molecules with relatively strong intermolecular interactions (large enthalpies of sublimation; Searle & Williams (1992)). By analogy, this entropy/enthalpy compensation will also occur in complexes of biological interest formed in solution. Here, large enthalpic barriers to dissociation can be equated with physical barriers to solvation (figure 3*a*), as polar groups become separated in the transition state. Where separated polar groups are inaccessible (or poorly accessible) to solvent, the transition state is more akin to a gas phase dissociation where there is poor stabilization of polar functional groups. Consequently the electrostatic well may be deep ($\gg RT$) such that only small amplitude motions are possible in the associated state; thus, much entropy is to be gained in the dissociation process, but a large adverse entropy is the price to pay in the association step. The limiting value of $T\Delta S_{\mathrm{T+R}}$ corresponds to the maximum possible loss of entropy for formation of a 'rigid' complex, and is likely to be achieved only in the case of very large binding exothermicities, that may well lie outside the range possible for complexes formed through non-covalent interactions. The situation is most readily discussed in terms

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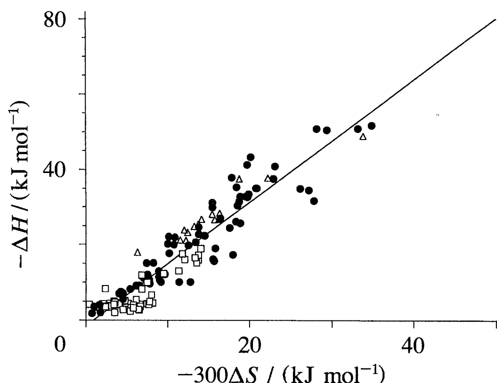

Figure 4. Plot of enthalpy ($-\Delta H$) against entropy ($-300\Delta S$) of weak (non-covalent) associations in non-polar organic solvents collated from the literature. Data include (●) charge transfer complexes of iodine, and (□) collision complexes data from Foster (1969) and references therein; (△) lactam dimerisations (Chen & Swenson 1969; Wagner 1975; Walmsley *et al.* 1976). The line of best fit is given by $y = 1.63x - 1.46$, correlation coefficient $R = 0.94$.

of a bimolecular association in which there is no barrier to the association step (figure 3*b*). In this regard, we have modelled enthalpic barriers to dissociation in aqueous solution by collating thermodynamic enthalpies and entropies for weak associations in non-polar solvents. Here, the hydrophobic effect is irrelevant, and we make the assumption that solvent release from polar functional groups, at least in the cases where the interfacial surface area in an A·B complex is relatively small, is of relatively small thermodynamic significance. This should be a useful approximation where there is poor solute solvation (by non-polar solvent molecules) of one or two pairs of polar groups which come together in the complex. Thus, the experimental parameter $T\Delta S$ is a rough measure of the cost of a bimolecular association in terms of the loss of translational and rotational entropy. A large body of experimental data from the literature from studies of enthalpies and entropies of weak associations (lactam dimerisations, collision complexes and charge transfer complexes), that satisfy the criteria of having few or no internal rotors to restrict, and whose thermodynamic parameters are measured at temperatures close to 300 K, are presented in figure 4. Interestingly, similar relations are obtained between the same variables in, for example, the sublimation of organic crystals (Searle & Williams 1992). Even under conditions where ΔH_{ass} is large, the limiting value for $T\Delta S_{\mathrm{T+R}}$ of 50–60 kJ $\mathrm{mol^{-1}}$, proposed for a 'rigid' covalent transformation, is not achieved. However, we conclude that 'sloppy' associations with small exothermicities can give rise to remarkably small adverse entropies on binding.

(*c*) *Enthalpic barriers to dissociation: ristocetin complexes*

One of our goals is to utilize the relation between the exothermicity of association and its adverse entropy (as expressed in the data of figure 4), in presenting a complete description of the binding energies for weak associations in aqueous solution. Such a generalization would, of course, be very approximate, since such a relation would be expected to hold strictly only if the shapes of the electrostatic wells were very similar in all cases, and the density of states within the wells were similar. It also treats the entropies of the separated components in a non-rigorous manner, but the important point is that diverse sets of experimental data (figure 4) suggest that the generalization is well-based. In the absence of a better current guide, we suggest that

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Table 1. *A semi-quantitation of binding contributions to the association of* N-Ac-D-Ala-D-Ala *with ristocetin A*

term	origin	ΔG	$T\Delta S$	ΔH
ΔG_r	restriction of 4 rotors	16 ± 4	-16 ± 4	0
ΔG_h	burial of 214 Å^2 of hydrocarbon	-43 ± 11	43 ± 11	0
ΔG_p	formation of 2 amide–amide H-bonds	-4 ± 6	2 ± 3	-2 ± 3
ΔG_{T+R}	endothermic barrier to complex dissociation of 73 kJ mol^{-1}	45	-45	0
ΔG_p (CO_2^-)	carboxylate binding energy	-42 ± 21	19 ± 18	-23 ± 3
Experimental parameters from Rodriguez-Tebar *et al.* (1986)		-28	-3	-25

it may be useful, perhaps giving equilibrium constants in many cases to within 1 or 2 orders of magnitude for lower exothermicities (up to *ca.* -45 kJ mol^{-1}), and to within 2 or 3 orders of magnitude for the larger exothermicities (subject to the restrictions outlined above).

In this section, we illustrate the principles involved by considering a description of the binding of N-ac-D-Ala-D-Ala to ristocetin A. In doing this, we utilize approximate numbers that are plausible based upon the experimental results summarized in earlier sections of this paper. The case for doing this is to present for examination a model upon which we can build. The actual numbers used, often derived from large and often opposing variables, are at present most important simply as a vehicle to illustrate principles.

In illustrating these principles, we use -0.2 ± 0.05 kJ mol^{-1} Å^{-2} for the surface area-dependence of the free energy contribution from hydrophobic effect; -2 ± 3 kJ mol^{-1} for the strength of the amide–amide hydrogen bond; and 3–5 kJ mol^{-1} for each rotor restricted upon binding. Given these approximate values, the elements of the binding interation which have not been considered are (i) what is the loss in translational and rotational free energy (ΔG_{T+R}) upon binding, and (ii) what is the intrinsic binding energy (ΔG_p) associated with the binding of the carboxylate group into the pocket of three amide NHs (figure 1)?

In table 1, the values of ΔG_r, ΔG_p and ΔG_h, for the association are given. The value of ΔG_{T+R} is estimated to be in the region of 45 kJ mol^{-1} for an endothermic barrier to dissociation of the complex of 73 kJ mol^{-1} (measured by nmr; Williamson *et al.* (1984)) using the correlation of entropy against enthalpy for the solution associations presented in figure 4. We appreciate that the ΔH values derived from figure 4 are thermodynamic values, but they may usefully approximate enthalpy barriers to dissociation where non-polar solvents poorly solvate polar entities which are uncovered in dissociation (cf. figure 3). Additionally, 45 kJ mol^{-1} may well represent a lower limit for ΔG_{T+R} since in the transition state for the ristocetin A/N-Ac-D-Ala-D-Ala association, the cell wall analogue appears likely to have already lost some of its translational and rotational entropy. This loss is expected as a consequence of the association pathway being one in which polar interactions are maintained as much as possible: as interactions to water are gradually lost, polar interactions between antibiotic and peptide are gradually developed.

Since the experimental ΔG for binding for N-Ac-D-Ala-D-Ala to ristocetin A is known (table 1), the intrinsic binding energy of the carboxylate anion [ΔG_p (CO_2^-)] is the only unknown, and is estimated by the above approach to be

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-42 ± 21 kJ mol^{-1}. The uncertainties in all parameters are large with the consequence that the ΔG_p value deduced is subject to large error limits, with a most probable value of *ca.* 10^7 M^{-1}. Additional experimental observations suggest that a value close to *ca.* 10^6 to 10^7 M^{-1} is a reasonable estimate. CDP-I, an analogue of vancomycin in which the carboxylate binding pocket, consisting of three amide NHs, has been partly destroyed (leaving only one NH), binds di-Ac-Lys-D-Ala-D-Ala (binding constant of 10^6 M^{-1} with ristocetin and vancomycin) with an imperceptibly small binding constant (< 10 M^{-1}; S. E. Holroyd *et al.*, unpublished results). Thus, destroying the majority of the carboxylate binding interaction in this way appears to be implying a ΔG_p value for the carboxylate-group–ristocetin-A interaction of at least 10^6 M^{-1}. Some indication of the exothermicity of the carboxylate interaction is indicated by the binding of the acetate anion to ristocetin A. Despite only a small binding constant of *ca.* 10 M^{-1}, the exothermicity, although subject to considerable uncertainty, appears to be large (in the range 30 ± 17 kJ mol^{-1}; Williams *et al.* (1991)), representing a substantial proportion of the total binding exothermicity of N-Ac-D-Ala-D-Ala.

5. Conclusion

We recognize that the parameters derived using the above approach are approximate only, and may indeed vary from one environment to another, and may be additionally complicated by cooperativity. The ΔG_p values discussed correspond to the intrinsic free energy of binding at the pertaining value of ΔG_{T+R}; it is possible that ΔG_p values could be larger at a larger value of ΔG_{T+R}. It nevertheless seems worthwhile to attempt such a semi-quantitative description of binding even if only to give rough estimates of the relevant parameters, and underscore any limitations that may arise. Despite the uncertainties in the approach, the derived parameters provide guide-lines for assessing whether productive binding is possible, and for giving molecular recognition a numerical foundation.

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