Binding Energy of an Amide–Amide Hydrogen Bond in Aqueous and Nonpolar Solvents

Andrew J. Doig[†] and Dudley H. Williams*

Contribution from the Cambridge Centre for Molecular Recognition, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, England. *Received April 17, 1991*

Abstract: Thermodynamic data are available for the dimerization of small molecules containing peptide groups in water and tetrachloromethane. Dimerization is opposed by loss of translational and rotational entropy and the freezing out of internal rotations and is favored by the formation of two amide–amide hydrogen bonds. The magnitude of the translational entropy lost in solution can be estimated from the Sackur–Tetrode equation in the gas phase, which is corrected by Trouton's rule for condensation to a liquid and a dilution term to a 1 M solution. If the effects of the translation, rotation, and internal rotation are removed from the thermodynamic parameters for complexation, the free energy, entropy, and enthalpy for the formation of peptide hydrogen bonds are obtained. This free energy will be close to the theoretical maximum intrinsic binding energy of a hydrogen bond. It is found that the formation of a hydrogen bond is highly favorable in both aqueous and nonpolar solvents by a mean value of -26 kJ mol^{-1} in water and -32 kJ mol^{-1} in tetrachloromethane. These values may be too large by up to 30–50%, but even the smaller values (-13 and -22 kJ mol^{-1}, respectively) are much larger than previously realized. The process is largely entropy driven in water while both entropy and enthalpy contribute in tetrachloromethane. Hydrogen bond formation in nonpolar solvents is driven by electrostatic attraction between the polar peptide units and the introduction of a number of low-frequency vibrations in the bound complex. Hydrogen bond formation in water is largely driven by the release of bound water molecules. The results obtained from the dimerizations agree well with those obtained from studies of hydrogen bond formation in the antibiotics ristocetin and vancomycin, which were found by a different method. Amide–amide hydrogen bonds thus provide a large contribution to protein stability and other peptide–peptide associations.

Hydrogen bonds consist of two electronegative atoms bound to the same hydrogen. One good hydrogen bond donor is the NH of a polypeptide backbone; one excellent acceptor is the C=O group in the backbone. Hydrogen bonds between these donors and acceptors are the basis of the two major units of protein secondary structure, namely the α-helix and the β-sheet. It is therefore of fundamental importance to protein structure and the thermodynamics of protein folding to understand the strength of an amide–amide hydrogen bond and the entropy and enthalpy of its formation.

The definition of an intrinsic binding constant is the maximum binding energy possible between two functional groups when there is perfect complementarity between them and the entropic penalties associated with fixing the two groups into their relative optimum configurations (such as internal rotations and translational entropy) have been removed.[1,2] It is a free energy change from solvated to bound species. An estimation of the apparent binding energy of a peptide hydrogen bond ($\Delta G_{\mathrm{hbond}}$) can be made from thermodynamic measurements of the dimerization of species held together in the dimer solely by peptide hydrogen bonds. These include urea in water,[3] δ-valerolactam in water[4] and tetrachloromethane,[5] diketopiperazine in water,[6] γ-butyrolactam in CCl_4,[7] and ϵ-caprolactam in CCl_4.[8] Both of the hydrogen bonds formed in each of these dimers are identical, due to the symmetry of the dimer. *N*-Methylacetamide is not considered here as it appears to stack in water, rather than form hydrogen-bonded dimers,[9] and its mode of dimerization (and hence the number of hydrogen bonds which form) may be complicated by cis–trans isomerization about the peptide bond. The values of $\Delta G_{\mathrm{hbond}}$ measured from these dimers may be smaller than the maximum intrinsic binding energy because the presence of a second hydrogen bond means that the configuration of the first hydrogen bond may be moved from its optimum geometry. However, the dimers are essentially strain-free so the calculated values of $\Delta G_{\mathrm{hbond}}$ (apparent binding energies[10]) may be close to the intrinsic binding energy. The dimerization of these molecules is generally unfavorable in water (i.e. the free energy of dimerization is positive). Dimerization is slightly favorable in nonpolar solvents (i.e. the free energy of dimerization is negative). These results have been used as evidence that the free energy of formation of a peptide hydrogen bond is positive in water and negative in nonpolar solvents and that the contribution of hydrogen bonds to protein stability is small at best. However, as pointed out by Jencks[1] and Creighton,[11] other factors apart from hydrogen bonding will affect the equilibrium position, such as whether the bonding is intramolecular or intermolecular. In particular, in any bimolecular association, three degrees of translational and three degrees of rotational freedom must be lost, which is entropically highly unfavorable to dimerization. In order to calculate an apparent binding energy for a hydrogen bond the magnitudes of these additional factors must be estimated and removed from the free energy, entropy, and enthalpy of dimerization.

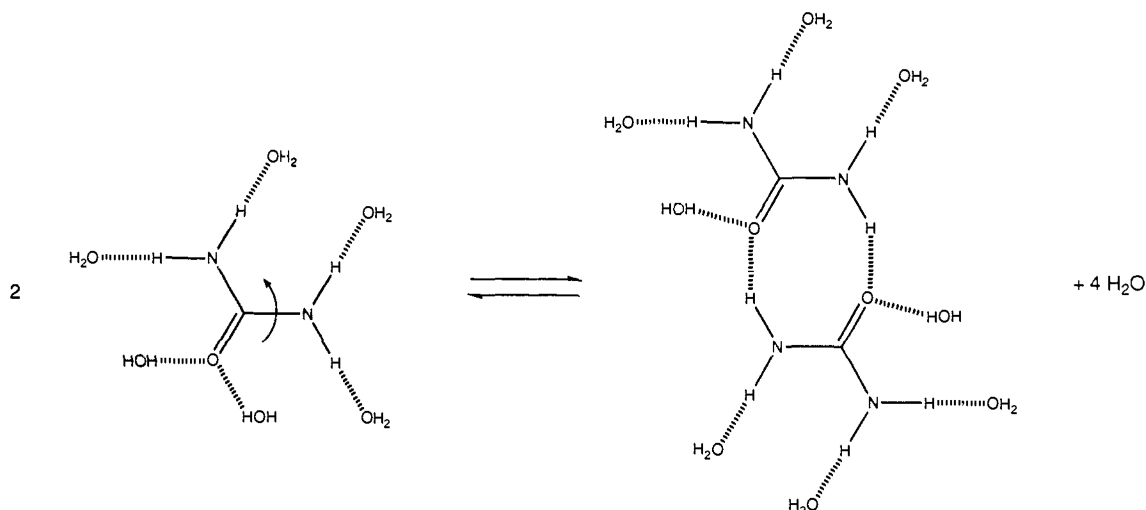
[†] Present address: Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305.

Methods and Results

The general reaction $2A \rightarrow A_2$, where A_2 is the bound complex, is disfavored unless there are significant favorable thermodynamic forces driving the binding. The major barrier to binding is that each of unbound A have large translational (S_{trans}) and rotational (S_{rot}) entropies in solution. These translational and rotational entropies have little dependence on the size of the molecule, so that the complex A_2 has a transitional and a rotational entropy not very different from those of A. This means that a large amount of translational and rotational entropy is lost in a bimolecular association. To overcome this loss of translational and rotational entropy, there need to be favorable non-covalent interactions within the complex (such as hydrogen bonds, salt bridges, and hydrophobic interactions) if the associated complex is to have a negative free energy of formation.

(1) Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 209–410.
(2) Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4046–4050.
(3) Schellman, J. A. *C. R. Trav. Lab. Carlsberg, Ser. Chim.* **1955**, *29*, 223–229.
(4) Susi, H.; Timasheff, S. N.; Ard, J. S. *J. Biol. Chem.* **1964**, *239*, 3051–3054.
(5) Tsuboi, M. *Bull. Chem. Soc. Jpn.* **1954**, *24*, 75–77.
(6) Gill, S. J.; Noll, L. *J. Phys. Chem.* **1972**, *76*, 3065–3068.
(7) Affsprung, H. E.; Christian, S. D.; Worley, J. D. *Spectrochim. Acta A* **1964**, *20*, 1415–1420.
(8) Lord, R. C.; Porro, T. J. *Z. Elektrochem.* **1960**, *64*, 672–676.
(9) Jorgensen, W. L. *Acc. Chem. Res.* **1989**, *22*, 184–189.
(10) Fersht, A. R. *Biochemistry* **1988**, *27*, 1577–1580.
(11) Creighton, T. E. *Biopolymers* **1983**, *22*, 49–58.

0002-7863/92/1514-338$03.00/0 © 1992 American Chemical Society



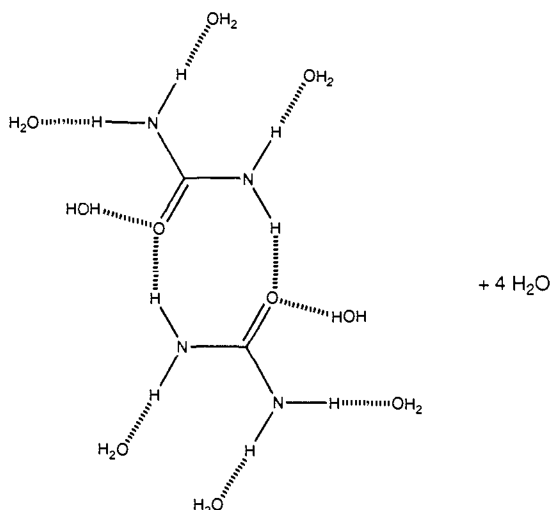


Figure 1. Urea dimerization in aqueous solution.

For a perfect gas, S_{trans} is given by the Sackur–Tetrode equation

$$S_{trans} = 5R/2 + (5R/2)\ln T - R\ln P + R\ln[(2\pi m/h^2)^{3/2}k^{5/2}] \quad (1)$$

where R is the ideal gas constant, T is the temperature, P is the pressure, m is the mass of the molecule, h is Planck's constant, and k is Boltzmann's constant. S_{trans} depends on the ln m (the natural logarithm of the mass of the molecule), which in practice means that there is little variation of translational entropy with mass. At 298 K and 1 atm, eq 1 simplifies to eq 2 where S_{trans} depends only on the relative molecular mass (RMM) of the molecule.

$$S_{trans} = 108.8 + 12.47\ln(\text{RMM})\ \text{J K}^{-1}\ \text{mol}^{-1} \quad (2)$$

S_{rot} is given by eq 3.

$$S_{rot} = R + R\ln[\pi^{7/2}(8kT/h^2)^{3/2}(I_AI_BI_C)^{1/2}] \quad (3)$$

The rotational entropy thus depends on the ln $(I_AI_BI_C)$ (where I_A, I_B, and I_C are the moments of inertia about three perpendicular axes). As with translation, this means that there is relatively little increase of rotational entropy as the size of the molecule increases.

First, we consider the dimerization of urea in aqueous solution. Urea contains a number of polar groups which bind strongly to solvent water molecules. As urea translates and rotates in solution, some water molecules will move with the urea. This will therefore increase the effective molecular mass and moment of inertia of the urea and hence S_{trans} and S_{rot}. When urea dimerizes, some waters which were bound to the peptide groups which participate in the new hydrogen bonds are lost (Figure 1). It is assumed that, after averaging over all waters which are ordered both strongly and weakly by the polar groups, each C=O retains two bound waters and each NH group one.[12,13] In order to calculate the change in translational and rotational entropy upon dimerization, the translational and rotational entropies of A and A_2 in the gas phase can be calculated using eqs 2 and 3 and a mass and moment of inertia which includes the "permanently" bound waters. Though it is not certain how many water molecules translate and rotate with the urea, S_{trans} and S_{rot} are relatively insensitive to any change in mass or moment of inertia since they both depend on the logarithms of these terms.

Equations 1, 2, and 3 are reasonably accurate for the gas phase. However, we are interested in changes in entropy for systems at 1 M concentration in water (since ΔG for complexation is calculated from K_c, which is defined with reference to 1 M solutions). The change in entropy on going from a gas to a 1 M aqueous solution can be broken down into two hypothetical stages: first, the gas is condensed into a liquid which consists entirely of the molecules present in the gas phase (i.e., the urea and the bound water). This "pure liquid" is then diluted to a concentration of 1 M. The entropy of condensation for a wide range of liquids is found to be approximately constant at 85 J K^{-1} mol^{-1} (Trouton's rule), and this shows little variation with boiling point.[14] However, for water it is 109 J K^{-1} mol^{-1}.[15] A larger value is found in the case of water due to strong hydrogen bonding in the solvent which leads to increased order. Since we take the peptides to be partially surrounded by water in the gas phase (Figures 1–3), we take an intermediate value for the entropy of condensation for hydrated urea as 100 J K^{-1} mol^{-1}. Thus, the change in translational entropy on dimerization (ΔS_{trans}) is decreased by 100 J K^{-1} mol^{-1} (Figure 4). The Hildebrand rule,[16] which gives the entropy of condensation at 298 K as a function of boiling point, unfortunately cannot be used as many peptides decompose before boiling and they are too polar for the rule to be appropriate. It is not necessary to directly consider the differing solvation energies of the hydrogen-bonded and isolated peptide units since this difference will form part of the binding energy of the hydrogen bond. The change in entropy (ΔS) on dilution is given by eq 4, where M_1 is the initial concentration and M_2 is the final concentration.

$$\Delta S = R\ln(M_1/M_2) \quad (4)$$

The initial concentration for a pure liquid is equal to 1000/RMM, assuming the density of the pure liquid is the same as water (1 g dm^{-3}) and the final concentration is 1 M. The change in entropy on dilution is therefore approximately

$$\Delta S = R\ln(1000/\text{RMM}) \quad (5)$$

Figure 4 shows the results of these calculations for urea dimerization. It is found that $\Delta S_{trans}(g) = -167$ J K^{-1} mol^{-1} and $\Delta S_{trans}(1\ \text{M,aq}) = -86$ J K^{-1} mol^{-1}. In other words, the change in translational entropy upon dimer formation approximately halves on transfer from the gas phase to 1 M aqueous solution.

The change in rotational entropy on dimer formation (ΔS_{rot}) was calculated using MacroModel V3.0[17] with bound waters energy minimized using AMBER[18] and was found to be −125 J K^{-1} mol^{-1}.

(12) Rossky, P. J.; Karplus, M. *J. Am. Chem. Soc.* **1979**, *101*, 1913–1937.
(13) Williams, D. H.; Cox, J. P. L.; Doig, A. J.; Gardner, M.; Gerhard, U.; Kaye, P.; Lal, A.; Nicholls, I. A.; Salter, C. J.; Mitchell, R. C. *J. Am. Chem. Soc.* **1991**, *113*, 7020–7030.
(14) Everett, D. H.; *J. Chem. Soc.* **1960**, 2566–2573.
(15) Atkins, P. W. *Physical Chemistry*; Oxford University Press: Oxford, 1982; pp 143–144.
(16) Hildebrand, J. H.; Scott, R. L. *The Solubility of Nonelectrolytes*; Reinhold: New York, 1950; pp 426–428.
(17) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Compn. Chem.* **1990**, *11*, 440–467.
(18) Weiner, S. J.; Kollman, P. A.; Case, D.; Singh, U. C.; Alagona, G.; Profeta, S.; Weiner, P. *J. Am. Chem. Soc.* **1984**, *106*, 765–784.

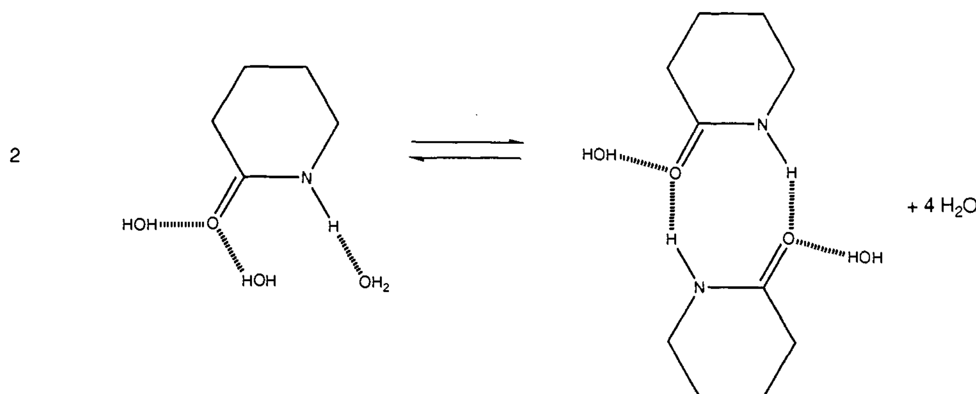


Figure 2. δ-Valerolactam dimerization in aqueous solution.

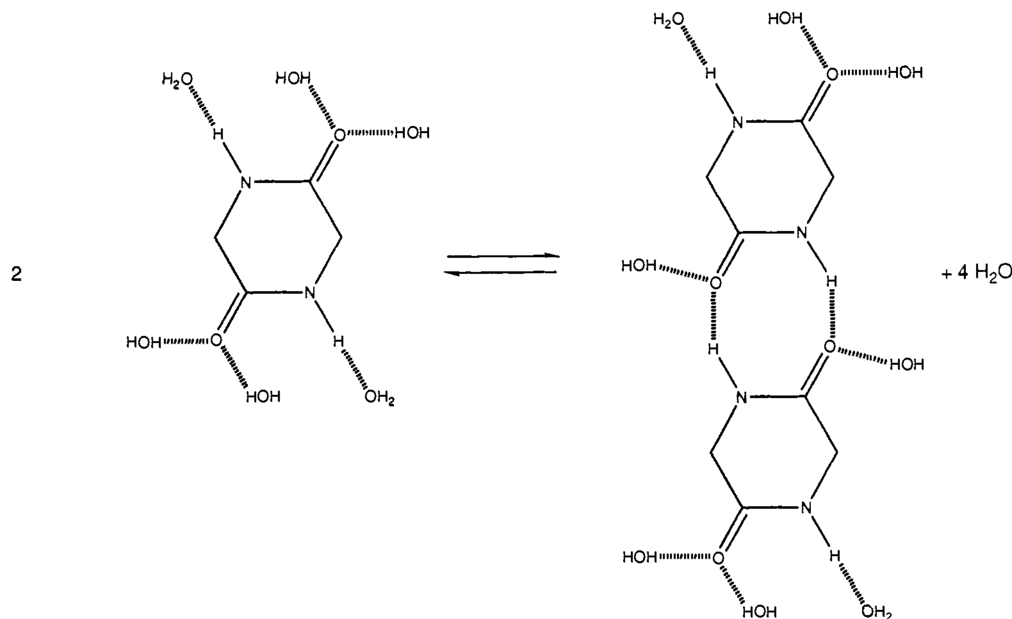


Figure 3. Diketopiperazine dimerization in aqueous solution.

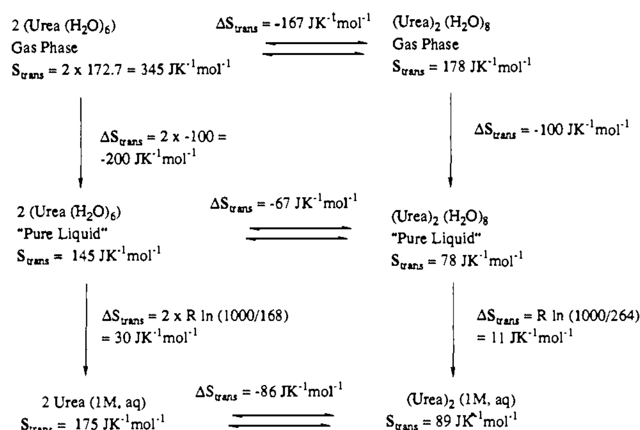


Figure 4. Calculation of ΔS_{trans} for urea dimerization in aqueous solution.

Another factor which may affect the entropy of dimerization is a change in the number of internal rotations present. An isolated urea molecule is able to rotate about the C–N bond shown in Figure 1. The free energy, enthalpy, and entropy of an internal rotation are functions of the potential energy barrier to the rotation and the moments of inertia of the groups on either end of the bond about which the rotation is taking place about that bond.[19] The potential barrier to the rotation was estimated using MacroModel[17] and the AMBER force field[18] with a dielectric constant of 80, as the free urea is in water. The energy of the urea was calculated with OCNH dihedral angles of 0° (the minimum energy conformation) and 90° (the maximum energy conformation). The dihedral bond was constrained to these angles and the rest of the molecule energy minimized. The constraint was then removed and the energy of the urea calculated. The difference between the two energies (the potential barrier to the rotation) was found to be 56 kJ mol^{-1}. This is about three times the barrier to a typical internal rotation about a single bond because delocalization between the carbonyl group and the nitrogen lone pair is lost when the dihedral angle is 90°. The moments of inertia about the bond were calculated using a Fortran program written by Mark Gardner (unpublished) and the energy minimized urea structure including the six bound water molecules depicted in Figure 1. It was found that the internal rotation had a free energy of −3.9 kJ mol^{-1}, an enthalpy of 2.2 kJ mol^{-1} and an entropy of 20.4 J K^{-1} mol^{-1}.

When an internal rotation is frozen out its free energy is not entirely lost as the rotation is replaced by a torsional vibration about the bond. Page and Jencks[20] have suggested that this vibration retains 20% of the free energy of the internal rotation which it replaces. In the case of the internal rotation frozen out in urea, this means that the free energy of the torsional vibration in the complex (G_{vib}) is $-3.9/5 = -0.78$ kJ mol^{-1}. If it is assumed

(19) Pitzer, K. S. *Quantum Chemistry*; Constable: London, 1953; pp 492–500.
(20) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678–1683.

Table I. Changes in Translational Entropy ($J\ K^{-1}\ mol^{-1}$) in Solution

compound	solvent	ΔS_{trans} (g)	$\Delta\Delta S_{trans}$ (condensation)	ΔS_{trans} (pure liquid)	$\Delta\Delta S_{trans}$ (dilution)	ΔS_{trans} (aq)
urea	water	−167	100	−67	−19	−86
δ-valerolactam	water	−166	100	−66	−19	−85
δ-valerolactam	CCl_4	−157	85	−72	−25	−97
γ-butyrolactam	CCl_4	−156	85	−71	−26	−97
ε-caprolactam	CCl_4	−159	85	−74	−24	−98
diketopiperazine	water	−169	100	−69	−18	−87

Table II. Thermodynamics of Hydrogen Bond Formation

compound	solvent	ΔG_{hbond} ($kJ\ mol^{-1}$)	ΔH_{hbond} ($kJ\ mol^{-1}$)	ΔS_{hbond} ($J\ K^{-1}\ mol^{-1}$)
urea	water	−27	0	92
γ-butyrolactam	CCl_4	−31	−11	68
δ-valerolactam	water	−27	−8	64
δ-valerolactam	CCl_4	−32	−18	48
ε-caprolactam	CCl_4	−32	−8	80
diketopiperazine	water	−25	−5	66
mean	CCl_4	−32	−12	65
mean	water	−26	−4	74
ristocetin/vancomycin	water	−24	−1	77

that the vibration behaves as a simple harmonic oscillator, eq 6 can be used to find the fundamental frequency of the vibration ($270\ cm^{-1}$ in this case). Equations 7 and 8 can then be used to find the entropic and enthalpic components of this free energy. If this is done it is found that $S_{vib} = 6.6\ J\ K^{-1}\ mol^{-1}$ and $H_{vib} = 1.2\ kJ\ mol^{-1}$.

$$G_{vib} = RT \ln (1 - e^{-\theta/T}) \quad (6)$$

$$S_{vib} = -R \ln (1 - e^{-\theta/T}) + R\theta/T(e^{\theta/T} - 1) \quad (7)$$

$$H_{vib} = R\theta/(e^{\theta/T} - 1) \quad (8)$$

where $\theta = h\nu/k$

and

ν = the fundamental frequency of the vibration

The total cost in free energy of freezing out the internal rotation in urea ($\Delta G_{int\text{-}rot}$) is therefore $3.9 - 0.78 \approx 3\ kJ\ mol^{-1}$, the total change in enthalpy of freezing out the internal rotation in urea ($\Delta H_{int\text{-}rot}$) is $1.2 - 2.2 \approx -1\ kJ\ mol^{-1}$, and the total change in entropy of freezing out the internal rotation in urea ($\Delta S_{int\text{-}rot}$) is $6.6 - 20.4 \approx -14\ J\ K^{-1}\ mol^{-1}$. It is assumed that the change in solvation of the portion of the molecule not involved in the new hydrogen bond is negligible. This may be untrue—however the fact that similar binding free energies are obtained for hydrogen bonds in urea and in the lactams supports this assumption.

The experimental change in entropy of dimerization is $-56\ J\ K^{-1}\ mol^{-1}$.[3] This is equal to $\Delta S_{trans} + \Delta S_{rot} + 2\Delta S_{hbond} + 2\Delta S_{int\text{-}rot}$.

$$\therefore \quad -56 = -86 - 125 + 2\Delta S_{hbond} - 28$$

$$\therefore \quad \Delta S_{hbond} = 92\ J\ K^{-1}\ moll^{-1}$$

In other words, this estimate gives the entropy of formation of a hydrogen bond (ΔS_{hbond}) in urea in aqueous solutions as $92\ J\ K^{-1}\ mol^{-1}$.

The enthalpy of formation of a hydrogen bond (ΔH_{hbond}) can be calculated in a similar manner. The change in internal energy associated with translation is $-3RT/2$. The additional $P\Delta V$ term required to change internal energy to enthalpy is negligible for a solution. The enthalpy of rotation of one molecule is also lost on dimerization. This heat release, which favors binding, is equal to $-3RT/2 = -3.7\ kJ\ moll^{-1}$ for a nonlinear molecule. The enthalpy change due to internal rotations is $-1\ kJ\ mol^{-1}$. The experimental value for ΔH of dimerization of urea in aqueous solution is $-8.8\ kJ\ mol^{-1}$.[3]

$$-8.8 = \Delta H_{trans} + \Delta H_{rot} + 2\Delta H_{hbond} + 2\Delta H_{int\text{-}rot}$$

$$\therefore \quad -8.8 = -3.7 - 3.7 + 2\Delta H_{hbond} - 2$$

$$\therefore \quad \Delta H_{hbond} = 0.3\ kJ\ mol^{-1}$$

Thus the enthalpy of formation of a hydrogen bond in urea in aqueous solution is close to zero at room temperature. From the Gibbs equation

$$\Delta G_{hbond} = \Delta H_{hbond} - T\Delta S_{hbond}$$

$$\Delta G_{hbond} = 0 - 298 \times 0.092 = -27\ kJ\ mol^{-1}$$

Similar calculations for the formation of peptide hydrogen bonds in water can be performed on δ-valerolactam and diketopiperazine using the data of Susi et al.[4] and Gill and Noll,[6] respectively (Tables I and II). Data are also available for the dimerization of δ-valerolactam,[5] γ-butyrolactam,[7] and ε-caprolactam[8] via peptide hydrogen bonds in CCl_4. No CCl_4 molecules are assumed to translate and rotate with free peptide groups. No internal rotations are frozen out in the lactams upon dimerization. In nonpolar solvents the entropy change of condensation is $-85\ J\ K^{-1}\ mol^{-1}$ (Trouton's rule[15]). The results of each these calculations are given in Tables I and II.

The most uncertain terms which are used in deriving these thermodynamic changes for hydrogen bond formation are the translational and rotational entropies of a species in aqueous solution. Page and Jencks[20] have suggested that gas-phase translational entropies are also appropriate for liquids, at least for nonpolar molecules in nonpolar solvents. If this is the case, the free energies and entropies of the hydrogen bond will be even larger than those given in Table II. Finkelstein and Janin[21] suggest that about half the transitional and rotational entropy of a substrate is lost upon complex formation; however, this conclusion is reached by treating six vibrational degrees of freedom in the complex as part of the translational and rotational entropy. In the analysis in this paper, these vibrations are treated as part of the hydrogen bond binding energy as they arise as a result of the introduction of new hydrogen bonds.

These two alternative methods can be used to give a range for possible hydrogen bond entropies and free energies of formation. Enthalpy will not be affected. Table III shows the results of the calculations if it is assumed that translational and rotational entropies are identical in the gas phase and in solution, as suggested by Page and Jencks. The binding energy of the hydrogen bond is now found to be much larger (mean ΔG_{hbond} in water is $-38\ kJ\ mol^{-1}$; mean ΔG_{hbond} in CCl_4 is $-41\ kJ\ mol^{-1}$). Table IV gives hydrogen bond entropies and free energies of formation if it is assumed that translational and rotational entropies halve on transfer from the gas phase to solution, as suggested by Finkelstein and Janin, and that no solvent water molecules translate and rotate with the polar groups. Even in this latter "worst case" situation, the free energy of formation of the hydrogen bond is still large in both solvents (mean ΔG_{hbond} in water is $-13\ kJ\ mol^{-1}$; mean ΔG_{hbond} in CCl_4 is $-22\ kJ\ mol^{-1}$). The method used earlier in this paper for the estimation of translational and rotational entropies in solution gives hydrogen bond free energies of intermediate strength. The mean binding energy of the amide–amide hydrogen bond at $-13\ kJ\ mol^{-1}$ in water is still significantly larger than

(21) Finkelstein, A. V.; Janin, J. *Protein Eng.* **1989**, *3*, 1–3.

Table III. Thermodynamics of Hydrogen Bond Formation Based on Page and Jencks' Estimate of Translational and Rotational Entropies in Solution

compound	solvent	ΔS_{trans} (J K^{-1} mol^{-1})	ΔS_{rot} (J K^{-1} mol^{-1})	ΔS_{hbond} (J K^{-1} mol^{-1})	ΔG_{hbond} (kJ mol^{-1})
urea	water	−167	−125	132	−39
γ-butyrolactam	CCl_4	−156	−87	98	−40
δ-valerolactam	water	−166	−123	105	−39
δ-valerolactam	CCl_4	−157	−92	78	−41
ε-caprolactam	CCl_4	−159	−96	111	−41
diketopiperazine	water	−169	−128	107	−37
mean	CCl_4			96	−41
mean	water			115	−38

Table IV. Thermodynamics of Hydrogen Bond Formation Based on Finkelstein and Janins' Estimate of Translational and Rotational Entropies in Solution and Assuming No Water Molecules Remain Bound during Translation and Rotation

compound	solvent	ΔS_{trans} (J K^{-1} mol^{-1})	ΔS_{rot} (J K^{-1} mol^{-1})	ΔS_{hbond} (J K^{-1} mol^{-1})	ΔG_{hbond} (kJ mol^{-1})
urea	water	−76	−35	42	−13
γ-butyrolactam	CCl_4	−78	−44	38	−22
δ-valerolactam	water	−79	−46	23	−15
δ-valerolactam	CCl_4	−79	−46	16	−23
ε-caprolactam	CCl_4	−80	−48	47	−22
diketopiperazine	water	−80	−45	21	−11
mean	CCl_4			34	−22
mean	water			29	−13

previous estimates of this quantity, despite the fact that the value of −13 kJ mol^{-1} is generated by making assumptions for the uncertain quantities in the calculations which give the smallest binding energy possible. The conclusion therefore that the binding energy of the amide–amide hydrogen bonds is much larger than hitherto realized seems inescapable.

Discussion

Tables II–IV show that the binding energy of a hydrogen bond is large and negative in both solvents considered. The formation of a peptide hydrogen bond in these systems is therefore highly favorable no matter what its environment, though it is stronger in nonpolar solvents. The estimate of Roseman[22] of −3 kJ mol^{-1} for the free energy of transfer of a peptide hydrogen bond in *N*-methylacetamide from water to CCl_4 is reasonably close to the mean values of −6 kJ mol^{-1} in Table II and −3 kJ mol^{-1} in Table III, but significantly smaller than that (−9 kJ mol^{-1}) in Table IV.

The results obtained here are in striking disagreement with those of previous workers who concluded that hydrogen bonds between uncharged atoms contribute at most 8 kJ mol^{-1} and probably nothing to binding energy (for example, see refs 23–26). This confirms the general importance of this work, despite any uncertainties in the exact numbers. Hydrogen bonds between uncharged species have previously been considered to provide only 2 to 8 kJ mol^{-1} to binding energy and a factor of 2 to 20 to specificity.[10,27] Even the lower mean values in water [−13 and −26 kJ mol^{-1} (Tables IV and II)] indicate that amide–amide hydrogen bonds can provide factors of ca. 10^2–10^4 to specificity at room temperature.

It is possible to explain semiquantitatively how the entropic and enthalpic components of the binding energy arise, though such explanations must be made with caution, due to the major problem of enthalpy/entropy compensation in solvation.[28,29] The variations in entropy and enthalpy, seen in Table II, are much larger than the variation in free energy. The magnitude of the binding energy can be considered to arise from a number of factors including the difference in solvation between a free peptide group and a C=O···H–N group, a change in the number of vibrational modes, and electrostatics. One of the advantages of using binding energies in this manner is that a large number of factors, which may be difficult to estimate individually, are together taken care of in a single number, derived from experiment.

When each of these species dimerizes, a number of new vibrations are introduced. The presence of additional vibrations makes a favorable contribution to the free energy, particularly if the vibrations have a low frequency.[20] Thus, part of the energetic components of the binding constant can be attributed to the introduction of low-frequency vibrations. If the vibrational modes and frequencies in the dimers and monomers are known, the magnitudes of their contributions to the free energy, entropy, and the enthalpy of formation of the hydrogen bond can be estimated by assuming that the vibrations are harmonic oscillators and using eqs 6, 7, and 8.

The fundamental frequencies of the vibrations present in the peptide monomers and dimers were estimated using MacroModel V3.0[17] after energy minimization of the molecules using MM2.[30] It is assumed that the vibrational spectrum is identical in vacuum and CCl_4. This has been shown to be a good assumption in the case of acetic acid dimers.[31] It was found that the frequency distribution of the dimer vibrations was very similar to that of the two monomeric units combined but with the addition of a number of low-frequency vibrations, presumably involving the hydrogen bonds. The contributions of these additional vibrations to free energy, entropy, and enthalpy were estimated using eqs 6, 7, and 8 and are given in Table V. Thus, the introduction of new vibrations favors dimerization entropically and opposes it enthalpically. Overall, new vibrations favor the formation of hydrogen bonds in nonpolar solvents in these cyclic peptides by approximately −12 kJ mol^{-1} per hydrogen bond. These results must be treated with caution since the calculation assumes that the change in energy with distance from the center of the vibrational potential well is parabolic (i.e., the vibration is a simple harmonic oscillator), an assumption which may be poor for low-frequency vibrations involving weak bonds and many atoms.

(22) Roseman, M. A. *J. Mol. Biol.* **1988**, *201*, 621–623.
(23) Baldwin, R. L. *Trends Biochem. Sci.* **1989**, *14*, 291–294.
(24) Dill, K. A. *Biochemistry* **1990**, *29*, 7133–7155.
(25) Pace, C. N.; Heinemann, U.; Hahn, U.; Saenger, W. *Angew. Chem.* **1991**, *30*, 343–360.
(26) Murphy, K. P.; Gill, S. J. *Thermochim. Acta* **1989**, *172*, 11–20.
(27) Fersht, A. R.; Shi, J.-P.; Knill-Jones, J.; Lowe, D. M.; Wilkinson, A. J.; Blow, D. M.; Brick, P.; Carter, P.; Waye, M. M. Y.; Winter, G. *Nature* **1985**, *314*, 235–238.
(28) Roseman, M. A.; Jencks, W. P. *J. Am. Chem. Soc.* **1975**, *97*, 631–640.
(29) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; pp 354.
(30) Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127–8134.
(31) Jakobsen, R. J.; Mikawa, Y.; Brasch, J. W. *Spectrochim. Acta* **1966**, *23A*, 2199–2209.

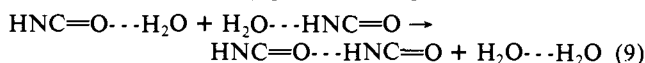
Table V. Contribution of New Vibrations to Thermodynamic Parameters per Hydrogen Bond Formed

compound	solvent	ΔG_{vib} (kJ mol^{-1})	ΔH_{vib} (kJ mol^{-1})	ΔS_{vib} (J K^{-1} mol^{-1})
γ-butyrolactam	CCl_4	−11	6	57
δ-valerolactam	CCl_4	−12	6	61
ε-caprolactam	CCl_4	−13	6	65

However, the numbers obtained by this procedure can give a rough indication of the contribution of vibrations to the hydrogen bond binding energy in CCl_4. If the values of ΔS_{hbond} for hydrogen bond formation in CCl_4 in Table II are compared to ΔS_{vib} in Table V, it is seen that the introduction of new vibrations can account for essentially all of the entropic component of the binding energy in CCl_4. In water, some vibrations due to bonds between water molecules and peptide groups are lost, so the contribution of changes in vibrational modes and frequencies will be of less importance. The difficulties introduced by transient bonding to water molecules mean that it is not possible to estimate the change in vibrational energy due to dimerization in water by this method.

It is likely that the hydrogen bonds in the interior of proteins will be more rigid than those in the dimers considered in this paper. This will mean that the frequencies of the vibrations involving the hydrogen bonds in proteins will be higher and hence have a lower entropy. This is likely to be offset by hydrogen bonds in proteins being stronger enthalpically than in the dimers as they are held closer to their optimum geometry. In conclusion, it is probable that amide–amide hydrogen bonds in proteins will be enthalpically favored and entropically disfavored compared to the hydrogen bonds in the dimers. The overall change is free energy is uncertain. Additionally, if the local dielectric is lower within proteins, compared to within the dimers in water, the hydrogen bond will be enthalpically stronger.

A favorable enthalpy change in water cannot be attributed to a gain in the number of hydrogen bonds in the dimer, since the formal number of hydrogen bonds, taking the solvent into consideration, is invariant (eq 9; ref 32; Figures 1–3).



$$HNC{=}O\cdots H_2O + H_2O\cdots HNC{=}O \rightarrow HNC{=}O\cdots HNC{=}O + H_2O\cdots H_2O \quad (9)$$

However, CCl_4 cannot form any hydrogen bonds to peptide groups; thus, there is a formal increase of one hydrogen bond upon dimerization in this case (eq 10).

$$HNC{=}O + HNC{=}O \rightarrow HNC{=}O\cdots HNC{=}O \quad (10)$$

The increase in the formal number of hydrogen bonds will make a large contribution to the favorable enthalpy change upon hydrogen bond formation in CCl_4 as a result of simple electrostatics. In contrast, hydrogen bond formation in water has a smaller enthalpy change. It may well be that all four types of hydrogen bond involved in eq 9 are of different strengths which could give rise to a small change in enthalpy.

A favorable entropy change in aqueous solution can be attributed to peptide groups binding solvent molecules as in Figures 1–3. The release of these bound water molecules is entropically favorable and provides a strong driving force for the formation of the peptide hydrogen bond.[33,34] The maximum amount a given water molecule can contribute to the entropy change in this manner is equivalent to the entropy change of ice melting to liquid water[1] which is 22 J K^{-1} mol^{-1}.[15] The figures for ΔS_{hbond} in Table II imply that 3 to 4 equiv of ice-like water are ordered by a solvated peptide group, assuming that no other factors contribute to ΔS_{hbond}. If this explanation is correct, it implies that, in aqueous solution, ΔS_{hbond} for a hydrogen bond between an amide group and a hydroxyl group will be about half the value of ΔS_{hbond} for an amide–amide hydrogen bond and that ΔS_{hbond} for a hydroxyl–hydroxyl hydrogen bond will be approximately zero.

Throughout this paper, we have assumed that the bound dimer contains two hydrogen bonds. It is conceivable that the dimer is bound by only a single hydrogen bond.[3,23,32] However, this is highly unlikely since the formation of the second hydrogen bond is much easier than the formation of the first. This occurs because the first bond must fully overcome the very large loss of translational and rotational free energy (≈55 kJ mol^{-1}). This is offset to a small extent (≈5 kJ mol^{-1}) by the introduction of an internal rotation about the new bond. In contrast, the formation of the second hydrogen bond is opposed solely by the freezing of this rotor. Thus, the second hydrogen bond is much easier to introduce than the first. An alternative way to look at this is that the formation of the first hydrogen bond is intermolecular and the second is intramolecular, which gives the second a much higher effective concentration. The assumption that there are two hydrogen bonds present in the dimer is therefore justified.

One of the aims of this work is to obtain binding energies of various functional group interactions which can be used to help predict constants between peptides in aqueous solution. We have previously determined the apparent binding energy of an amide–amide hydrogen bond from data for complexation between the peptide antibiotics ristocetin and vancomycin and the peptides *N*-Ac-Gly-D-Ala and *N*-Ac-D-Ala.[13] The binding free energies, entropies, and enthalpies in these peptide/peptide complexes were derived by an independent method to that used in this paper which did not require the estimation of translational and rotational entropies in solution. It is gratifying that the results obtained are similar (Table II).

In the dimers, the hydrogen bond angles are approximately 120°. In water, a bond angle of ≈120° has the advantage of being able to retain an additional hydrogen bond between the carbonyl and a solvent water molecule, so a bond angle of 120° may be of lower free energy than one which is close to being linear, as seen in vancomycin and ristocetin. The values of ΔH_{hbond} for ristocetin and vancomycin and ΔH_{hbond} from peptide dimerizations in water are small in both cases. This implies that the local dielectric constant is high in the antibiotics, as it is in water, since a low dielectric constant would lead to a stronger hydrogen bond in the bound complex and hence a more negative value of ΔH_{hbond}. As the antibiotic/peptide complex is a model for a portion of a protein, this suggests that the dielectric constant within hydrogen-bonded regions of proteins is also high.

Conclusion

Using thermodynamic data for the dimerization of a number of molecules by amide–amide hydrogen bonds in water and CCl_4, it is possible to calculate the apparent binding energy of each of the hydrogen bonds. The results show that the formation of the hydrogen bonds is highly favorable in both solvents, though slightly more favorable in CCl_4 than water. Hydrogen bond formation in water is largely entropy driven; the binding energy in CCl_4 has enthalpic and entropic components. The negative free energy change for the hydrogen bond formation can be attributed to the introduction of new vibrations in the dimer in CCl_4, a gain in the number of hydrogen bonds in CCl_4, and the release of bound polar solvent molecules in water. The results clearly demonstrate that amide–amide hydrogen bonds are capable of providing a large driving force to stabilize folded protein structures and other peptide–peptide complexes. Even using lower limits for the size of translational and rotational entropy in solution, the binding energy is still much larger than previously realized.

Acknowledgment. We thank the S.E.R.C. (U.K.), The Upjohn Company (Kalamazoo), SmithKline Beecham, and I.C.I. Pharmaceuticals for financial support. Andrew Doig thanks I.C.I. for a student scholarship.

Registry No. Urea, 57-13-6; γ-butyrolactam, 616-45-5; δ-valerolactam, 675-20-7; ε-caprolactam, 105-60-2; diketopiperazine, 106-57-0; ristocetin, 1404-55-3; vancomycin, 1404-90-6.

(32) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1–63.
(33) Chothia, C. *Nature* **1975**, *254*, 304–308.
(34) Fersht, A. R. *Enzyme Structure and Mechanism*; Freeman: New York, 1985; pp 301–302.