Stability of an Amide–Hydrogen Bond in an Apolar Environment*

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ABSTRACT: Free-energy changes for dissociating an N–H···O=C bond to N–H (aqueous solution) and C=O (aqueous solution) are not significantly different if the initial amide–hydrogen bond is in an aqueous or an apolar solvent. Literature data lead to the same conclusion for O–H···O=C hydrogen bonds. Evidently the intrinsic interaction between donor and acceptor groups is not sensitive to the adjacent molecular environment. This is of interest in regard to unfolding of ordered protein conformations.

Ever since N–H ··· O=C hydrogen bonds have been assumed to exist in protein molecules, efforts have been made to evaluate the stability of this bond. Using $CH_3CONHCH_3$ as the smallest model compound representative of the peptide amide group, CONH, Klotz and Franzen (1962) examined the equilibria

$$\text{N–H (aqueous solution)} + \text{O=C (aqueous solution)} \longrightarrow \text{N–H···O=C (aqueous solution)} \quad (1)$$

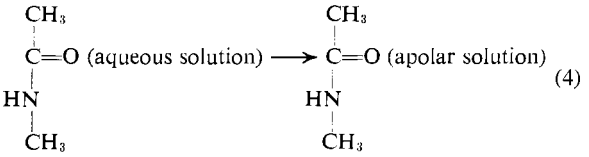
$$\text{N–H (apolar solution)} + \text{O=C (apolar solution)} \longrightarrow \text{N–H···O=C (apolar solution)} \quad (2)$$

For reaction 1 at 25°, ΔG° was found to be 3.1 kcal/mole; for reaction 2 it is −0.92 kcal/mole. Thus it is clear, at least for the model amide, that N–H and O=C in an aqueous solution do *not* tend to form N–H···O=C bonds immersed in an aqueous environment. Similarly it is evident that N–H and O=C groups in an apolar solution *do* tend to form N–H···O=C hydrogen bonds immersed in an apolar environment.

In the denaturation of proteins some N–H···O=C bonds in the apolar interior of specific native conformations become exposed to water in the disordered denatured conformations. It becomes relevant to ask, therefore, what is ΔG° for reaction 3, since this corresponds to the refolding of a disordered to a native conformation in the protein.

$$\text{N–H (aqueous solution)} + \text{O=C (aqueous solution)} \longrightarrow \text{N–H···O=C (apolar solution)} \quad (3)$$

There are several experimental avenues to the evaluation of ΔG° for eq 3. One of the easiest would be to study the distribution equilibrium



(4)

From such distribution experiments, ΔG° for the following transfer reaction could be evaluated

$$\text{N–H (aqueous solution)} + \text{O=C (aqueous solution)} \longrightarrow \text{N–H (apolar solution)} + \text{O=C (apolar solution)} \quad (5)$$

This combined with ΔG° for eq 2 would give ΔG° for reaction 3.

Experimental Section

Materials. *N*-Methylacetamide from commercial sources was distilled at atmospheric pressure through a 15-in. Vigreaux column and the fraction boiling at 208–209° was collected. Carbon tetrachloride was Fisher Spectranalyzed Certified Reagent grade. It was dried by storage over Drierite followed by vapor phase contact with phosphorus pentoxide for at least 30 hr. When used in the reference cell of the spectrophotometer, the CCl_4 was kept in continuous (vapor phase) contact with P_2O_5 by means of an equilibrator fitted directly into the ground-glass joint at the filling part of the cell. This procedure reduced the content of water in CCl_4 from original values as high as 25% of saturation to negligible quantities. All salts were Fisher Certified Reagent grade.

Spectroscopic Measurements. Absorbance measurements in the region of 1.2–1.9 μ were made with the Cary 14R spectrophotometer. Cell compartments and cell holders were thermostatted, the temperature being controlled to ±0.5°. The instrument and cell compartments were purged with dry nitrogen. Absorption cells with a light path of 10.00 cm were used. Absorbance in the 2.6–3.7-μ range was measured with Beckman IR-5 and IR-9 double-beam spectrophotometers, using quartz window cells of about 0.5 cm thickness.

Equilibration Experiments. Samples (10 ml) of aqueous solutions of *N*-methylacetamide, at a series of concentrations from 1 to 10 M, were equilibrated by shaking at constant temperature with equal volumes of CCl_4 in 50-ml glass-stoppered flasks. The equilibrium concentrations of *N*-methylacetamide in CCl_4, as well as of the dissolved water in this solvent phase, were determined spectrophotometrically. To establish the time necessary for equilibrium partition between water and

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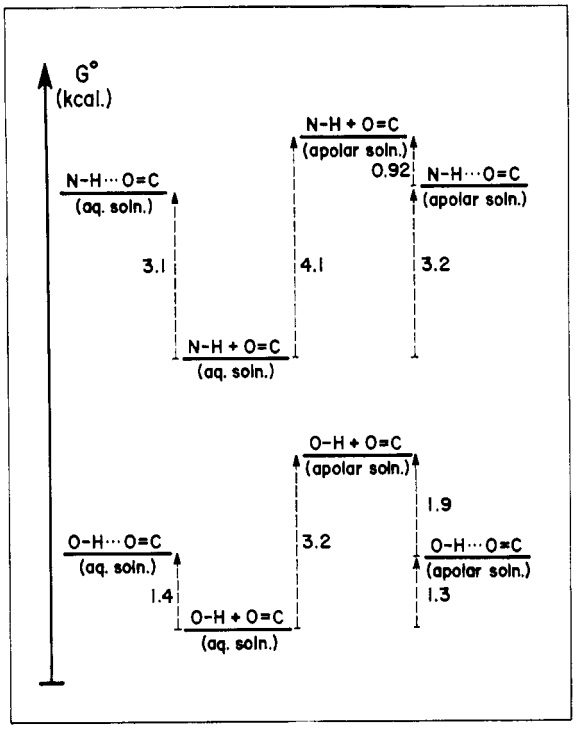

FIGURE 1: Relative free energies for amide N–H···O=C hydrogen bonds (at 25°) and for carboxylic acid O–H···O=C hydrogen bonds (at 15°) in aqueous and apolar solutions.

CCl_4 phases, the concentration of *N*-methylacetamide in the CCl_4 was measured by its absorbance at 3.45 μ at times from 5 min to 22 hr. Less than 10 min sufficed for equilibrium to be reached.

Results

In the simplest treatment K_D' for the distribution in eq 4 may be defined by

$$K_D' = \frac{f_{amide}^{CCl_4}}{C_{amide}^{H_2O}} \tag{6}$$

where $f_{amide}^{CCl_4}$ represents the formal (molar) concentration of amide in all its forms in the CCl_4 solvent in equilibrium with water and $C_{amide}^{H_2O}$ represents the equilibrium (molar) concentration of amide in the aqueous phase. In water the amide is essentially all hydrated monomer except at very high concentrations (Klotz and Franzen, 1962). In CCl_4, however, some dimer as well as monomer exists and both species may be partly hydrated by some of the water dissolved in this phase; all of these species are included in $f_{amide}^{CCl_4}$. The total $f_{amide}^{CCl_4}$ was measured experimentally by reading the absorbance at 3.45 μ, the position of the C–H stretching vibration in CH_3CONH-CH_3. Having determined the quantity of amide in CCl_4, one can easily compute the concentration remaining in the aqueous phase and thereafter K_D'. The results of one such series of equilibrations are summarized in Table I. Extrapolation to zero concentration gives $K_D^0 = 8 \times 10^{-4}$.

TABLE I: Equilibration of *N*-Methylacetamide between Water and CCl_4 at 25°.

Original (M) Concn of Amide in H_2O	Equilibrium		Distribution Ratio
	$C_{amide}^{H_2O}$	$f_{amide}^{CCl_4}$	
(0.000)			(0.0008)
1.982	1.980	0.002	0.0010
3.998	3.993	0.005	0.0013
5.529	5.521	0.008	0.0015
6.991	6.979	0.012	0.0017
8.015	7.999	0.016	0.0020
8.601	8.582	0.019	0.0022
9.204	9.180	0.024	0.0026

The extrapolation to zero concentration should effectively remove any contribution of amide dimer to $f_{amide}^{CCl_4}$ since the fraction of dimer drops below 0.1 when the total concentration of amide in CCl_4 is lowered to 0.01 M (Klotz and Franzen, 1962). On the other hand, since the CCl_4 and H_2O phases are in equilibrium at all amide concentrations, it seemed desirable to try to evaluate the effect of the water dissolved in the organic phase. For this purpose the amount of water dissolved in the CCl_4 solutions was measured by infrared absorbance at 1.396 μ, where interference from *N*-methylacetamide absorption is negligible. For calibration carbon tetrachloride solutions of known water concentration were prepared isopiestically (Christian *et al.*, 1963a) by equilibration with saturated aqueous salt solutions of known activity. Since the overtone infrared was being used for the water concentration, absorbance at 1.689 μ was used to determine *N*-methylacetamide in the same CCl_4 solutions.

Assuming that amide dimers in CCl_4 become increasingly negligible with lowering of solute concentration, we may write

$$f_{amide}^{CCl} = C_{amide}^{CCl_4} + C_{amide\cdot H_2O}^{CCl_4} \tag{7}$$

where amide·H_2O represents the amide hydrate. If

$$K_D = \frac{C_{amide}^{CCl_4}}{C_{amide}^{H_2O}} \tag{8}$$

then it follows directly from eq 6 that

$$K_D = K_D' - \frac{C_{amide\cdot H_2O}^{CCl_4}}{C_{amide}^{H_2O}} \tag{9}$$

The concentration of amide·H_2O hydrate was obtained from the difference between total water concentration and free water in the CCl_4 phase. Total water was calculated from the absorbance at 1.396 μ. Free water concentration was computed on the assumption that its activity in the CCl_4 phase was reduced to the same extent

as in the aqueous phase.[1] The error in $C^{CCl_4}_{amide \cdot H_2O}$ is large since it is obtained from a difference between two numbers of nearly equal magnitude. Nevertheless it became apparent that the contribution of the second term on the right-hand side of eq 9 is less than 10% of the first. With these corrections, the series of experiments using overtone infrared analytical procedures gave $K_D = 14 \times 10^{-4}$.

This may be compared with the previously described K_D^0 of 8×10^{-4}. Since the equilibrium concentration of amide in the CCl_4 phase is so small compared with that in the aqueous phase, it is difficult to detect accurately. Hence more precise values of K_D cannot be obtained. Nevertheless in the average value of K_D, 11 ± 3 ($\times 10^{-4}$), the average deviation corresponds to an uncertainty in ΔG° of 0.15 kcal.

Discussion

From an average K_D of 1.1×10^{-3} it follows that ΔG° is 4.1 kcal/mole for the transfer[2] described by eq 5. Combining this information with the ΔG° value of -0.92 kcal/mole for eq 2, we obtained $\Delta G^\circ = 3.2$ kcal/mole for the hydrogen-bond formation described by eq 3, *i.e.*, for taking free N–H and O=C groups from an aqueous environment and forming an N–H···O=C hydrogen bond in an apolar environment.

With this free energy now available we can compare the relative stabilities of the N–H···O=C hydrogen bond in a variety of environments. This can be visualized conveniently by the chart in Figure 1.

It was already known from previous work (Klotz and Franzen, 1962) that the N–H···O=C bond is unstable with respect to separated N–H and O=C in aqueous solution by 3.1 kcal but stable in CCl_4 solution by 0.92 kcal. The interesting new result, which is shown so strikingly in Figure 1 is that the N–H···O=C bond is intrinsically no more stable in an apolar environment than in an aqueous one. Thus a transfer of bonded N–H···O=C from apolar to aqueous surroundings occurs with a ΔG° of essentially zero (at least as measured by the model compound $CH_3CONHCH_3$). Stated in an alternative way, the ΔG° of dissociating an N–H···O=C bond to N–H (aqueous) and O=C (aqueous) is essentially the same whether the original amide–hydrogen bond is an aqueous or an apolar phase.

Literature data indicate that a similar conclusion is valid for O–H···O=C bonds in carboxylic acids. For example, from studies of the dimerization of acetic acid in benzene (Christian *et al.*, 1963b) one can calculate the following free-energy change (at 15°) for forming one hydrogen bond, assuming the carboxylic acid dimer in this solvent has a double bridge (eq 10).

$$\text{O–H (apolar solution)} + \text{O=C (apolar solution)} \longrightarrow \text{O–H···O=C (apolar solution)} \quad (10)$$
$$\Delta G^\circ = -1.88 \text{ kcal}$$

Published data for partition of acetic acid between water and benzene (Christian *et al.*, 1963b) give ΔG° for the following transfer

$$\text{O–H (aqueous solution)} + \text{O=C (aqueous solution)} \longrightarrow \text{O–H (apolar solution)} + \text{O=C (apolar solution)} \quad (11)$$
$$\Delta G^\circ = +3.21 \text{ kcal}$$

From these results it follows that

$$\text{O–H (aqueous solution)} + \text{O=C (aqueous solution)} \longrightarrow \text{O–H···O=C (apolar solution)} \quad (12)$$
$$\Delta G^\circ = +1.33 \text{ kcal}$$

Data for formation of acetic acid dimers in aqueous solution are much less accurate even though they have been estimated by several groups of workers (Katchalsky *et al.*, 1951; Martin and Rossotti, 1961; Schrier *et al.*, 1964). An average value of these observations gives

$$\text{O–H (aqueous solution)} + \text{O=C (aqueous solution)} \longrightarrow \text{O–H···O=C (aqueous solution)} \quad (13)$$
$$\Delta G^\circ = 1.4 \text{ kcal}$$

if one assumes, as has been suggested by Martin and Rossotti (1961), that only one O–H···O=C hydrogen bond exists in acetic acid dimers in aqueous solution. A comparison of eq 12 with eq 13, or of the graphical equivalent in Figure 1, shows that the solvent phase has little effect on stability of the O–H···O=C hydrogen bond.

Thus for either an N–H or O–H bonded to O=C the interaction is not affected by marked differences in the

[1] Since the chemical potential of free water in both phases of the equilibrating system is the same, the mole fraction of water in the aqueous phase (a first approximation to its activity in this phase), multiplied by the saturation concentration of water in the CCl_4–H_2O two-component solution, gives the free water concentration in the CCl_4–H_2O–amide phase.

[2] It must be recognized, of course, that the actual experimental transfer is of $CH_3CONHCH_3$ from water to an apolar solvent (eq 4), *i.e.*, two methyl groups are also transferred in addition to the N–H and CO groups. It is not very clear what ΔG° should be assigned to the methyl transfer. Tanford (1962) has found that the difference in transfer free energies for glycine and alanine leads to a ΔG° of -0.73 kcal mole^{-1} for carrying a methylene group from water to an apolar environment. On the other hand, corresponding data for glutamine and asparagine give a ΔG° of only $+0.09$ kcal mole^{-1} for the same process; from glutamic and aspartic acids one calculates a ΔG° of -0.01 kcal mole^{-1}. It seems, therefore, that the ΔG° of transfer of a methylene group depends upon the substituents to which it is attached.

Using the glutamine-asparagine ΔG° of transfer as a measure of the contribution of each CH_3 group in the process of eq 4 we conclude that ΔG° for eq 5 should be 3.9 kcal mole^{-1} (instead of 4.1). Correspondingly, for the hydrogen-bonding process of eq 3 we obtain 3.0 kcal mole^{-1} (instead of 3.2). These are essentially insignificant changes. Likewise the glutamic-aspartic data would not alter the ΔG° values of eq 3 and 4 significantly. On the other hand, the alanine-glycine data lead to a ΔG° value of 5.6 kcal mole^{-1} for eq 5 and of 4.7 kcal mole^{-1} for eq 3, which is appreciable. Nevertheless, the net result of even this correction is to *accentuate* the thermodynamic instability of an N–H···O=C hydrogen bond in an apolar environment if it can come in contact with water, *i.e.*, to raise the height of the free-energy level at the upper right of the chart in Figure 1. Thus no matter which data we use to correct for the transfer of methylene groups, we conclude that the N–H···O=C bond (in an apolar environment) is thermodynamically unstable in the presence of an aqueous solvent.

nature of solvent. Furthermore it seems that there is little influence on bond stability due to neighboring CH_3 substituents, for if methyl group effects were present these should be modified by a transfer from an aqueous to an apolar solvent. The intrinsic interaction between hydrogen-donor and hydrogen-acceptor groups is thus not sensitive to its immediate (noncovalent) environment.

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Structure of Macromolecular Aggregates. I. Aggregation-Induced Conformational Changes in Polypeptides*

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ABSTRACT: The interactions between the polyacids poly-α-L-glutamic acid and poly-α-L-aspartic acid and the polybases poly-L-lysine and poly-L-ornithine in a variety of solvents have been studied with optical rotatory dispersion and circular dichroism. The data obtained suggest that poly-α-L-glutamic acid and poly-L-lysine form a β-pleated-sheet structure with 1:1 stoichiometry at pH 4 and 7 in aqueous 0.01 M NaF, although poly-L-lysine by itself is in a random coil conformation under the same conditions and poly-α-L-glutamic acid by itself is in a random coil conformation at pH 7 and an α-helix conformation at pH 4. No interaction was detected in the poly-α-L-glutamic acid–poly-L-lysine mixture at pH 11 where poly-L-lysine is helical and poly-α-L-glutamic acid is a random coil. The rotatory spectra of the other polyacid–polybase mixtures, poly-α-L-glutamic acid–poly-L-ornithine, poly-α-L-aspartic acid–poly-L-ornithine, and poly-α-L-aspartic acid–poly-L-lysine, at pH 7 are considerably reduced in amplitude, but cannot be identified with any specific geometric structure. Since β structure is observed only with the polyacids and polybases having the longest hydrocarbon side chains, hydrophobic interactions are apparently of importance in the stabilization of the aggregate. Rotatory spectra characteristic of α-helical structure are observed in methanol–water–0.01 M NaF (pH 7) solutions of mixtures of poly-α-L-aspartic acid–poly-L-ornithine and poly-α-L-aspartic acid–poly-L-lysine at methanol concentrations where none of these polypeptides are helical by themselves. An investigation of the stoichiometry suggests that primarily the polybase is being converted into an α helix. The nature of the poly-α-L-glutamic acid–poly-L-ornithine and poly-α-L-glutamic acid–poly-L-lysine interactions in methanol–water solutions could not be determined, apparently because of very extensive aggregation. These results indicate extreme conformational changes can occur upon aggregation of macromolecules. The nature of the aggregate is a sensitive function of the polypeptide side chains as well as of solvent composition. The effects of aggregation on rotatory spectra are also considered.

Many of the functional properties of biological systems are dependent upon the molecules in the system existing in a specific state of aggregation, for example, protein coats of viruses, the quaternary structure of enzymes and membranes. In order to better understand the factors influencing the structure of protein aggregates and the properties of such aggregates, we have investigated the structure of polypeptide aggregates formed under a variety of conditions. Optical rotatory dispersion and circular dichroism measurements were made on solutions of mixtures of the polyacids and polybases PAA, PGA, PO, and PL.[1] In aqueous solution (0.01 M NaF), PGA and PL appear to interact to form a β-pleated sheet when both polypeptides are initially random coils (pH 7) and when PGA is initially in an α-he-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PAA, poly-α-L-aspartic acid; PGA, poly-α-L-glutamic acid; PO, poly-L-ornithine; PL, poly-L-lysine.