Thermochimica Acta, 172 (1990) 11-20
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

GROUP ADDITIVITY THERMODYNAMICS FOR DISSOLUTION OF SOLID CYCLIC DIPEPTIDES INTO WATER *

K. P. MURPHY and S. J. GILL**

Department of Chemistry and Biochemistry
University of Colorado
Boulder, Colorado 80309-0215

SUMMARY

The thermodynamics of dissolution of a set of cyclic dipeptide solids into water has been studied in order to establish the energetic contributions of various groups which determine protein folding. The techniques are described and the results are interpreted in terms of a group additivity scheme. It is shown that this kind of group additivity leads to linear correlations (either entropy change or enthalpy change with heat capacity change) which have been observed for protein denaturation and the dissolution of apolar compounds [1].

INTRODUCTION

A knowledge of the interactions which stabilize globular protein structures is a prerequisite to understanding and modeling protein folding. Because of the complexity of protein structures, many investigators have turned to model compound studies in order to probe key features that determine protein stability. The unfolding of a globular protein involves the transfer of amino acid groups from the anhydrous interior of the molecule into solvent water, and thus most model compound studies have focused on the transfer from some initial phase into water. Most studies to date have utilized a liquid initial phase. Such studies have included the transfer of amino acids or amino acid analogues from an organic phase into water [2, 3]. Hydrophobic properties have been examined from the dissolution of pure hydrocarbon liquids into water [4, 5]. Other

---

* Presented at the 7th International Symposium on Microcalorimetric Applications in Biology, Egham, U.K., 9-11 April 1990, and Dedicated to Ingemar Wadsö on the Occasion of his 60th Birthday.

** To whom correspondence should be addressed.

0040-6031/90/$03.50

studies have investigated the thermodynamics of transfer from the gas phase [6-9].

Considerable evidence exists however, to suggest that the interior of a globular protein is essentially a solid [10]. The packing density [11] and compressibility [12] of proteins is similar to that of organic solids rather than organic liquids. Likewise, the fact that many protein structures have been determined to high resolution by X-ray diffraction demonstrates their crystalline nature. Consequently, a solid model compound system would seem more appropriate in attempting to understand the energetics of protein stability. Additionally, the crystal structures of many model solids are available, and this information can prove to be valuable in the interpretation of results.

In this paper we outline two techniques we have utilized in studying the thermodynamics of dissolution of a series of solid cyclic dipeptides and summarize the results of these studies [13]. The cyclic dipeptides all have the basic structure illustrated in Figure 1. The compounds studied were c(GG), c(AG), c(AA), c(LG), c(VV), c(GF), c(LP), and c(SY), where the "c" indicates "cyclo" and the letters are the standard one letter abbreviations for the amino acids in the L conformation (G=glycine, A=alanine, L=leucine, V=valine, F=phenylalanine, P=proline, S=serine, and Y=tyrosine). The results are discussed in terms of a group additivity scheme and it is shown that this group additivity leads to the entropy - heat capacity correlations which have been observed for several systems [1].

METHODS

Phase equilibrium perturbation calorimetry

The molar enthalpy change for the transfer of a compound from the solid to the aqueous phase can be determined by calorimetry.

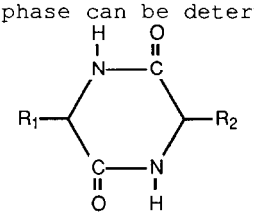

Figure 1. Basic cyclic dipeptide structure. R_1 and R_2 can be any side chain of the amino acids.

Previously the predominant method was flow calorimetry [14, 15]. This method requires the precise weighing of a dry sample which is then dissolved in a continuous flow of solvent. Recently we have developed an alternative approach to measuring the heat of dissolution of slightly soluble solids -- phase equilibrium perturbation calorimetry (PEPC) [16]. The PEPC technique utilizes a titration microcalorimeter [17]. The sample cell is loaded with an equilibrium mixture of the crystalline solid and saturated solution. A small volume, v, of pure solvent is injected into the cell which perturbs the equilibrium by diluting the saturated solution. The heat is measured as additional solid dissolves to reestablish the equilibrium. The heat from an injection is simply the molar enthalpy change times the number of moles which must dissolve to reestablish the equilibrium:

$$q = v\ K\ \Delta H^{o} \qquad (1)$$

where K is the solubility constant and ΔH^{o} is the molar enthalpy change for the transition from solid to solution. Heats of dilution can be used where necessary to correct for nonideallity of the saturated solution [16, 18].

Since both K and ΔH^{o} vary in a known way with temperature, measurements over a range of temperatures can be used to separate the two terms and also to determine the heat capacity change, ΔCp^{o}. In practice however, when it is feasible, a separate determination of the solubility is desirable.

Differential refractive index

The low solubility of many solids makes reliable solubility determination difficult. When a suitable chromophore is present in the molecule, optical techniques, such as UV absorbance, are appropriate. In the case of the cyclic dipeptides there is not a suitable chromophore for absorbance measurements and another technique is required. One such technique is a dry weight determination in which the dried residue from a known volume of saturated solution is weighed. Another technique, differential refractive index (DRI) determination is more versatile and yields solubilities over a range of temperatures.

The refractive index of a solution is proportional to the concentration of solute in the solution and can be used even in the absence of specific chromophores. A differential measurement, in which the refractive index of the solution is measured with respect

to that of the solvent, ensures linearity over a wide range of concentrations and eliminates the temperature dependence observed in the non-differential measurement. Additionally, since light scattering is not a problem as it is in absorbance measurements, equilibration with excess solid can be performed in the sample cell. This differential approach was used by Debye to measure the refractive index of polymer solutions [19] and by Gill et al. to determine solubilities of diketopiperazine in urea solutions [20].

A new sample cell design which we have used is shown in Figure 2. The cell is made from Pyrex with 1/16th in. optical flats (Esco, Oak Ridge, NJ) at the ends of each chamber. The flats are attached to the cell walls using a UV setting optical adhesive (Norland Products, Inc., New Brunswick). The solid/solution equilibrium mixture is loaded into the sample cell and pure water into the solvent chamber. A small magnetic stir bar in the sample chamber ensures rapid equilibration upon reloading or a change in temperature. The cell is placed in an aluminum block which is thermostated with 1/4 in. stainless steel tubing embedded lengthwise. Water from a circulating water bath is flowed through the tubing and the temperature is monitored with a thermocouple.

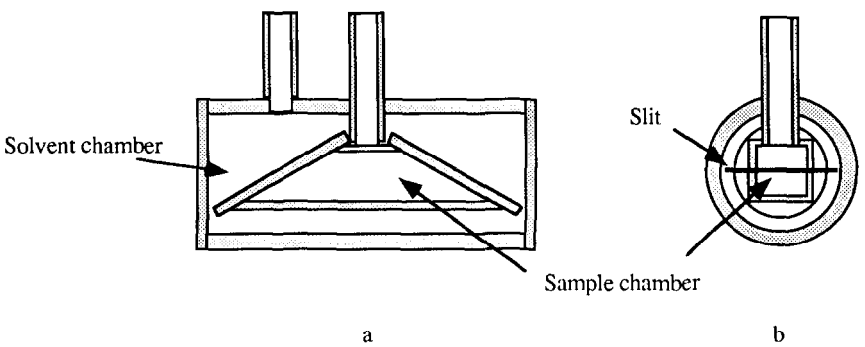

Figure 2. Differential refractive index cell shown to scale. a) side view. b) end-on view.

Monochromatic light from a Helium/Neon laser is expanded with a small lens and passed through a horizontal slit. The slit image is collimated with another lens and passed through the sample cell. A third lens then focuses the image onto a filar micrometer field. As the slit image passes through both the sample and solvent chambers (see Figure 2b), a split image is observed in the micrometer field. The displacement of the sample image from the

reference (solvent) image is a measure of the DRI. The molar refractivity can be calibrated by use of a solution of known concentration.

The molar enthalpy change can be determined from the temperature dependence of the solubility and consequently the DRI and PEPC data can be used together to determine K, ΔH^{o} and ΔCp^{o} without a solubility calibration. The thermodynamic parameters are determined from the data using a non-linear least squares fitting algorithm [21] in which multiple data sets can be fit simultaneously.

RESULTS AND DISCUSSION

The thermodynamic parameters at 298 K for the dissolution of the eight cyclic dipeptides into water are given in Table 1. The free energy change is based on a standard state of unit mole fraction. The confidence intervals were estimated by F-testing based on a 67% confidence level.

The eight compounds studied demonstrate the effects of several functional groups. The peptide backbone is present in all the compounds. The amino acids G, A, V, and L all have purely apolar side chains; an aromatic ring is found in F and Y; an -OH group is present in S and Y; and P has a blocked amino group on the peptide backbone.

Group additivity

The crystal structures of many cyclic dipeptides show a common motif of a hydrogen bonded chain with apolar channels [22-24]; thus these functional groups might be expected to contribute independently to the observed thermodynamics. Such group additivity has been observed for ΔH^{o} and ΔCp^{o} for the dissolution of gases [25] and for the partial molar heat capacity of aqueous solutions [26]. The groups which make up the cyclic dipeptides are then the peptide, -CONH-, the hydroxyl, -OH, the aromatic ring, Φ, and apolar hydrogens, -CH. This last group has been shown to be proportional to the thermodynamic parameters for the dissolution of apolar liquids [27] and is known to be proportional to the accessible apolar surface area or to the number of primary shell water molecules [28]. Note that a benzene ring under this scheme consists of one aromatic ring and six apolar hydrogens (i.e six -CH.groups).

TABLE.1.
Thermodynamic parameters for the dissolution of cyclic dipeptides at 298 K. The standard state is unit mole fraction. The errors shown are one standard deviation estimates based on F-testing. Taken from [13].

Compound	ΔG°	ΔH°	ΔCp°
	kJ mol^{-1}	kJ mol^{-1}	J K^{-1} mol^{-1}
c(GG)	14.7[a]	26.2 ± 0.2[b,c]	-15 ± 18[b]
c(AG)	11.4 ± 0.2[b]	17.7 ± 0.3[b,c]	53 ± 44[b]
c(AA)	14.1 ± 0.5[b]	13.7 ± 0.1[2,3]	100 ± 33[b]
c(LG)	17.0 ± 0.3	13.3 ± 1.6	230 ± 70
c(VV)	25.2 ± 0.5[2]	8.1 ± 0.2[b,c]	320 ± 25[b]
c(LP)	16.6 ± 0.1	7.5 ± 0.2	316 ± 13
c(GF)	22 ± 1	19.3 ± 0.2	320 ± 150
c(SY)	22.5 ± 0.3	26 ± 2	230 ± 100

[a] S. J. Gill, J. Hutson, J. R. Clopton, M. Downing, *J. Phys. Chem.* 65, 1432 (1961).
[b] K. P. Murphy, S. J. Gill, *J. Chem. Thermodynamics* 21, 903 (1989).
[c] Value given is 6% lower than previously published due to an error in calibration of injection syringe.

For a group additivity scheme the thermodynamics of each compound is assumed to be the sum of the thermodynamics of its constituent groups. For example ΔH° for c(GG) is equal to twice ΔH° for the -CONH- plus four times ΔH° for -CH. The average contribution of each group to the overall thermodynamics is determined using a linear least squares fit to the experimental quantities. The crystal structure of c(GG) reveals that the diketopiperazine ring is planar [29]. This is in contrast to the chair conformation which is almost always observed in cyclic dipeptide structures [22-24]. In fact we observed that the c(GG) enthalpy data did not fit the simple correlation found for the aliphatic side group molecules of Table 1. Likewise, the blocked peptide amino group in c(LP) yields a structure which differs from the common motif [30] and it is not included in key set that was used to obtain the group parameters.

The best fit values for the thermodynamic group contributions are given in Table 2. The errors are one standard deviation as determined from the fit [21]. As observed from other studies of apolar compound dissolution [25, 27], the -CH and Φ groups make a positive contribution to ΔCp°. The polar groups, in contrast, make

a negative contribution, as has also been observed [25]. The contribution of -OH is less certain however, since in the present data set this group is only present in c(SY) and the crystal structure of this compound reveals one water per molecule [23]. For this reason the -OH group values are given in parentheses to indicate the tentative interpretation.

TABLE 2
Group contributions to thermodynamics of dissolution for cyclic dipeptides. Adapted from [13].

Group	ΔG^o/kJ mol^{-1}	ΔH^o/kJ mol^{-1}	ΔS^o/J K^{-1} mol^{-1}	ΔCp^o/J K^{-1} mol^{-1}
-CONH-	1.6 ± 1.2	11.0 ± 1.0	32 ± 7	-56 ± 8
-CH	1.3 ± 0.2	-0.8 ± 0.2	-7 ± 1	27 ± 1
Φ	5.7 ± 1.8	5.7 ± 1.8	-9 ± 11	158 ± 13
(-OH)	(0.3 ± 1.1)	(3.4 ± 1.1)	(10.4 ± 7)	(-45 ± 8)

There are several important conclusions which can be drawn from the parameters in Table 2. One important point is that the peptide group, which is involved in hydrogen bonds, stabilizes the crystalline state and, by analogy, the native state of globular proteins. This is in contrast to a widely held view that a hydrogen bond on the interior of a protein will be exactly compensated by a hydrogen bond to water upon unfolding; however it does agree with the conclusion reached by Privalov and Gill [31] that enthalpic contributions are the principal determinant of native protein structures. It is also seen that the apolar groups provide a strong stabilizing force since the ΔG^o value of 1.3 is multiplied by the number of apolar hydrogens. Additionally, aromatic groups strongly favor the solid state. This is possibly due to the favorable aromatic-aromatic interactions which have been observed by Burley and Petsko [32]. Finally, the -OH appears to provide some stability to the crystal, but as mentioned above, this conclusion is quite tentative.

Correlations of ΔS^o and ΔH^o with ΔC_p^o

We have previously noted [1] that protein denaturation and apolar compound dissolution have the common feature of a reference temperature, T^*, at which all compounds of a given class have the same value of ΔS^o designated as ΔS^*. Furthermore, it has also been observed for protein denaturation, that the denaturational ΔH^o

values, normalized to the number of amino acid residues, also attain a common value, ΔH^* near this same temperature. At the temperature T^* the hydration effect, attributed to the interaction of apolar groups with water, is zero and thus the intercept values, ΔH^* and ΔS^*, represent the thermodynamic contribution of the polar groups [1, 31]. Insight into the thermodynamic basis of these observations can be found in the details of group additivity properties.

First, the observation of a convergence temperature can be noted by plotting ΔH° or ΔS° for a given temperature, T, typically 298 K, versus ΔC_p° for a given class of compounds [1]. This can be seen from Equations (2) and (3).

$$\Delta H^\circ(T) = \Delta H^* + \Delta C_p^\circ \ (T - T^*) \quad (2)$$

$$\Delta S^\circ(T) = \Delta S^* + \Delta C_p^\circ \ \ln \ (T / T^*) \quad (3)$$

where the starred terms are the thermodynamic values at the reference temperature T^*. Such a plot will have an intercept of the common value (ΔH^* or ΔS^*) and a slope of $T-T^*$ or $\ln T / T^*$.

Second, the occurrence of the linear correlation of ΔS° and ΔH° with ΔC_p° can be seen to result from the group additivity observed for the cyclic dipeptide dissolution. For any given cyclic dipeptide the total ΔS° value is the sum of the group terms. Thus, for the purely apolar, non-aromatic compounds ΔS° can be represented as:

$$\Delta H^\circ = 2 \ \Delta H^\circ_{(-CONH-)} + N_{-CH} \ \Delta H^\circ_{(-CH)} \quad (4)$$

$$\Delta S^\circ = 2 \ \Delta S^\circ_{(-CONH-)} + N_{-CH} \ \Delta S^\circ_{(-CH)} \quad (5)$$

where N_{-CH} is the number of apolar hydrogens and subscripts indicate group values. Similarly, the total ΔC_p° is given as:

$$\Delta C_p^\circ = 2 \ \Delta C_p^\circ_{(-CONH-)} + N_{-CH} \ \Delta C_p^\circ_{(-CH)} \quad (6)$$

Eliminating N_{-CH} in (4) or (5) and (6) and setting the resulting equations equal to each other yields:

$$\Delta H^\circ = 2\left\{\Delta H^\circ_{(-CONH-)} - \Delta C_p^\circ_{(-CONH-)}\left(\frac{\Delta H^\circ_{(-CH)}}{\Delta C_p^\circ_{(-CH)}}\right)\right\} + \Delta C_p^\circ\left(\frac{\Delta H^\circ_{(-CH)}}{\Delta C_p^\circ_{(-CH)}}\right) \quad (7)$$

$$\Delta S^\circ = 2\left\{\Delta S^\circ_{(-CONH-)} - \Delta C_p^\circ_{(-CONH-)}\left(\frac{\Delta S^\circ_{(-CH)}}{\Delta C_p^\circ_{(-CH)}}\right)\right\} + \Delta C_p^\circ\left(\frac{\Delta S^\circ_{(-CH)}}{\Delta C_p^\circ_{(-CH)}}\right) \quad (8)$$

Equations (7) and (8) have the same form as (2) and (3) and the bracketed terms can be identified with ΔH^* and $T-T^*$ and with ΔS^* and $\ln (T/T^*)$. It can thus be seen that the value of T^* results from the ratios of the apolar enthalpy or entropy change to the apolar heat capacity change and that the intercept quantities, ΔH^* and ΔS^* are determined by polar group contributions.

In summary it is seen that the thermodynamics of dissolution of solid cyclic dipeptides follow a group additivity relationship and that this group additivity results in the thermodynamic correlations which have been observed in protein denaturation and the dissolution of apolar model compounds. Description of the thermodynamics with reference to T^* provides a means of separating the contribution of the apolar thermodynamics from the total values.

Acknowledgement We wish to acknowledgement the support of NSF grant CHE-891145 and NIH grant GM44276 for various aspects of this work.

REFERENCES

1. K.P. Murphy, P.L. Privalov and S.J. Gill, Common Features of Protein Unfolding and Dissolution of Hydrophobic Compounds, Science, 247 (1990) 559-561.
2. Y. Nozaki and C. Tanford, The Solubility of Amino Acids and Two Glycine Peptides in Aqueous Ethanol and Dioxane Solutions, J. Biol. Chem., 246 (1971) 2211.
3. J.L. Fauchere and V. Pliska, Hydrophobic parameters Π of amino acid side chains from the partitioning of N-acetyl-amino acid amides, Eur. J. Chem.-Chim. ther., 18 (1983) 369-375.
4. S.J. Gill, N.F. Nichols and I. Wadsö, Calorimetric Determination of Enthalpies of Solution of Slightly Soluble Liquids I. Application to Benzene in Water, J. Chem. Thermodynamics, 7 (1975) 175.
5. S.J. Gill, N.F. Nichols and I. Wadsö, Calorimetric Determination of Enthalpies of Solution of Slightly Soluble Liquids II. Enthalpy of Solution of Some Hydrocarbons in Water and Their Use in Establishing the Temperature Dependence of Their Solubilities, J. Chem. Thermodynamics, 8 (1976) 445-452.
6. R. Wolfenden, Interaction of the Peptide Bond with Solvent Water: A Vapor Phase Analysis, Biochem., 17 (1978) 201-204.
7. R. Wolfenden, L. Andersson, P.M. Cullis and C.C.B. Southgate, Affinities of Amino Acid Side Chains for Solvent Water, Biochem., 20 (1981) 849-850.
8. G. Olofsson, A.A. Oshodj, Qvarnström and I. Wadsö, Calorimetric Measurements on Slightly Soluble Gases in Water. Enthalpies of Solution of Helium, Neon, Argon, Krypton, Xenon, Methane, Ethane, Propane, n-Butane and Oxygen at 288.25, 298.15, and 308.15 K., J. Chem. Thermodynamics, 16 (1984) 1041-1052.
9. S.F. Dec and S.J. Gill, Heats of Solution of Gaseous Hydrocarbons in Water at 15, 25, and 35 °C, J. Sol. Chem., 14 (1985) 827-836.
10. C. Chothia, Principles that Determine the Structure of Proteins, Annu. Rev. Biochem., 53 (1984) 537-572.
11. B. Lee and F.M. Richards, The Interpretation of Protein Structures: Estimation of Static Accessibility, J. Mol. Biol., (1971)
12. B. Gavish, E. Gratton and C.J. Hardy, Adiabatic Compressibility of Globular Proteins, Proc. Natl. Acad. Sci USA, 80 (1983) 750-754.

13. K.P. Murphy, Probing the Energetics of Protein Stability through Solid Model Compound Studies, Ph. D. thesis, University of Colorado (1990).
14. S.J. Gill and M.L. Siebold, Flow Calorimeter Cell for Measuring Heats of Solutions of Solids, Rev. Sci. Instr., 47 (1976) 1399-1401.
15. S.-O. Nilsson and I. Wadsö, A flow-microcalorimetric vessel for solution of slightly soluble solids, J. Chem. Thermodynamics, 18 (1986) 1125-1133.
16. K.P. Murphy and S.J. Gill, Calorimetric Measurement of the Enthalpy of Dissolution of Diketopiperazine in Water as a Function of Temperature, Thermochim. Acta, 139 (1989) 279-290.
17. I.R. McKinnon, L. Fall, A. Parody-Morreale and S.J. Gill, A Twin Titration Microcalorimeter for the Study of Biochemical Reactions, Anal. Biochem., 139 (1984) 134-139.
18. K.P. Murphy and S.J. Gill, Thermodynamics of Dissolution of Cyclic Dipeptide Solids Containing Hydrophobic Side Groups, J. Chem. Thermodynamics, 21 (1989) 903-913.
19. P. Debye, Light Scattering in Solutions, J. Appl. Phys., 15 (1944) 338-342.
20. S.J. Gill, J. Hutson, J.R. Clopton and M. Downing, Solubility of Diketopiperazine in Aqueous Solutions of Urea, J. Phys. Chem., 65 (1961) 1432-1435.
21. P. Bevington, Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York, 1969.
22. L.E. Webb and C.-F. Lin, Conformations of Cyclic Dipeptides. Structure of *cyclo*-Glycyl-L-tyrosyl (L-3-(4-Hydroxybenzyl)-2,5-piperazinedione), J. Amer. Chem. Soc., 93 (1971) 3818-3819.
23. L.E. Webb and C.-F. Lin, Crystal Structures and Conformations of the Cyclic Dipeptides *cyclo*-(Glycyl-L-tyrosyl) and *cyclo*-(L-Seryl-L-tyrosyl) Monohydrate, J. Amer. Chem. Soc., 95 (1973) 6803-6808.
24. E. Benedetti, R.E. Marsh and M. Goodman, Conformational Studies on Peptides. X-Ray Structure Determinations of Six N-Methylated Cyclic Dipeptides Derived from Alanine, Valine, and Phenylalanine, J. Am. Chem. Soc., 98 (1976) 6676-6684.
25. S. Cabani, P. Gianni, V. Mollica and L. Lepori, Group contributions to the thermodynamic properties of non-ionic organic solutes in dilute aqueous solution, J. Solution Chem., 10 (1981) 563-595.
26. N. Nichols, R. Sköld, C. Spink and I. Wadsö, Additivity relations for the heat capacities of non-electrolytes in aqueous solution, J. Chem. Thermodynamics, 8 (1976) 1081-1093.
27. S.J. Gill and I. Wadsö, An Equation of State Describing Hydrophobic Interactions, Proc. Natl. Acad. Sci., 73 (1976) 2955-2958.
28. W.L. Jorgensen, J. Gao and C. Ravimohan, Monte Carlo Simulations of Alkanes in Water: Hydration Numbers and the Hydrophobic Effect, J. Phys. Chem., 89 (1985) 3470-3473.
29. R.B. Corey, The Crystal Structure of Diketopiperazine, J. Am. Chem. Soc., 60 (1938) 1598-1604.
30. I.L. Karle, Crystal Structure and Conformation of the Cyclic Dipeptide cyclo-L-Prolyl-L-leucyl, J. Amer. Chem. Soc., 94 (1972) 81-84.
31. P.L. Privalov and S.J. Gill, Stability of Protein Structure and Hydrophobic Interaction, Adv. Protein Chem., 39 (1988) 191-234.
32. S.K. Burley and G.A. Petsko, Aromatic-Aromatic Interaction: A Mechanism of Protein Structure Stabilization, Science, 229 (1985) 23-28.