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# Energetics of hydrogen bonding in proteins: A model compound study

SUSAN M. HABERMANN AND KENNETH P. MURPHY

Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242

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## Abstract

Differences in the energetics of amide-amide and amide-hydroxyl hydrogen bonds in proteins have been explored from the effect of hydroxyl groups on the structure and dissolution energetics of a series of crystalline cyclic dipeptides. The calorimetrically determined energetics are interpreted in light of the crystal structures of the studied compounds. Our results indicate that the amide-amide and amide-hydroxyl hydrogen bonds both provide considerable enthalpic stability, but that the amide-amide hydrogen bond is about twice that of the amide-hydroxyl. Additionally, the interaction of the hydroxyl group with water is seen most readily in its contributions to entropy and heat capacity changes. Surprisingly, the hydroxyl group shows weakly hydrophobic behavior in terms of these contributions. These results can be used to understand the effects of mutations on the stability of globular proteins.

**Keywords:** calorimetry; enthalpy; heat capacity; hydrogen bonding; hydroxyl; model compounds; solvation

Hydrogen bonds are ubiquitous in biological molecules and their contribution to the stability of these structures is of fundamental importance. Numerous studies have been performed in an attempt to quantitate the energetics of hydrogen bonding in proteins and other biological macromolecules. In spite of the considerable effort that has gone into such studies, there remains disagreement in the current literature regarding not only the magnitude, but even the sign of the contribution of hydrogen bonds to the thermodynamics, and especially the enthalpy, of proteins.

In 1955, Schellman, analyzing the non-ideality of urea solutions, arrived at a value of  $-6.3 \text{ kJ mol}^{-1}$  for the  $\Delta H^\circ$  of formation of an amide hydrogen bond (i.e., a hydrogen bond between NH and C=O groups from amide linkages) in aqueous solution (Schellman, 1955). However, a subsequent study by Klotz and Franzen (1962) looked at the dimerization of *N*-methyl-acetamide in aqueous solution and concluded that the  $\Delta H^\circ$  of formation of an amide hydrogen bond in water was zero. The conclusion of Klotz and Franzen has been appealing because one could argue that the loss of amide-amide hydrogen bonds is compensated by the formation of amide-water hydrogen bonds (Klotz & Franzen, 1962; Klotz, 1993). However, Némethy and Scheraga (1962) suggested that hydrophobic interactions, which would accompany *N*-methyl-acetamide dimerization and carry a  $\Delta H^\circ$  opposite to hydrogen bond formation, resulted in the net zero  $\Delta H^\circ$ .

Némethy and Scheraga's (1962) suggestion was supported in subsequent studies on the association in solution of  $\delta$ -valerolactam (Susi et al., 1964) and diketopiperazine (Gill & Noll, 1972), both of which have *cis* peptide bonds and would thus have less likelihood of hydrophobic interactions in solution. These studies indicated a  $\Delta H^\circ$  of hydrogen bond formation in water between  $-8$  and  $-13 \text{ kJ mol}^{-1}$ , consistent with the earlier report of Schellman. Recent calorimetric studies on the unfolding of an alanine-based  $\alpha$ -helix peptide also support a favorable  $\Delta H^\circ$  of amide hydrogen bond formation in water (Scholtz et al., 1991).

Although there now is general agreement that the  $\Delta H^\circ$  of hydrogen bond formation in water can be favorable, it has been suggested recently that the unfavorable  $\Delta H^\circ$  of dehydrating polar groups upon transfer to the protein interior or into a protein-protein interface might result in an overall unfavorable  $\Delta H^\circ$  for the burial of hydrogen bonded polar groups (Yang et al., 1992; Makhatadze & Privalov, 1993). These conclusions are based on experimental values for the  $\Delta H^\circ$  of transferring model compounds from vapor into water or on theoretical calculations of this process. The precision of such approaches is questionable because they generally require taking the difference between large numbers. Nevertheless, recent experimental evidence for an enthalpically unfavorable hydroxyl-carbonyl hydrogen bond in the interface between the immunosuppressive drug FK506 and the protein FKBP (Connelly et al., 1994) appears to support this viewpoint, although the  $\Delta G^\circ$  of this hydrogen bond is still favorable due to a favorable  $\Delta S^\circ$  of dehydration. Notably, a tightly bound water is observed interacting with the hydroxyl group in the crystal structure of the unligated protein (Connelly

Reprint requests to: Kenneth P. Murphy, Department of Biochemistry, University of Iowa, Iowa City, Iowa, 52242; e-mail: k-murphy@uiowa.edu.

et al., 1994). The removal of this tightly bound water should carry an enthalpic penalty, but be entropically favorable and may explain their result.

A serious problem with the above mentioned studies of model compound interactions in solution is that the structures of the interacting complexes are unknown, hence assignment of the observed energetics to specific interactions is problematic. For example, the  $\Delta H^\circ$  assigned by Schellman to hydrogen bonding actually applies to all solute-solute interactions that give rise to urea non-ideality. These may include, but are unlikely limited to, hydrogen bonding. Furthermore, these interactions take place in aqueous solution so that the role of changes in hydration is not addressed. We have sought to avoid these problems by studying the energetics of transfer of cyclic dipeptides (i.e., diketopiperazines) from the crystal into water (Murphy & Gill, 1989a, 1989b, 1990). Because the compounds are cyclic, the contributions of ionic end effects are avoided. The crystal structures of all but two of the compounds discussed in the current study are known.

The dissolution of these amino acid-containing crystals involves the disruption of the interactions present in the crystal followed by the solvation of the previously interacting groups. Because the protein interior and the interface in protein-protein interactions are similar to crystals in terms of volumes and packing densities (Richards, 1977; Chothia, 1984; Harpaz et al., 1994), this transfer is analogous to the transfer of such groups from the protein interior or interface that accompanies protein unfolding or dissociation (Murphy & Gill, 1991). Additionally, the results of such studies provide important information on the interaction between solute functional groups and water through the changes in entropy and heat capacity. Such information has been invaluable in understanding the hydrophobic effect (Gill & Wadsö, 1976; Baldwin, 1986; Murphy et al., 1990) and in modeling the temperature dependence of biological processes in solution (Privalov & Makhadze, 1992; Gómez et al., 1995).

Our previous studies focused primarily on the effects of hydrophobic side chains on the dissolution energetics of cyclic dipeptides (Murphy & Gill, 1989b). In the current study, we examine the effects of hydroxyl groups on the structure and energetics of this class of compounds in an effort to examine differences between amide-amide and amide-hydroxyl hydrogen bonds and to investigate the solvation of hydroxyl groups. By comparing the structure and energetics of cyclic dipeptides that contain aliphatic side chains with three new compounds that contain hydroxyl groups (specifically, c(AS),<sup>1</sup> c(SS), and c(GS)), we can study the effects of different hydrogen bond interactions.

## Results

### Solubilities

The solubilities at 298 K were determined by differential refractive index measurements as described in the Materials and methods. The results, in molar units, are given in Table 1, along with the solubilities of the aliphatic compounds determined previ-

<sup>1</sup> Cyclic dipeptides are indicated using the standard, one-letter abbreviations for the amino acid residues. All residues are L isomers (except glycyl).

**Table 1.** Solubility data of cyclic dipeptides at 298 K<sup>a</sup>

Compound	Solubility (mol L <sup>-1</sup> )	Solubility mole fraction
c(GG)	0.145 ± 0.003 <sup>b,c</sup>	0.0026
c(AG)	0.559 ± 0.011 <sup>d</sup>	0.0105
c(AA)	0.560 ± 0.005 <sup>c</sup>	
c(LG)	0.187 ± 0.007 <sup>d</sup>	0.0034
c(LG)	0.179 ± 0.004 <sup>c</sup>	
c(LG)	0.061 ± 0.001 <sup>d</sup>	0.0011
c(LG)	0.0481 ± 0.0002 <sup>b</sup>	
c(AS)	1.466 ± 0.025 <sup>e</sup>	0.0310
c(SS)	0.165 ± 0.003 <sup>c</sup>	0.0028
c(GS)	0.949 ± 0.051 <sup>c</sup>	0.0175

<sup>a</sup> Partial molar volume values used in calculation of mole fraction were from Zamyatnin (1984). Reported errors are the standard deviations from repeat measurements.

<sup>b</sup> Data from Gill et al. (1961).

<sup>c</sup> Data from van de Kleut et al. (1994).

<sup>d</sup> Data from Murphy and Gill (1989b).

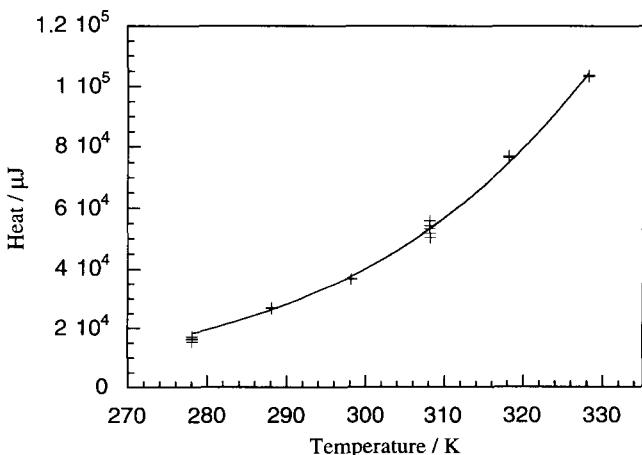
<sup>e</sup> This work.

ously (Murphy & Gill, 1989b; van de Kleut et al., 1994). The mole fraction solubilities,  $X_2$ , were calculated as described in the Materials and methods. These are also given in Table 1.

### Dissolution energetics

The dissolution energetics were measured by Phase Equilibrium Perturbation Calorimetry (PEPC) (Murphy & Gill, 1989a) as described in the Materials and methods. Data collected over a range of temperatures are fitted to the equations given in the Materials and methods in order to obtain  $\Delta H^\circ$  at 298 K and  $\Delta C_p$ . An example fit is given in Figure 1. The  $\Delta G^\circ$  is calculated from the solubility as:

$$\Delta G^\circ = -RT \ln X_2, \quad (1)$$



**Fig. 1.** Results of a typical fit for c(SS). The solid curve represents the best fit of the values using a nonlinear least-squares fitting procedure as described in the text. Note that at least five determinations are shown at each temperature.

and  $\Delta S^\circ$  as:

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T}, \quad (2)$$

where  $R$  is the gas constant and  $T$  is the absolute temperature.

The dissolution energetics of the three hydroxyl-containing cyclic dipeptides at 298 K are summarized in Table 2, along with values published previously for compounds containing aliphatic side chains (c(GG), c(AG), c(AA), and c(LG)). Although the energetics of c(VV) dissolution have also been studied (Murphy & Gill, 1989b; van de Kleut et al., 1994), its low solubility has led to disagreement in the literature over the correct values. Consequently, it is not included in this analysis.

## Discussion

### Structural comparison

The dissolution energetics of the cyclic dipeptides must be interpreted in terms of the crystallographic structures. Of the compounds whose energetics are listed in Table 2, the crystal structures of five are known. The chemical structure and numbering scheme of the atoms in these compounds is indicated in Figure 2A, B, C, and D.

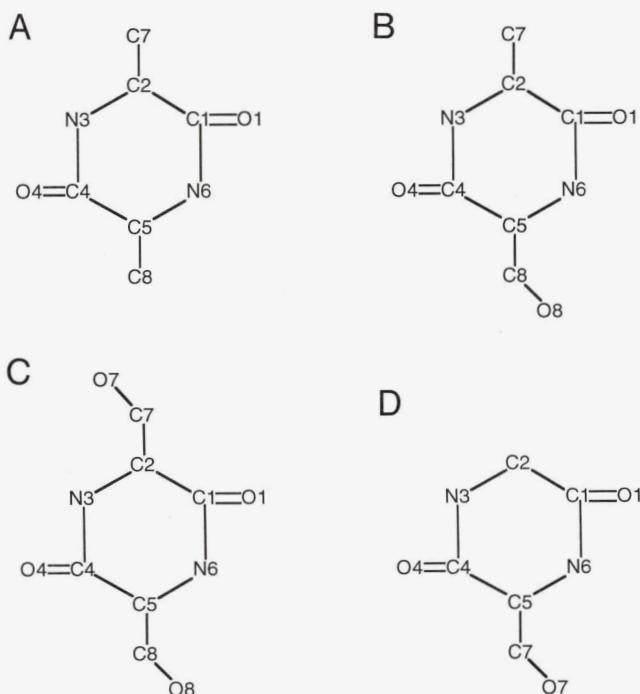
Known structures for the *cis* cyclic dipeptides (i.e., the L, L isomers) that contain non-hydrogen bonding side chains all contain the same hydrogen bonding pattern (Swenson et al., 1996). The peptide backbone is hydrogen bonded to the two adjacent diketopiperazine rings to form continuous, hydrogen bonded chains and the side chains are sequestered into channels. Each cyclic dipeptide molecule has two amide-amide hydrogen bonds as illustrated for c(AA) (Fig. 3). It should be noted that, although each molecule participates in four hydrogen bonds, these hydrogen bonds are shared with other molecules so that the number of hydrogen bonds per molecule is two. The same interactions are observed in the structure of c(GG) (Corey, 1938; Degeilh & Marsh, 1959) (not shown). The crystal structures for c(AG) and c(LG) have not been determined, however, we assume that these compounds contain the same basic interactions as the six other cyclic dipeptides that contain non-hydrogen bonding side chains (see Swenson et al., 1996).

**Table 2.** Dissolution energetics of cyclic dipeptides at 298 K<sup>a</sup>

Compound	$\Delta H^\circ$ (kJ mol <sup>-1</sup> )	$\Delta C_p$ (J K <sup>-1</sup> mol <sup>-1</sup> )	$\Delta S^\circ$ (J K <sup>-1</sup> mol <sup>-1</sup> )	$\Delta G^\circ$ (kJ mol <sup>-1</sup> )
c(GG) <sup>b</sup>	26.2 ± 0.2	-15 ± 18	38.6 ± 0.7	14.7 ± 0.05
c(AG) <sup>b</sup>	17.7 ± 0.3	53 ± 44	21.1 ± 1.0	11.4 ± 0.05
c(AA) <sup>b</sup>	13.7 ± 0.1	100 ± 33	-1.3 ± 0.5	14.1 ± 0.09
c(LG) <sup>b</sup>	13.3 ± 1.6	230 ± 70	-12.4 ± 5.4	17.0 ± 0.04
c(AS)	15.8 ± 0.2	371 ± 18	23.8 ± 0.7	8.7 ± 0.04
c(SS)	22.7 ± 0.3	106 ± 16	27.8 ± 1.0	14.4 ± 0.05
c(GS)	17.6 ± 0.5	107 ± 21	25.6 ± 1.7	9.9 ± 0.13

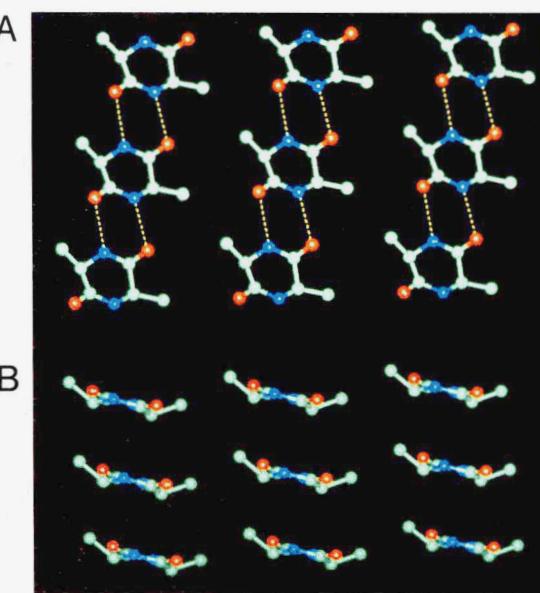
<sup>a</sup> Confidence intervals for  $\Delta H^\circ$  and  $\Delta C_p$  are the errors from the fit, those for  $\Delta G^\circ$  are propagated from the errors in solubility, and those for  $\Delta S^\circ$  are propagated from errors in  $\Delta H^\circ$  and  $\Delta G^\circ$ .

<sup>b</sup> Data from Murphy and Gill (1990).

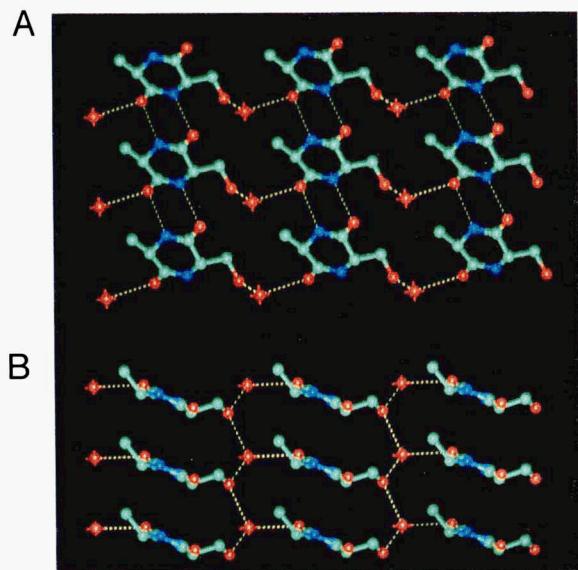


**Fig. 2.** Chemical structure and numbering scheme of cyclic dipeptides. **A:** c(AA). **B:** c(AS). **C:** c(SS). **D:** c(GS).

The addition of a single serine side chain, comparing c(AS) to c(AA), results in little change in the hydrogen bonding pattern (Swenson et al., 1996). As seen in Figure 4, the peptide backbone of each c(AS) molecule is still hydrogen bonded to two



**Fig. 3.** Crystal structure of c(AA). Coordinates are from Sletten (1970). Yellow dashed lines indicate hydrogen bonding interactions. **A:** View perpendicular to the backbone hydrogen bond chains. **B:** View looking down the backbone hydrogen bond chains. Carbon is shown in green, nitrogen in blue, and oxygen in red. Insight II 2.3 (Biosym Technologies, Inc., San Diego, California) was used to produce Figures 5, 6, 7, and 8.



**Fig. 4.** Crystal structure of c(AS) (Swenson et al., 1996). **A:** View perpendicular to the backbone hydrogen bond chains. **B:** View looking down the backbone hydrogen bond chains. The serine hydroxyls are hydrogen bonded via bridging water molecules (indicated as crosses).

adjacent diketopiperazine rings with the side chains sequestered into channels. In order to satisfy the hydrogen bond requirement of the serine hydroxyl, there is a single water per molecule that bridges adjacent hydroxyls in the side-chain channel. This wa-

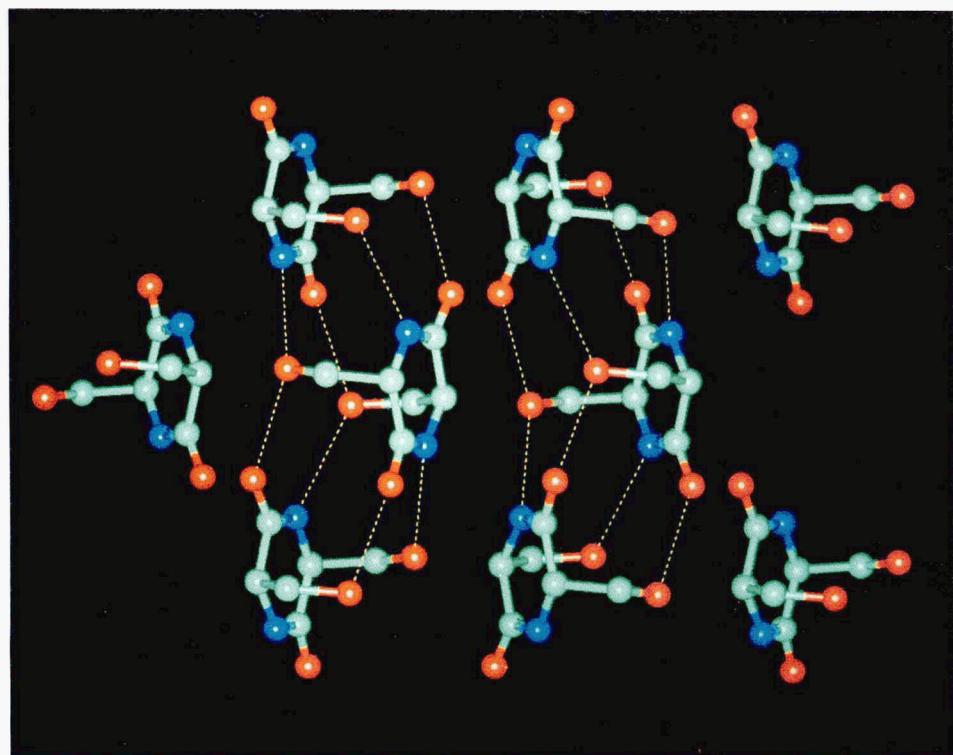
ter also hydrogen bonds to a carbonyl oxygen. Thus, in addition to the two amide-amide hydrogen bonds of the backbone, each molecule has a hydroxyl-water hydrogen bond and a carbonyl-water hydrogen bond (Swenson et al., 1996).

In c(SS), the addition of the second serine side chain results in a significantly different hydrogen bonding pattern (Fava et al., 1981). In this case, the serine hydroxyls are hydrogen bonded to the peptide backbone of adjacent molecules while the peptide backbone is hydrogen bonded to adjacent serine hydroxyls (Fig. 5). Each c(SS) molecule has four amide-hydroxyl hydrogen bonds rather than the two amide-amide hydrogen bonds observed in cyclic dipeptides without hydrogen bonding side chains.

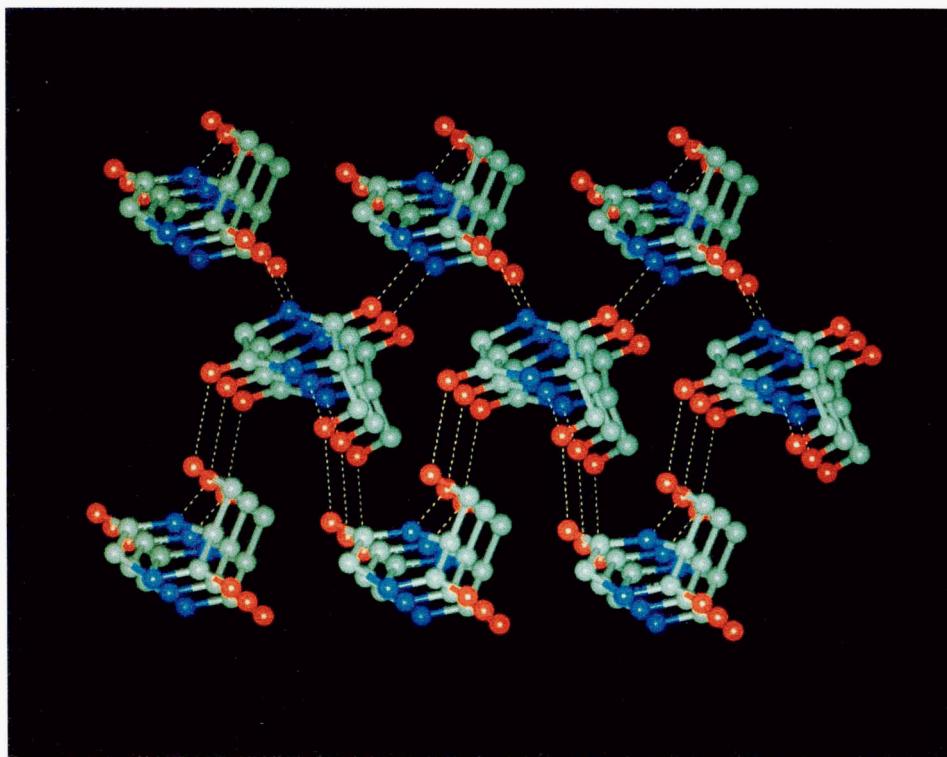
The crystal structure of c(GS), in contrast to c(AS), is anhydrous and is held together by a three-dimensional network of hydrogen bonds (Swenson et al., 1996) (Fig. 6). The serine hydroxyl of c(GS) is involved in two amide-hydroxyl hydrogen bonds, one to a neighboring carbonyl group and a second to an amino group in a separate molecule (below the ring in Fig. 6). This accounts for the hydrogen bonding of one of the peptide groups in each molecule. The second peptide group forms amide-amide hydrogen bonds to two additional molecules. The net result is a single amide-amide and two amide-hydroxyl hydrogen bonds per molecule.

A summary of the hydrogen bond interactions of the cyclic dipeptides is given in Table 3.

Although the number and types of hydrogen bonds are expected to affect the dissolution energetics, other factors, such as van der Waals interactions and hydrogen bond geometries, are likewise expected to be important. The packing densities,



**Fig. 5.** Crystal structure of c(SS) (Fava et al., 1981). The serine hydroxyls are hydrogen bonded to adjacent peptide backbones.



**Fig. 6.** Crystal structure of c(GS) (Swenson et al., 1996). Each serine hydroxyl is involved in two hydrogen bonds to neighboring peptide backbones of separate molecules.

which are related to van der Waals interactions, are given in Table 4, along with hydrogen bond lengths and angles. Packing densities were calculated using the amino acid residue van der Waals volumes of Richards (1977), and the molar volume in the crystal determined from the crystallographic structures. The water molecule in the crystal of c(AS) was assumed to be spherical, with a radius of 1.4 Å. There is no correlation of the dissolution energetics with packing density except for  $\Delta H^\circ$  (see below).

The three new compounds studied can each be considered as the addition of a hydroxyl to a parent compound having an al-

anine side chain. Thus, c(GS) can be viewed as adding a hydroxyl to c(GA), c(AS) as adding a hydroxyl to c(AA), and c(SS) as adding a hydroxyl to c(AS). It is interesting to note that, in each of these three cases, we observe a different effect on the hydrogen bonding pattern, either a substantial rearrangement or the incorporation of a hydration water, which would have been difficult to predict a priori.

#### Structural energetics

Transfer energetics of model compounds are generally interpreted in terms of groups additivity in which the contribution of a functional group is assumed to be independent of neighboring functional groups (Nichols et al., 1976; Cabani et al., 1981; Makhatadze & Privalov, 1990; Spolar et al., 1992). Group additivity has been applied previously to analyzing cyclic dipeptide dissolution energetics (Murphy & Gill, 1990).

Three functional groups will be considered in the following analysis: peptides (CONH), apolar hydrogens (aH), and hydroxyls (OH). The peptide consists of the amide linkage, including the carbonyl carbon, carbonyl oxygen, amide nitrogen, and amide hydrogen. An apolar hydrogen is any hydrogen atom bonded to carbon. The number of apolar hydrogens scales with the hydrophobic surface area (Gill & Wadsö, 1976; Murphy & Freire, 1992) and with the number of waters in the first solvation shell (Jorgensen et al., 1985).

#### Contributions to $\Delta C_p$

Group additivity is most clearly observed in the  $\Delta C_p$  data. Figure 7A shows the  $\Delta C_p$  values for the cyclic dipeptides in Ta-

**Table 3.** Summary of hydrogen bond interactions and functional groups in cyclic dipeptide crystals<sup>a</sup>

Compound	Hydrogen bonds	$N_{\text{aH}}$	$N_{\text{CONH}}$	$N_{\text{OH}}$	$N_{\text{H}_2\text{O}}$
c(GG)	2 Amide-amide	4	2	0	0
c(AG)	2 Amide-amide <sup>a</sup>	6	2	0	0
c(AA)	2 Amide-amide	8	2	0	0
c(LG)	2 Amide-amide <sup>a</sup>	12	2	0	0
c(AS)	2 Amide-amide 1 H <sub>2</sub> O-hydroxyl 1 H <sub>2</sub> O-carbonyl	7	2	1	1
c(SS)	4 Amide-hydroxyl	6	2	2	0
c(GS)	1 Amide-amide 2 Amide-hydroxyl	5	2	1	0

<sup>a</sup> These interactions are assumed by analogy with other known structures as described in the text.

**Table 4.** Packing densities and hydrogen bond lengths and angles in cyclic dipeptide crystals

Compound	Packing density	Hydrogen bond	Length D-A (Å)	Angle D-H...A (°)
c(GG) <sup>b</sup>	0.807	N-H...O	2.85	170
c(AA) <sup>c</sup>	0.744	N3-H3...O1	2.90	174
c(AS) <sup>d</sup>	0.747	N6-H6...O4	2.89	168
		O8-H8...OWat	2.69	171
		N3-H3...O1	2.96	175
		N6-H6...O4	2.93	173
c(SS) <sup>e</sup>	0.792	OWat-HWat...O1	2.82	159
		O8-H8...O1	2.69	164
		O7-H7...O4	2.68	174
		N6-H6...O7	2.98	159
c(GS) <sup>d</sup>	0.775	N3-H3...O8	2.96	154
		N3-H3...O4	2.84	162
		N6-H6...O7	2.91	167
		O7-H7...O1	2.73	171

<sup>a</sup> The lengths are indicated between the hydrogen bond donor (D) and acceptor (A) atoms.

<sup>b</sup> Data of Degeilh and Marsh (1959).

<sup>c</sup> Data of Benedetti et al. (1969).

<sup>d</sup> Data of Swenson et al. (1996).

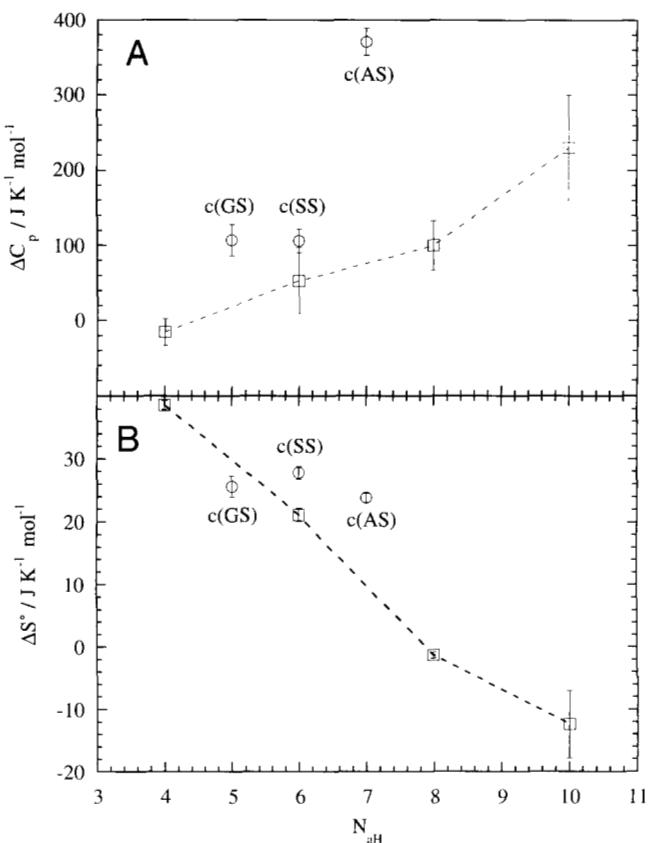
<sup>e</sup> Data of Fava et al. (1981).

ble 2 versus the number of apolar hydrogens,  $N_{\text{aH}}$ , taken from Table 3. For cyclic dipeptides that contain aliphatic side chains,  $\Delta C_p$  increases linearly as the number of apolar hydrogens is increased (Murphy & Gill, 1990). The extrapolation to zero apolar hydrogens is interpreted as the contribution of the peptide group to the dissolution  $\Delta C_p$ , which is negative (Murphy & Gill, 1990). The observation that this polar group makes a negative contribution to  $\Delta C_p$  has been important in interpreting protein unfolding thermodynamics (Murphy & Gill, 1991; Murphy & Freire, 1992; Privalov & Makhatadze, 1992; Spolar et al., 1992). The  $\Delta C_p$  for each of the compounds containing hydroxyl groups lies above this line, suggesting that the hydroxyl makes a positive contribution to  $\Delta C_p$ . When the hydroxyl group is hydrated, as in c(AS), there appears to be an additional large, positive contribution to the  $\Delta C_p$  of dissolution.

In terms of group additivity, the dissolution  $\Delta C_p$  can be described as the sum of its constituent contributions by the following equation:

$$\Delta C_p = N_{\text{aH}} \Delta c_{p\text{AH}} + N_{\text{CONH}} \Delta c_{p\text{CONH}} + N_{\text{OH}} \Delta c_{p\text{OH}} + N_{\text{H}_2\text{O}} \Delta c_{p\text{H}_2\text{O}}, \quad (3)$$

in which the contribution of each group, indicated by  $\Delta c_p$ , is multiplied by the number of such groups in the compound,  $N$ . Linear regression of the dissolution  $\Delta C_p$  data from Table 2 using Equation 3 and the functional groups in Table 3 yields the values in Table 5. Note that, because the number of peptide groups is the same in each compound, the peptide parameter is, in essence, the intercept value and will contain any contributions that are common to the dissolution process but not reflected in the group additivity scheme.



**Fig. 7.** Dissolution (A)  $\Delta C_p$  and (B)  $\Delta S^\circ$  at 298 K versus the number of apolar hydrogens for the cyclic dipeptides that contain aliphatic side chains (squares) and hydroxyl groups (circles). Dashed line connecting the aliphatic compounds is not a fit, but merely provided to show the trend for these compounds.

The apolar group makes a positive contribution ( $28.1 \text{ J K}^{-1} \text{ mol}^{-1}$ ) to the  $\Delta C_p$  (Murphy & Gill, 1990), reflecting the hydrophobic effect (Gill & Wadsö, 1976; Dill, 1990). This value is in good agreement with published values for apolar groups (Gill et al., 1985). The peptide group makes a negative contribution ( $-56.5 \text{ J K}^{-1} \text{ mol}^{-1}$ ) as noted previously (Murphy & Gill, 1990; Privalov & Makhatadze, 1992; Spolar et al., 1992). In contrast to the peptide group, the hydroxyl group makes a positive contribution ( $36.3 \text{ J K}^{-1} \text{ mol}^{-1}$ ). The water apparently makes a large, positive contribution ( $250 \text{ J K}^{-1} \text{ mol}^{-1}$ ); however, in this study, only the c(AS) crystal contains water, so that the generality of this contribution requires further study.

**Table 5.** Functional group contributions to  $\Delta C_p$  and  $\Delta S^\circ$  of dissolution at 298 K for cyclic dipeptides

Group	$\Delta C_p / \text{J K}^{-1} \text{mol}^{-1}$	$\Delta S^\circ / \text{J K}^{-1} \text{mol}^{-1}$
aH	$28.1 \pm 4.9$	$-7.0 \pm 1.0$
Peptide	$-56.5 \pm 19.5$	$31.1 \pm 3.8$
Hydroxyl	$36.3 \pm 16.8$	$-12.5 \pm 3.3$
$\text{H}_2\text{O}$	$251.0 \pm 33.7$	$8.1 \pm 6.6$

### Contributions to $\Delta S^\circ$

The dissolution  $\Delta S^\circ$  values, using a mole fraction standard state, are shown in Table 2. There has been considerable recent discussion on the appropriate standard state for analyzing transfer energetics (DeYoung & Dill, 1990; Sharp et al., 1991), with the primary concern being to eliminate contributions from changes in translational entropy so that solvation entropy can be addressed. These arguments do not pertain to transfer from the crystalline phase, however, because the molecules in the crystal do not have appreciable translational entropy (Chan & Dill, 1994). Regardless, use of different standard states in the analysis here will result only in a different intercept value (see below).

The  $\Delta S^\circ$  of dissolution at 298 K is plotted versus the number of apolar hydrogens in Figure 7B. For cyclic dipeptides that contain aliphatic side chains,  $\Delta S^\circ$  decreases linearly as the number of apolar hydrogens is increased (Murphy & Gill, 1990). Like the increase in  $\Delta C_p$ , the decrease in  $\Delta S^\circ$  is a hallmark of the hydrophobic effect (Gill & Wadsö, 1976; Dill, 1990). Our results indicate that the dissolution  $\Delta S^\circ$  of cyclic dipeptides that contain hydroxyl groups does not vary significantly.

There are several contributions to  $\Delta S^\circ$  of dissolution. These include the restructuring of solvent around the solute, the gain in translational and rotational entropy of the solute, the gain in entropy of crystallographic waters, and the gain in configurational entropy of the solute that arises from rotations around side-chain bonds ( $\Delta s_{conf}$ ). Consequently, the  $\Delta S^\circ$  can be written as:

$$\begin{aligned} \Delta S^\circ = & N_{\text{aH}} \Delta s_{\text{AH}} + N_{\text{CONH}} \Delta s_{\text{CONH}} \\ & + N_{\text{OH}} \Delta s_{\text{OH}} + N_{\text{H}_2\text{O}} \Delta s_{\text{H}_2\text{O}} + \Delta s_{conf}. \end{aligned} \quad (4)$$

Note that the gain in translational and rotational entropy upon dissolution is not included in Equation 4. This contribution should be essentially the same for each compound and thus cannot be separated from the contribution of the peptide backbone. Consequently, the parameter for the peptide backbone also includes this contribution.

The final term in Equation 4,  $\Delta s_{conf}$ , will vary according to the side chains in the compound. For Gly and Ala, this latter term is zero, however, it does contribute to Ser and Leu in the considered compounds. Lee et al. (1994) have calculated the configurational entropy for all amino acid side chains in an  $\alpha$ -helix, and we use their values here. Assuming that the side chain in the crystal occupies one conformer, for Ser,  $\Delta s_{conf} = 15.4 \text{ J K}^{-1} \text{ mol}^{-1}$ , and for Leu,  $\Delta s_{conf} = 6.8 \text{ J K}^{-1} \text{ mol}^{-1}$ . Linear regression of the  $\Delta S^\circ$  data in Table 2 according to Equation 4, using the above values for  $\Delta s_{conf}$ , gives the results in Table 5. The negative contribution ( $-7.0 \text{ J K}^{-1} \text{ mol}^{-1}$ ) for the apolar hydrogen is similar to that published previously (Gill & Wadsö, 1976). The negative contribution of the hydroxyl, on the other hand, is considerably less in magnitude than that reported by Privalov and Makhatadze (1990) based on gas dissolution studies. Their value of  $-47 \text{ J K}^{-1} \text{ mol}^{-1}$  is approximately four times larger than the  $-12.5 \text{ J K}^{-1} \text{ mol}^{-1}$  we observe. This difference may reflect contributions other than solvation to the gas dissolution value, as suggested previously (Murphy, 1994).

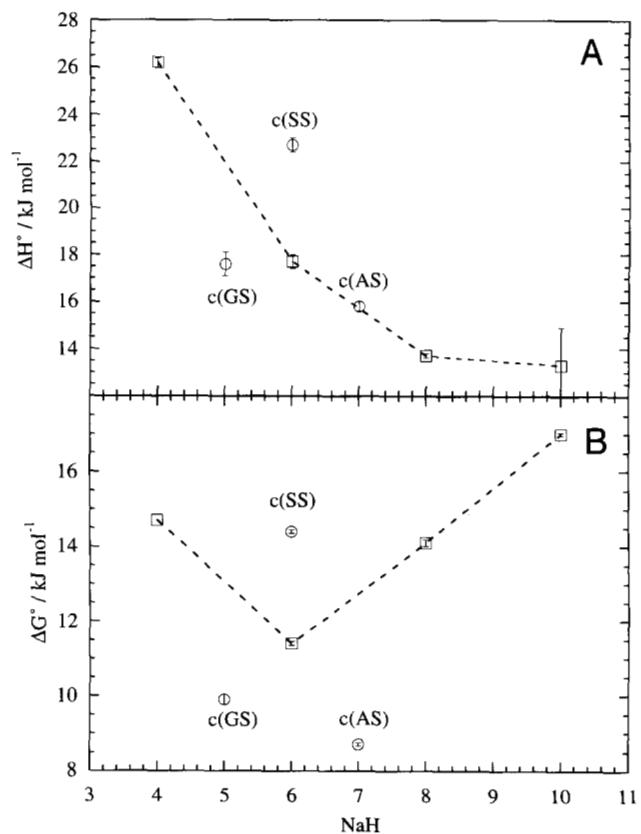
The negative  $\Delta S^\circ$  and positive  $\Delta C_p$  for the hydroxyl group suggests that it behaves very much like a hydrophobic group in terms of its solvation. This "hydrophobic" behavior of a small

polar group is in agreement with theoretical calculations (Rashin & Bukatin, 1994). However, as seen below, the enthalpic contribution of this group is not similar to that of hydrophobic groups. The unfavorable  $\Delta S^\circ$  of exposing the hydroxyl to water provides a favorable free energy to the burying of this group. However, the added configurational entropy of Ser relative to Ala effectively offsets this hydration entropy at 298 K.

### Contributions to $\Delta H^\circ$

The dissolution  $\Delta H^\circ$  values at 298 K are given in Table 2 and plotted versus the number of apolar hydrogens in Figure 8A. For cyclic dipeptides that contain aliphatic side chains,  $\Delta H^\circ$  decreases as the number of apolar hydrogens is increased (Murphy & Gill, 1990). Although the correlation is not as linear as those observed for  $\Delta C_p$  and  $\Delta S^\circ$ , the general trend is clear. The negative trend with increasing  $N_{\text{aH}}$  suggests that the exposure of aliphatic surface to solvent is enthalpically favorable relative to the crystal. Analogously, burial of aliphatic surface should enthalpically disfavor the native state of globular proteins.

Given the decrease in  $\Delta H^\circ$  with increasing number of apolar hydrogens, the positive  $\Delta H^\circ$  values observed suggest that the overall enthalpy associated with disrupting and solvating the hydrogen bonding amide groups is unfavorable. Such interactions therefore should enthalpically stabilize the native state of proteins. The contribution of the peptide backbone includes the loss



**Fig. 8.** Dissolution (A)  $\Delta H^\circ$  and (B)  $\Delta G^\circ$  at 298 K versus the number of apolar hydrogens for the cyclic dipeptides that contain aliphatic side chains (squares) and hydroxyl groups (circles). Dashed line connecting the aliphatic compounds is not a fit, but merely provided to show the trend for these compounds.

of the amide-amide hydrogen bonds as well as the solvation of these groups, suggesting that the solvation enthalpy is insufficient to overcome the hydrogen bonding interactions. This observation, that the amide-amide interaction is more favorable energetically than the amide-water interaction, is supported by recent ab initio calculations (Nilar & Pluta, 1995).

The addition of a hydroxyl group in c(AS) results in a dissolution  $\Delta H^\circ$  at 298 K similar to the cyclic dipeptides with non-hydrogen bonding side chains. This might be expected because c(AS) contains the same backbone interactions as aliphatic cyclic dipeptides and the hydroxyl is already hydrated in the crystal. However, the  $\Delta C_p$  of this compound is quite large, so that its  $\Delta H^\circ$  differs from that for the aliphatic compounds at other temperatures.

In the case of c(GS), where the added hydroxyl results in one amide-amide hydrogen bond and two amide-hydroxyl hydrogen bonds, there is a decrease in the  $\Delta H^\circ$ . For c(SS), there is an increase in the dissolution  $\Delta H^\circ$ , but, in this case, there are four amide-hydroxyl hydrogen bonds as compared to two amide-amide hydrogen bonds in cyclic dipeptides that contain non-hydrogen bonding side chains.

A simple group additivity analysis is sufficient to interpret the dissolution  $\Delta H^\circ$  values for the aliphatic cyclic dipeptides because the hydrogen bond interactions in the crystal are the same for all of the compounds (Murphy & Gill, 1990, 1991). In this study, however, the hydrogen bond interactions vary for many of the compounds, so that the contribution of peptide and hydroxyl groups is expected to be different in different compounds. Rather than interpret the  $\Delta H^\circ$  values in terms of group additivity, they can be described as the sum of contributions from the apolar transfer and the various hydrogen bonding interactions:

$$\Delta H^\circ = N_{\text{aH}} \Delta h_{\text{aH}}^\circ + N_{\text{am-am}} \Delta h_{\text{am-am}}^\circ + N_{\text{am-OH}} \Delta h_{\text{am-OH}}^\circ + N_{\text{OH-H}_2\text{O}} \Delta h_{\text{OH-H}_2\text{O}}^\circ, \quad (5)$$

where  $N_{\text{aH}}$  is the number of apolar hydrogens, each of which contributes  $\Delta h_{\text{aH}}^\circ$  to the enthalpy;  $N_{\text{am-am}}$  is the number of amide-amide hydrogen bonds, each of which contributes  $\Delta h_{\text{am-am}}^\circ$  to the enthalpy, and likewise, for amide-hydroxyl ( $\text{am-OH}$ ) and amide-water ( $\text{am-H}_2\text{O}$ ) hydrogen bonds. A linear regression of the  $\Delta H^\circ$  data in Table 2 to Equation 5 yields the values in Table 6.

The enthalpy of the amide-amide hydrogen bond appears to make a strong, favorable contribution ( $13.5 \text{ kJ mol}^{-1}$ ) to the stabilization of the crystalline state of cyclic dipeptides. The amide-hydroxyl hydrogen bond also makes a favorable contri-

bution; however, it is approximately half the contribution of the amide-amide hydrogen bond ( $7.1 \text{ kJ mol}^{-1}$ ). The hydroxyl- $\text{H}_2\text{O}$  hydrogen bond makes a negligible contribution to the  $\Delta H^\circ$  of dissolution within experimental error. It should be noted that the water-carbonyl hydrogen bond contribution cannot be separated from the water-hydroxyl contribution in c(AS), and should be considered as part of that term.

The enthalpic difference between amide-amide and amide-hydroxyl hydrogen bonds observed here is important in several respects. The majority of hydrogen bonds in globular proteins are amide-amide hydrogen bonds involved in regular, secondary structure (Baker & Hubbard, 1984). However, site-directed mutagenesis experiments aimed at understanding the contribution of hydrogen bonding to protein stability (Shirley et al., 1992; Blaber et al., 1993) can only investigate the contribution of hydrogen bonds involving side chains (see below). Although providing important data, mutagenesis studies are unable to address the contribution of backbone hydrogen bonding, which is the most relevant to understanding protein stability.

The  $\Delta H^\circ$  of dissolution also scales with packing density. A plot of  $\Delta H^\circ$  versus packing density for the five compounds with packing densities listed in Table 4 gives a straight line ( $R = 0.97$ ) with a slope of  $181 \text{ kJ mol}^{-1}$  and an intercept of  $-120 \text{ kJ mol}^{-1}$ . However, if the  $\Delta H^\circ$  of dissolution of c(GQ) (unpubl. data) is included in the plot, the correlation coefficient drops to 0.90, suggesting a lack of generality. Packing density does appear to be an important determinant of the dissolution  $\Delta H^\circ$ , but additional data are required to understand its contribution.

#### Contributions to $\Delta G^\circ$

The dissolution  $\Delta G^\circ$  values at 298 K are given in Table 2 and plotted versus the number of apolar hydrogens in Figure 8B. It is clear from the figure that group additivity does not hold for the aliphatic compounds because no clear trend of  $\Delta G^\circ$  with  $N_{\text{aH}}$  can be discerned. As discussed previously (Murphy & Gill, 1990), it appears that c(GG) behaves anomalously in terms of  $\Delta G^\circ$ , because the other three compounds with aliphatic side chains show a linear increase of  $\Delta G^\circ$  with  $N_{\text{aH}}$ .

Even though  $\Delta G^\circ$  for the aliphatic compounds does not obey group additivity well, it is instructive to consider the results of a regression analysis as was done for the  $\Delta H^\circ$  data. The dissolution  $\Delta G^\circ$  can also be described as the sum of contributions from the apolar transfer and the various hydrogen bonding interactions:

$$\Delta G^\circ = N_{\text{aH}} \Delta g_{\text{aH}}^\circ + N_{\text{am-am}} \Delta g_{\text{am-am}}^\circ + N_{\text{am-OH}} \Delta g_{\text{am-OH}}^\circ + N_{\text{OH-H}_2\text{O}} \Delta g_{\text{OH-H}_2\text{O}}^\circ. \quad (6)$$

Because of the contribution of  $\Delta H^\circ$  to  $\Delta G^\circ$ , Equation 6 is given in terms of interactions rather than individual group contribution. A linear regression of the  $\Delta G^\circ$  data in Table 2 to Equation 6 gives the values in Table 6. The amide-amide hydrogen bond free energy appears to make a favorable contribution ( $4.9 \text{ kJ mol}^{-1}$ ) to the stabilization of the crystalline state of cyclic dipeptides, even though this term includes the general "melting" entropy associated with the dissolution process. The amide-hydroxyl hydrogen bond also appears to make a favorable contribution ( $2.4 \text{ kJ mol}^{-1}$ ), approximately half the contribution of the amide-amide hydrogen bond, in spite of the unfavorable configurational entropy of the Ser side chain. Cor-

**Table 6.** Contributions to the dissolution  $\Delta H^\circ$  and  $\Delta G^\circ$  of cyclic dipeptides at 298 K<sup>a</sup>

Interaction	$\Delta H^\circ/\text{kJ mol}^{-1}$	$\Delta G^\circ/\text{kJ mol}^{-1}$
aH	$-1.3 \pm 0.7$	$0.6 \pm 0.4$
Amide-amide	$13.5 \pm 2.7$	$4.9 \pm 1.6$
Amide-hydroxyl	$7.1 \pm 1.3$	$2.4 \pm 0.8$
$\text{H}_2\text{O}$	$-2.1 \pm 4.6$	$-5.0 \pm 2.8$

<sup>a</sup> The reported uncertainties are the standard errors from the regression.

rection for the side-chain configurational entropy was not included in the  $\Delta G^\circ$  regression, because it has very little effect (data not shown). Recall, however, that these regression values are only qualitative because of the lack of group additivity in the aliphatic  $\Delta G^\circ$  data.

#### Comparison to protein data

The dissolution energetics of cyclic dipeptides we have presented can be used to rationalize the results of site-directed mutagenesis studies aimed at understanding the contribution of hydrogen bonding to the stability of proteins (Shirley et al., 1992; Blaber et al., 1993). Blaber et al. (1993) have studied the effects of introducing polar groups within T4 lysozyme in order to determine the energetics and structural consequences of burying a hydroxyl group. From their data and the  $\Delta C_p$  of the wild-type protein ( $10.5 \text{ kJ K}^{-1} \text{ mol}^{-1}$  [Zhang et al., 1991]), we have calculated  $\Delta H^\circ$  of unfolding at the  $T_m$  of the wild-type protein ( $51.55^\circ\text{C}$ ) and the difference in the unfolding  $\Delta H^\circ$  ( $\Delta\Delta H^\circ = \Delta H_{\text{mutant}}^\circ - \Delta H_{\text{wild-type}}^\circ$ ) (Table 7). A positive value of  $\Delta\Delta H^\circ$  indicates an enthalpic stabilization of the protein from the addition of the hydroxyl.

The magnitude and sign of  $\Delta\Delta H^\circ$  is indicative of the environment of the hydroxyl group. The complexity of the protein interior and the interactions therein is reflected in the broad range of  $\Delta\Delta H^\circ$  values observed in the 12 mutants. Of the 12 mutants studied, only 4 (positions 41, 42, 134, and 149) show a destabilizing  $\Delta\Delta H^\circ$ . In these cases, the hydroxyl does not form an intramolecular hydrogen bond, the side chain is in a strained conformation, or the hydroxyl is hydrogen bonded to a buried solvent molecule (Blaber et al., 1993). Two mutant side chains (82 and 93) are positioned poorly (i.e., mobile) (Blaber et al., 1993). Of the remaining mutants, the four alanine to serine mu-

tations show an average  $\Delta\Delta H^\circ$  of  $6.3 \pm 3.5 \text{ kJ mol}^{-1}$ , in good agreement with the amide-hydroxyl hydrogen bond contribution to the dissolution  $\Delta H^\circ$  of cyclic dipeptides ( $7.1 \pm 1.3 \text{ kJ mol}^{-1}$ ). If the two valine to threonine mutants are included, the average  $\Delta\Delta H^\circ$  is  $13 \pm 13 \text{ kJ mol}^{-1}$ . These results indicate the stabilizing contribution that the amide-hydroxyl hydrogen bond can make to the enthalpy.

Blaber et al. (1993) conclude that the introduction of hydrogen bonds into T4 lysozyme does not increase the stability of the protein. However, the destabilization is generally entropic, as indicated by the positive values of  $\Delta\Delta H^\circ$  in the table. It might be more precise to say that the addition of a hydroxyl is destabilizing in spite of the favorable hydrogen bond because of the entropic cost associated with this substitution. Using the configurational entropy values of Lee et al. (1994), the change in free energy due to configurational entropy for an Ala to Ser mutation should be destabilizing by approximately  $5.0 \text{ kJ mol}^{-1}$  at the  $T_m$  of the wild-type protein, accounting for much of the destabilization observed in these mutants.

In a related study, Shirley et al. (1992) mutated side chains that participate in intramolecular hydrogen bonds to look at their contribution to the stability of ribonuclease T1. Their Ser to Ala mutants are shown also in Table 7, where again we have calculated  $\Delta\Delta H^\circ$  at the  $T_m$  of the wild-type protein using the wild-type  $\Delta C_p$  of  $6.7 \text{ kJ mol}^{-1}$  (Plaza del Pino et al., 1992). In this case,  $\Delta\Delta H^\circ$  is equal to  $\Delta H_{\text{wild-type}}^\circ - \Delta H_{\text{mutant}}^\circ$ , so that a positive  $\Delta\Delta H^\circ$  also reflects an enthalpically stabilizing hydrogen bond. In the wild-type protein, Ser 12 is hydrogen bonded to the side-chain hydroxyl of Ser 14 and to the amide nitrogen of Asp 15, Ser 17 is hydrogen bonded to the carbonyl oxygen of Ser 13, and Ser 64 is hydrogen bonded to the amide nitrogen of Asp 66 (Shirley et al., 1992). Again, the average  $\Delta\Delta H^\circ$  per hydrogen bond ( $8.6 \text{ kJ mol}^{-1}$ ) suggests that the hydroxyl

**Table 7.** Energetics of wild-type and mutant T4 lysozyme (Blaber et al., 1993) and ribonuclease T1 (Shirley et al., 1992)

Protein	Mutant	$T_m$ ( $^\circ\text{C}$ )	$\Delta H^\circ$ ( $\text{kJ mol}^{-1}$ )	$\Delta\Delta H^\circ$ at wt $T_m$ ( $\text{kJ mol}^{-1}$ )	Hydrogen bond environment
T4 Lysozyme	wt	51.55	473	0.0	—
	A41S	49.78	444	-10.7	No HB
	A42S	44.06	372	-21.8	i - 3 Carbonyl, strained
	A49S	50.02	460	3.5	i - 4 Carbonyl, partly exposed
	A73S	50.28	464	5.0	i - 4 Carbonyl, partly exposed
	A82S	50.56	469	6.2	Solvent, mobile
	A93S	51.03	469	1.3	Solvent, mobile
	A98S	44.08	406	11.5	Distant carbonyl, strained
	A130S	48.66	448	5.2	i - 4 Carbonyl
	A134S	51.11	464	-3.7	i - 3 Carbonyl, strained and partly exposed
	V75T	47.85	473	38.8	i - 4 Carbonyl
	V87T	47.00	439	14.3	Side-chain carbonyl
	V149T	41.47	322	-44.8	Buried solvent
Rnase T1	wt	50.9	460	0.0	—
	S12A	47.4	414	12.3 <sup>a</sup>	Side-chain OH and amide NH
	S17A	52.6	456	15.6	Backbone carbonyl
	S64A	46.3	435	-5.7	Amide nitrogen

<sup>a</sup> This value is per hydrogen bond lost.

groups are generally involved in enthalpically favorable hydrogen bonds. Additionally, Shirley et al. conclude that the Ser hydroxyl hydrogen bonds stabilize the protein by an average of  $3.3 \text{ kJ mol}^{-1}$ , in good agreement with the value of  $2.4 \text{ kJ mol}^{-1}$  estimated for the contribution of the hydroxyl-amide hydrogen bond to the  $\Delta G^\circ$  of dissolution of the cyclic dipeptides indicated in Table 6.

### Conclusion

In summary, the amide-amide and amide-hydroxyl hydrogen bonds both provide considerable enthalpic stability to the crystalline state of cyclic dipeptides, with the amide-amide hydrogen bond contributing twice as much. From the contribution of the hydroxyl group to  $\Delta S^\circ$  and  $\Delta C_p$ , it appears that it interacts with solvent much like a hydrophobic group, in contrast to the peptide backbone.

There is good agreement between the energetics of amide-hydroxyl hydrogen bonds determined in this study and those observed from the effects of point mutations on the stability of T4 lysozyme and ribonuclease T1. This agreement indicates that the dissolution of cyclic dipeptides is an excellent model system for studying the energetics of proteins.

## Materials and Methods

### Dissolution energetics

Cyclic dipeptides, c(AS), c(GS), and c(SS), were purchased from Bachem Bioscience (Philadelphia, Pennsylvania) and used without further purification. Distilled-deionized water was used in all experiments.

The dissolution energetics were determined by Phase Equilibrium Perturbation Calorimetry (PEPC) as described previously (Murphy & Gill, 1989a). Briefly, a saturated solution with excess solid was placed in the cell of a CSC 4200 isothermal titration microcalorimeter (ITC) (Calorimetry Sciences Corporation, Pleasant Grove, Utah). The ITC was calibrated using both internal and external electrical heaters and verified at 298 K by the heat of protonation of Tris base (Christensen et al., 1976) and the heat of binding of  $\text{Ba}^{2+}$  to 18-crown-6 (Briggner & Wadsö, 1991).

A small volume (1–10  $\mu\text{L}$ ) of pure water, which dilutes the saturated solution, was injected into the cell, perturbing the equilibrium. Equilibrium was reestablished with the dissolution of excess solid and the dissolution heat was measured. The presence of excess solid was verified following each experiment. The heat effect  $q$  is given as:

$$q = vK_{sol}\Delta H^\circ, \quad (7)$$

where  $v$  is the volume of injected solvent,  $K_{sol}$  is the molar solubility of the compound, and  $\Delta H^\circ$  is the molar dissolution enthalpy change.  $K_{sol}$  and  $\Delta H^\circ$  are both temperature dependent. For  $\Delta H^\circ$ :

$$\Delta H^\circ = \Delta H^\circ(298) + \Delta C_p(T - 298), \quad (8)$$

where  $\Delta H^\circ(298)$  is the enthalpy change at 298 K,  $\Delta C_p$  is the heat capacity change, and  $T$  is the absolute temperature.

The solubility is related to the standard state free energy change as:

$$K_{sol} = \exp(-\Delta G^\bullet/RT), \quad (9)$$

where  $\Delta G^\bullet$  is the free energy change for a *molar* standard state as opposed to the free energy change for the mole fraction standard state given by Equation 1. The temperature dependence of  $\Delta G^\bullet$  is given as:

$$\begin{aligned} \Delta G^\bullet = \Delta H^\circ(298) - T &\left( \frac{\Delta H^\circ(298) - \Delta G^\bullet(298)}{298} \right) \\ &+ \Delta C_p \left[ (T - 298) - T \ln \frac{T}{298} \right], \end{aligned} \quad (10)$$

where  $\Delta G^\bullet(298)$  is the value of  $\Delta G^\bullet$  at 298 K.

Dissolution experiments were performed over a range of temperatures and the enthalpy and heat capacity change were determined by nonlinear least-squares fitting of the data using the program NONLIN (Michael L. Johnson, University of Virginia).

### Solubilities

The molar solubilities of the cyclic dipeptides were determined by differential refractive index (DRI), as described previously (Murphy & Gill, 1990), with a custom-made, differential refractive index apparatus. A differential measurement was made in which the refractive index of a saturated solution with excess solid of the cyclic dipeptide is measured with respect to that of the solvent, pure water. The instrument was calibrated for each compound by making a series of solutions of known concentration and determining the differential measurement at each concentration. At least three determinations were made for each compound.

The mole fraction solubility  $X_2$  was calculated from the molar solubility  $K_{sol}$  as:

$$X_2 = \frac{K_{sol}}{K_{sol} + (1 - K_{sol})\bar{v}_2/(1/\bar{v}_1)}, \quad (11)$$

where  $\bar{v}_2$  is the partial molar volume of the solute, calculated from the amino acid residue values of Zamyatnin (1984), and  $\bar{v}_1$  is the partial molar volume of water, taken as  $0.018 \text{ L g}^{-1}$ .

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