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CALORIMETRIC MEASUREMENT OF THE ENTHALPY OF DISSOLUTION OF DIKETOPIPERAZINE IN WATER AS A FUNCTION OF TEMPERATURE *

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ABSTRACT

Direct measurement of the heat of dissolution of solid diketopiperazine (DKP), which mimics hydrogen bonding and other interactions in proteins, has been made using a high-sensitivity titration microcalorimeter (McKinnon et al., Anal. Biochem., 139 (1984) 134). A value of $\Delta H_s^{\ominus} = 28.0 \pm 0.2$ kJ mol^{-1} at 25 °C was obtained with a $\Delta C_{p,s}^{\ominus} = -57 + 21$ J mol^{-1} K^{-1} in the range 20–40 °C. The thermodynamic properties of self-association of DKP in aqueous solution were also studied calorimetrically. The results were analyzed using a simple dimerization model and yielded an association constant of 0.19 ± 0.08 M$^{-1/2}$ at 25 °C with an enthalpy of dimer formation of -29 ± 7 kJ mol^{-1} dimer. The results extend earlier van't Hoff studies to enable calculations at high temperatures.

The enthalpy of solution of DKP, scaled either to its surface area or to two glycyl residues, may be compared with the enthalpies of denaturation of proteins (lysozyme, ribonuclease and myoglobin) scaled either to "buried" area or "buried" residues. The comparison is made with reference to the temperature (110 °C) where hydrophobic solvation is essentially enthalpic. Values of 11.6 kJ mol^{-1} Gly residue and 93.8 J mol^{-1} Å^{-2} are obtained for DKP. This agrees well with values of 9.3, 9.2 and 9.1 kJ mol^{-1} "buried" residue and 57.7 J mol^{-1}, 56.5 J mol^{-1} and 51.9 J mol^{-1} Å^{-2} for the proteins ribonuclease, lysozyme and myoglobin respectively. This energetic evidence supports the view that the unsolvated protein core region behaves as an intramolecular solid.

INTRODUCTION

The study of model compounds provides a means for understanding the interactions responsible for stabilizing the native conformation of proteins. Relevant interactions include hydrogen bonding, van der Waals forces, hydrophobic interactions and electrostatic forces. Studies of model compounds have included solubility of amino acids in different solvents [1–3],

* Dedicated to Professor E.F. Westrum, Jr., in honor of his contribution to Calorimetry and Thermal Analysis, and on the occasion of his 70th birthday.

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vapor pressure and calorimetric studies of hydrophobic compounds and amino acid analogs [4–7] and studies of hydrogen-bonding compounds such as δ-valerolactam [8], ϵ-caprolactam [4], *N*-methylacetamide [9,10], urea [11,12] and diketopiperazine (DKP) [13–15]. The primary goal of these explorations has been to characterize the energetics of hydrophobic interaction and hydrogen bonding in aqueous solutions.

Practically all of the abovementioned studies have focused on the solution properties of model compounds, and thus were consistent with the view that the protein core could be regarded as an organized liquid rather than an organized solid. However, there now exists considerable evidence that the interior of a globular protein, while not static, is more like an organic solid than a liquid (see ref. 16 and references cited therein). Lee and Richards [17,18] have demonstrated that the packing density of proteins is in the range seen for organic solids, i.e. much higher than that seen in organic liquids. Similarly, the adiabatic compressibility of proteins is even lower than that observed for organic solids [19], and the very fact that X-ray crystallography can be used to determine accurately the position of virtually all the atoms in protein crystals clearly argues for their solid-like nature. Consequently, it would appear that the thermodynamics of dissolution of solid model compounds would provide relevant properties to understand the salient features of protein structure formation.

Furthermore, a vast amount of detailed structural information is available for crystals of small molecules. This information, which describes intermolecular contacts, has not been utilized in understanding the gross thermodynamic properties of dissolving such crystals in water or other solvents. One of the purposes of the current study is to initiate the assembly of thermodynamic information on such materials, which information presumably reflects interactions governing protein folding. This paper compares the gross quantitative enthalpic contribution of the thermal denaturation of several proteins with carefully determined heats of solution of the model compound DKP.

Such studies require the determination of the dissolution properties of sparingly soluble compounds. Solubility and heats of solution as a function of temperature provide the key quantities of change in free energy, enthalpy and heat capacity. In this study we have adapted a titration microcalorimetry approach to determine the heat of solution of DKP as a function of temperature. Earlier solubility determinations have been made on this compound [13,14], and its aqueous solution properties have been characterized by flow calorimetry [15]. The new methods developed in the present study confirm and extend this earlier work so that high-temperature prediction of the dissolution properties can be made.

DKP was chosen as a model of typical interactions found in proteins because it is the cyclic dimer of glycine. There are thus no end charge effects and hydrogen bonding occurs between peptide linkages.

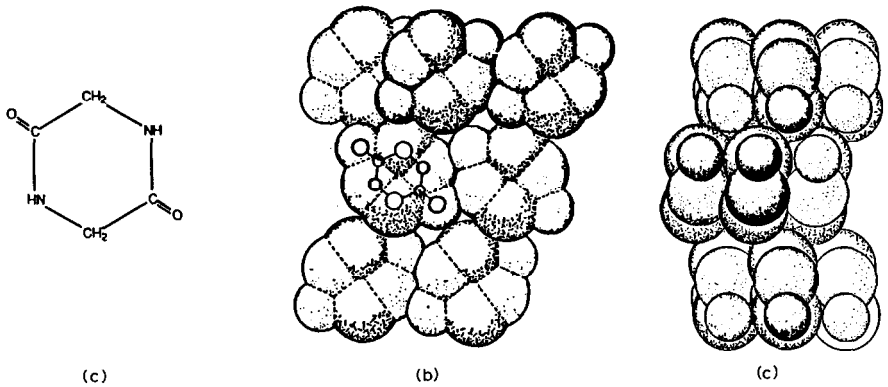

Fig. 1. (a) A representation of the structure of a single diketopiperazine molecule. (b) A packing drawing of some diketopiperazine molecules in the crystal viewed perpendicular to the plane of the molecule. The hydrogen-bonded chains extend along the horizontal. (c) A packing drawing viewed in the direction of the molecular chains. Figures 1(b) and 1(c) are taken from ref. 20. Used by permission.

Figure 1 depicts the crystal structure of DKP as solved by Corey in 1938 [20]. Figure 1(b) illustrates the packing of DKP molecules in the crystal. Infinite chains of hydrogen-bonded molecules extend along the horizontal axis. Figure 1(c) illustrates the packing of DKP molecules in the crystal, viewed in the direction of the H-bonded chains. The orientation of the atoms shown in Fig. 1(a) is shown in one molecule of Fig. 1(b).

The hydrogen-bonded structure makes DKP a good model for the hydrogen bonding which takes place within the peptide backbone of a protein. The close packing of DKP molecules in the solid also brings into play van der Waals interactions between groups, which can be presumed to mimic those found in proteins. The influence of various side groups of proteins is not addressed by this simple compound and will be the focus of further work.

EXPERIMENTAL

The dissolution process for a solid going to an infinitely dilute solution can be separated into two steps: transfer of a molecule from the crystal to a saturated solution, and transfer of a molecule from the saturated solution to a solution of infinite dilution. The first process is referred to here as dissolution and the second as dilution.

In a dissolution experiment, a small volume of pure solvent is injected into a saturated solution of DKP which contains excess solid and the heat generated as the system returns to equilibrium is measured. By knowing the solubility of DKP (1.66% w/w at 25 °C [13]) and the injection volume, the

amount of DKP dissolved and hence the molar enthalpy of dissolution can be calculated *.

In a dilution experiment, a series of small-volume injections of saturated DKP solution containing no excess solid are made into initially pure solvent. Heats of dilution yield information about solute–solute interactions in solution.

All experiments were run in a titration microcalorimeter which has been previously described in detail [21]. Briefly, the reaction takes place within a small glass cell with an effective volume of 218 μl (as determined by acid–base titration as has been described [22]). Titrant is injected into the cell by means of a glass needle which also serves to stir the solution. Since the reaction cell is filled with solution at all times, an equal volume of solution is ejected from the cell. The heat generated or absorbed by the reaction is largely compensated for by adjusting the baseline current in a heater which is wrapped around the reaction cell, and this current is recorded every second. The uncompensated heat generates a voltage across a thermopile which is located between the reaction cell and an aluminum block, and this voltage is also recorded every second. The thermopile is twinned to a second thermopile beneath a reference cell. From the currents and the voltage across the thermopile, the total heat generated or absorbed each second is calculated. The sensitivity of this system for a given titration step is better than 4 μJ. The temperature of the calorimeter is maintained by a water bath to within 0.001° C utilizing a Tronac temperature control unit.

For the dissolution experiments the reaction cell was filled with saturated solution and excess solid and the injection syringe was filled with distilled–deionized water. The system was equilibrated under constant stirring until a steady baseline was obtained. The injection volume was 10 μl. The reaction at all temperatures studied required less than 5 minutes to return to a steady baseline (see Fig. 2).

For dilution experiments, the reaction cell was filled with water and the syringe was filled with saturated DKP solution which had been filtered to remove solids. A series of ten 10 μl injections were performed at 25, 30 and 35° C, and additional series of 15 and 20 μl injections were performed at 25° C. There was no indication of any precipitation from the filtered saturated solutions although they were exposed to room temperature in the course of making the calorimetric measurements.

* In principle, an alternative approach may be used without independent knowledge of the solubility (Gill and Noll, J. Phys. Chem., 76 (1972) 3065) by measuring heats of solution at several temperatures and fitting the van't Hoff equation using a solubility parameter chosen at a specific temperature.

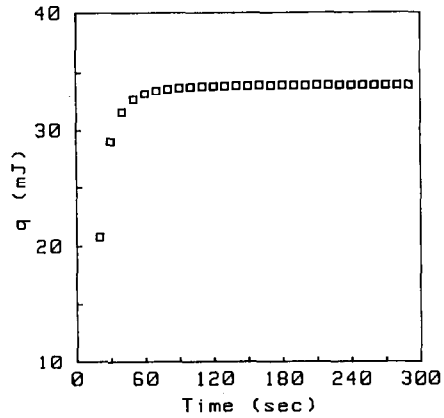

Fig. 2. The total heat absorbed upon the dissolution of DKP as a function of time. The injection volume is 10 μl and the temperature is 293.15 K. Note that equilibrium is rapidly reestablished and that the sensitivity of the instrument is more than adequate.

The concentration is related to the step number according to the following equation:

$$C_i = C_{\text{inj}}(1 - D^i) \tag{1}$$

in which C_i is the concentration in the reaction cell after step i, C_{inj} is the concentration of DKP in the injection syringe and D is a dilution factor equal to 1 minus the ratio of the injection volume v and the reaction cell volume V [23].

DKP was obtained from Sigma and recrystallized twice from water before use. The DKP solid was then ground using a mortar and pestle to ensure maximum surface area. Saturated DKP solution was prepared in distilled–deionized water by adding sufficient DKP solid so that a large excess of solid was visible. All solutions and distilled–deionized water were degassed with a water aspirator before use.

THEORY

We are primarily concerned with developing a relation for the heat of dissolution of a crystalline solid on forming a saturated solution. The saturated solution can consist of a set of complex species and thus exhibit non-ideal behavior.

Heat of dissolution

The non-ideality of a saturated DKP solution is evident from the heats of dilution. The structure of DKP suggests that hydrogen-bonded oligomers

can form. The dimeric species, however, is presumably the dominant oligomer [15] and for simplicity is regarded as the only significant species here. The equilibria that are relevant are

$$M(s) \xrightarrow{K_s} M \xrightarrow{K_d} \tfrac{1}{2}M_2 \tag{2}$$

where M is aqueous DKP monomer and M_2 is aqueous DKP dimer. K_s and K_d are equilibrium constants defined as

$$K_s = [M] \tag{3}$$

and

$$K_d = \frac{[M_2]^{1/2}}{[M]} \tag{4}$$

In both of these expressions the activity coefficients for the relevant species are assumed to be constant and are thus included in the defined constants.

The macroscopic partition function [24] is defined as follows

$$Q = [M] + [M_2] \tag{5}$$

or, in terms of the appropriate mass law relations,

$$Q = K_s + K_d^2 K_s^2 \tag{6}$$

Use of the solubility constant effectively represents a choice of the solid crystal phase as the reference state of the system. The total concentration of DKP in solution is given as:

$$[M]_t = [M] + 2[M_2] \tag{7}$$

or

$$[M]_t = K_s + 2K_d^2 K_s^2 \tag{8}$$

The relative apparent heat content per mole of DKP is related to the reciprocal temperature derivative by [24]

$$\overline{H} - \overline{H}^{\ominus} = -\frac{R}{[M]_t}\frac{\partial Q}{\partial(1/T)} \tag{9}$$

where $\overline{H}^{\ominus}$ represents the molar enthalpy of the crystal reference state and $\overline{H}$ represents the molar enthalpy of the dissolved material. R is the ideal gas constant and T is the temperature.

Application of this general equation to the specific problem at hand requires taking the appropriate temperature derivative of the equilibrium constants in eqn. (6). The van't Hoff equation, $\partial \ln K/\partial(1/T) = -\Delta H^{\ominus}/R$, applied to each equilibrium constant, K_s and K_d, then yields the result

$$\overline{H} - \overline{H}^{\ominus} = \frac{K_s \Delta H_s^{\ominus} + 2K_s^2 K_d^2 \Delta H_s^{\ominus} + 2K_s^2 K_d^2 \Delta H_d^{\ominus}}{K_s + 2K_s^2 K_d^2} \tag{10}$$

where $\Delta H_s^{\ominus}$ is the molar heat for the transfer of solid to aqueous monomer and $\Delta H_d^{\ominus}$ is the molar enthalpy for dimer formation at temperature T.

Experimentally, the heat evolved upon the injection of a volume v of solvent into a saturated solution is given by the product of the number of moles of solid dissolved in that volume ($v[M]_t$) and the enthalpy change per mole, as given by eqn. (10), yielding

$$q = vK_s\left[\Delta H_s^{\ominus} + 2K_sK_d^2\left(\Delta H_s^{\ominus} + \Delta H_d^{\ominus}\right)\right] \tag{11}$$

The temperature dependence of the enthalpy of dissolution $\Delta H_s^{\ominus}$ will be assumed to vary linearly with a slope of $\Delta C_{p,s}^{\ominus}$ for the dissolution process, i.e.

$$\Delta H_s^{\ominus}(T) = \Delta H_s^{\ominus}(T_0) + \Delta C_{p,s}^{\ominus}(T - T_0) \tag{12}$$

where T_0 is an arbitrary reference temperature, here 298.15 K.

The solubility constant K_s varies with temperature according to

$$K_s(T) = K_s(T_0)\exp\left[-\frac{\Delta H_s^{\ominus}(T_0)}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right] \times \exp\left[\frac{\Delta C_{p,s}^{\ominus}}{R}\left(\frac{T_0}{T} - 1\right)\right]\left(\frac{T}{T_0}\right)^{-R\Delta C_{p,s}^{\ominus}} \tag{13}$$

For simplicity we shall assume that the heat capacity change associated with solution dimerization is zero, so that the dimerization constant K_d varies with temperature as

$$K_d(T) = K_d(T_0)\exp\left[-\frac{\Delta H_d^{\ominus}}{R}\left(\frac{1}{T} - \frac{1}{T_0}\right)\right] \tag{14}$$

Heats of dilution

The heat of dilution of a solution provides information useful in the detailed analysis of the crystal dissolution process. In terms of simple dimer formation, the heat of dilution has been described in detail elsewhere [25] and will only be presented here briefly.

The experimental heat q for a given titration step i is given by

$$q = \overline{H}_i C_i V - \overline{H}_{i-1} C_{i-1}(V - v) - \overline{H}_{inj} C_{inj} v \tag{15}$$

This relation describes the difference between the enthalpy content of the state i and of the previous state, $i - 1$, of the system.

The macroscopic partition function with reference to the monomer is

$$Q = [M] + K_d^2[M]^2 \tag{16}$$

since, in the absence of solid, [M] is not given by K_s. Then

$$[M]_t = [M] + 2K_d^2[M]^2 \tag{17}$$

where $[M]_t$ is equivalent to C_i at any step.

The partial derivative of the partition function with respect to $1/T$ yields

$$\overline{H} - \overline{H}^{\ominus} = \frac{\Delta H_d^{\ominus} K_d^2 [M]^2}{[M]_t} \tag{18}$$

From eqn. (17), the concentration of the monomer can be expressed as

$$[M] = \frac{-1 + (1 + 8K_d^2[M]_t)^{1/2}}{4K_d^2} \tag{19}$$

Substitution of eqn. (19) into eqn. (18) gives the molar enthalpy at any step. These values are then substituted into eqn. (15) to give the heat at any step in the titration.

RESULTS

The average heat absorption for dissolution runs at 5 different temperatures is shown in Table 1. Six data points were taken at each temperature. These data are also plotted in Fig. 3(a).

The results of dilution runs at 3 temperatures are shown in Fig. 3(b)–(d).

The solid curves shown were generated by simultaneously fitting all the data using the relationships developed above. The best-fit values for the parameters at 25 °C are as follows: $\Delta H_s^{\ominus} = 28.0 \pm 0.2$ kJ mol^{-1}, $\Delta C_{p,s}^{\ominus} = -57 \pm 21$ J mol^{-1} K^{-1}, $K_d = 0.19 \pm 0.08$ M$^{-1/2}$ and $\Delta H_d^{\ominus} = -29 \pm 7$ kJ mol^{-1}. Errors reported are the 67% confidence intervals established by

TABLE 1

The heat absorbed upon the dissolution of DKP in the temperature range 293 to 313 K (the injection volume is 10 μl)

Temperature (K)	q (mJ)	Average q (mJ)
293.15	33.8 34.0 34.1 33.8 34.0 34.0	34.0 ± 0.1
298.15	40.3 40.1 41.1 41.0 40.9 40.7	40.7 ± 0.4
303.15	48.5 48.6 48.3 48.2 49.2 49.4	48.7 ± 0.5
308.15	56.3 57.3 56.5 56.8 57.0 57.0	56.8 ± 0.4
313.15	68.0 67.9 68.0 68.0 67.6 67.3	67.8 ± 0.3

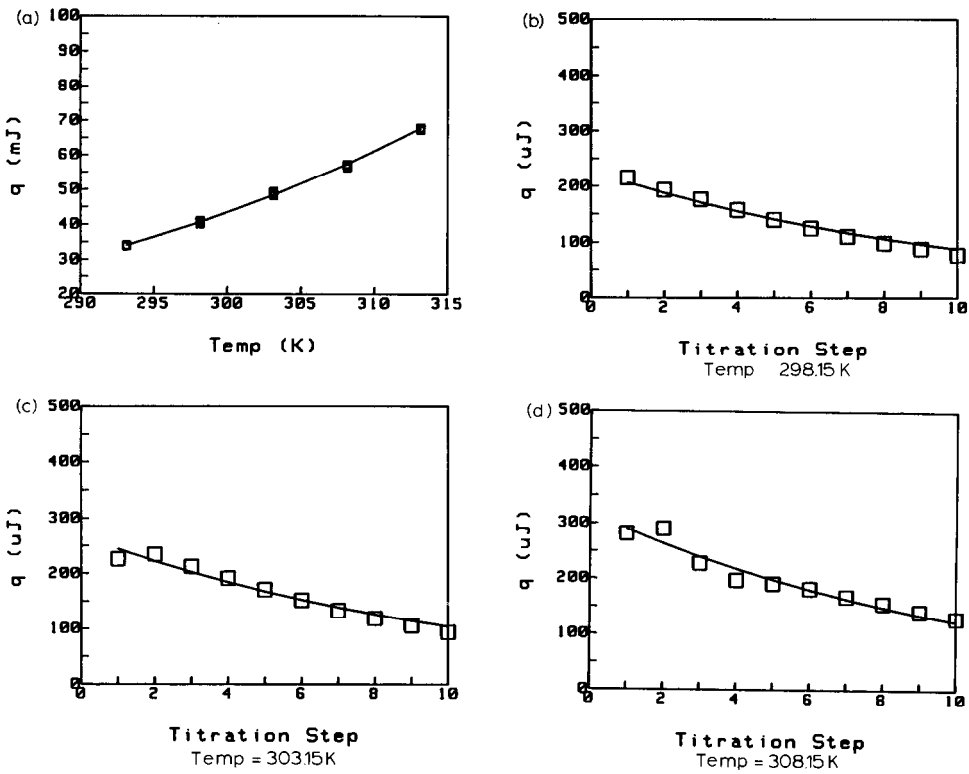

Fig. 3. (a) The heat absorbed q for dissolution of DKP as a function of temperature. The injection volume is 10 μl. Data are from Table 1. (b)–(d) The stepwise heat absorbed for a series of ten 10 μl injections of saturated DKP into initially pure water at the indicated temperatures. The concentration of DKP is a function of the step number as described by eqn. (1) in the text. The solid curves were generated by simultaneously fitting all four data sets using a non-linear least-squares fitting procedure according to the model described in the text.

F-testing of the fit. A value of $[M]_t = 0.145$ M at 25 °C as given in ref. 13 was utilized in this fitting procedure.

DISCUSSION

Proteins are complex systems, and it has proven difficult to unravel the relative contributions of the various interactions which determined protein structure in the presence of water.

For the process of denaturing proteins thermally, it has been argued that the large heat capacity change that occurs is due to the exposure of hydrophobic groups [26]. The enthalpy change of denaturation is therefore strongly temperature dependent. The temperature of maximum stability of

hydrophobic interaction between non-polar groups is found to be approximately 110 °C [26]. At this temperature the stability of hydrophobic group interaction is essentially enthalpic. The temperature of 110 °C therefore serves as a reference temperature where the heats of thermal denaturation of proteins may appropriately be compared with the heats of dissolution of small model compounds.

In order to determine the contribution of an "average" residue to the denaturational enthalpy of a protein, the enthalpy per residue must be divided by the fraction of the chain surface in the folded state which is inaccessible to solvent relative to an extended chain, since surfaces which are already in contact with solvent are assumed not to contribute to the heat of denaturation. Lee and Richards [17] have estimated the fraction of surface area which is accessible to solvent for ribonuclease-S (RNase-S), lysozyme and myoglobin as 0.35, 0.32 and 0.30 respectively. The inaccessible fraction is 1 minus this value. We assume that the fraction of inaccessible area is equal to the fraction of inaccessible residues. Assuming a constant ΔC_p the heat of denaturation of ribonuclease-A (RNase-A) at 110 °C is 6.07 kJ mol^{-1} residues, while those for lysozyme and myoglobin are 6.23 and 6.36 kJ mol^{-1} residues respectively [27]. Normalization of these values to the fraction of inaccessible residues yields 9.33, 9.16 and 9.08 kJ mol^{-1} "buried" residue for the three proteins respectively, assuming that the fraction of inaccessible surface area is the same for RNase-S and RNase-A.

The enthalpy of dissolution of DKP can be normalized similarly by noting that DKP is the cyclic anhydride of glycine and thus "equivalent" to two glycyl residues. This yields, at 110 °C, a value of 11.6 kJ mol^{-1} glycyl residue, a result quite similar to the values calculated for the three proteins.

Alternatively, the enthalpies of protein denaturation could be normalized to the change in the accessible surface area of the protein upon denaturation. This might more accurately reflect the energetics of contacts within the protein core. The accessible surface area of denatured RNase-S, represented as the extended chain, is 20 028 $Å^2$, while that of the folded molecule is 7010 $Å^2$, resulting in a change of 130 018 $Å^2$ [17]. This gives an area-scaled denaturational enthalpy of 57.7 J mol^{-1} $Å^{-2}$. The values obtained for lysozyme and myoglobin are found to be 56.5 and 51.9 J mol^{-1} $Å^{-2}$ respectively.

The water-accessible surface area of DKP is 247 $Å^2$ (calculation courtesy of Dr. B.K. Lee), and the area-scaled enthalpy of dissolution is thus 93.8 J mol^{-1} $Å^{-2}$. This value is again comparable with the values calculated for the three proteins. A higher value for DKP is not inconsistent with ones expectation of a higher density of interactions within the highly regular crystal lattice of the small-molecule compound. Furthermore, in view of the multiplicity of residues in the protein core, this order of magnitude agreement is as much as one could expect based on the assumption that the energetics of the crystal and the protein core are similar.

The rough quantitative agreement of this comparison of the energetics of crystal dissolution and protein denaturation is highly suggestive that this model compound contains the key elements of hydrogen bonding and van der Waals contacts which describe average enthalpies of interaction of amino acid residues in a folded polypeptide chain.

The procedure developed in this study can now be extended to the examination of a variety of solid small peptides with different side groups in order to provide quantitative information on the contributory role of various interactions that determine protein conformation.

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