A Simple Experimental Model for Hydrophobic Interactions in Proteins*

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The compound N-cyclohexyl-2-pyrrolidone contains a substantial apolar region as well as a peptide bond-like moiety. This solvent, therefore, provides a useful model for protein interiors. Under certain conditions of temperature and salt concentration, cyclohexylpyrrolidone forms a two-phase system with water. This permits partition coefficients and subsequent free energies of transfer of amino acid side chains from cyclohexylpyrrolidone to water to be simply determined. Free energies of transfer measured in this manner for 21 amino acids are found to be substantially less than those obtained from the commonly used ethanol/water solubility model. This suggests less of a contribution of hydrophobic interactions to the stabilization of protein structure than is conventionally assumed.

A role for apolar (hydrophobic) interactions in the formation and stabilization of protein structure is generally well accepted (1, 2). The relative contributions of hydrophobic interactions compared to other types of weak interactions such as direct electrostatic effects, however, remain unclear (3-5). This uncertainty arises at least partially from the lack of a reliable quantitative measure of apolar interactions within native proteins. Currently, the most widely employed experimental model is that developed by Nozaki and Tanford (6) in which the free energy of transfer (ΔG) of amino acid side chains from ethanol to water (as estimated from solubility measurements in each solvent) is taken as a measure of the contribution of each side chain to the total hydrophobic effect. This system provides a very useful "hydrophobicity scale" for apolar amino acid side chains, but the choice of ethanol or similar oxygen-containing organic solvents (e.g., dioxane) as a model for protein interiors and the unavailability of activity coefficients for most of the relevant solubility data limit its applicability (7). In this work, we describe a method supplementary to the solubility approach which employs a cyclohexylpyrrolidone-water two-phase system to estimate the relative tendencies of amino acid side chains to be found in protein interiors.

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MATERIALS AND METHODS

N-Cyclohexyl-2-pyrrolidone (99% minimum purity, area per cent GC) was obtained from GAF Corp. and further purified by multiple passages over a column of mixed bed ion exchange resin (Amberlite MB-1) and subsequent dehydration with anhydrous calcium sulfate. Sets of L-amino acids were purchased from United States Biochemical Corp. (kit 20L), Pierce (kit 22L), and Sigma (kit L7829) and were used without further purification. Arginine, lysine, histidine, and cysteine were routinely employed in their hydrochloride forms. Tritiated amino acids were obtained from New England Nuclear. For partitioning experiments, 1.0 ml of CHP was combined with 0.3 ml of an aqueous 2 M NaCl solution containing 1 mM unlabeled amino acid and 10 μl of tritiated amino acid (10-100 Ci/mmol). Phase systems were thoroughly shaken and then incubated for 15 h at 50°C. To determine partition coefficients, 10 μl of each phase was pipetted into separate vials containing 10 ml of scintillation fluid (15.2 g of 2,5-diphenyloxazole (PPO) + 1.14 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPPO) + 1 gallon of toluene). To compensate for potential quenching differences between the aqueous and organic phase containing counting mixtures, 10 μl of the opposite phase from an identical two-phase system lacking only labeled amino acid was added to the corresponding vial. The resultant mixtures were incubated overnight prior to counting. Partition coefficients (P) are defined as the ratio of the radioactivity in the bottom (aqueous) to the top (CHP) phase. Values given in the text reflect five determinations of amino acids from each of the three sources indicated above and are reported as plus or minus standard error of the mean. Relative solubilities of amino acids in CHP and CHP containing 5% water were measured by saturating both solvent systems with the same amount of amino acid (5-20 mg/ml) and an identical trace amount of the radiolabeled amino acid as described above. The 5% H2O/CHP solvent was obtained directly from the organic phase of a CHP/H2O two-phase system lacking amino acids as prepared above. Incubation was performed for 48 h and equilibrium was observed to be established. Free energies of transfer of amino acid side chains from CHP to CHP containing 5% H2O were calculated as (−RT ln SCHP⁻/SCHP⁺) − (∓RT ln SCHP⁻/SH₂O⁻) for CH₃COOH, and SCHP⁺ and SCHP⁻ are the amount of radioactivity of the subject amino acid found in the supernatant in the indicated solvent after centrifugation to remove suspended solid and SCHP⁻ and SCHP⁺ are the corresponding values for glycine. No attempt to correct for activity coefficients was made, although such corrections should be small since amino acids were only sparingly soluble in CHP and CHP containing 5% H2O. The concentration of water in the CHP phase was measured by H₂O partitioning, the NaCl content of the CHP phase by ⁴⁰Cl partitioning and the amount of CHP in the aqueous phase by carboxyl ultraviolet absorbance.

RESULTS AND DISCUSSION

The compound N-cyclohexyl-2-pyrrolidone (Fig. 1) provides a particularly useful model for the interior of typical proteins.
globular proteins for several reasons. By containing both a substantial apolar portion as well as a peptide bond-like moiety, liquid CHP provides a good approximation to the general electrostatic potential found within protein matrices. Furthermore, many of its physical properties at 25 °C (8) such as dielectric constant (~7), viscosity (11.5 cP), surface tension (42.3 dyne/cm), heat of vaporization (12.9 kcal/mol), and partial specific volume (0.97 cm³/g) are more representative of highly packed, cohesive protein interiors than the corresponding properties of organic compounds like ethanol (24.1, 1.1 cP, 21.9 dyne/cm, 9.2 kcal/mol, 1.25 cm³/g, respectively). The major weaknesses of this model are decreased hydrogen bonding ability from the lack of a free NH group and decreased resonance stabilization as a consequence of the cyclohexane group on the nitrogen. In general, however, the properties of CHP are probably as close to optimal for modeling protein interiors as will be encountered in a compound with the convenient ability to form an aqueous/organic two-phase system. Below a critical temperature (~50 °C), CHP displays miscibility with water. Above this temperature, an equilibrium two-phase system is slowly formed. This phase separation is enhanced by the presence of salts which lower the phase transition temperature (8). The tendency of an amino acid side chain to be found in a protein's interior can, therefore, be characterized simply by determination of the amino acid's partition coefficient with free energies of transfer calculated as \( \Delta G^T = -RT \ln P \) where \( P \) is the experimentally observed partition coefficient. In order that just the effect of the side chain be included, the value of \( \Delta G^T \) for glycine (\( \Delta G^T_{\text{Gly}} \)) is subtracted from \( \Delta G^T \) for each amino acid of side chain \( R \), i.e. \( \Delta G^T_R = \Delta G^T - \Delta G^T_{\text{Gly}} \), as originally described by Tanford (2). The use of a CHP/H₂O two-phase system in this manner permits very low concentrations of amino acids to be employed and ideality to be approached (activity coefficients ~1). Such a two-phase system also eliminates any need to refer to a solid phase which could differ in the two solvents used in conventional solubility experiments, a potential source of undetermined errors in \( \Delta G^T \) values in the ethanol/water model (6).

Salt concentration (2 M) and CHP concentration (4.7 M) were selected to provide the following system characteristics. The CHP upper phase contained 5 ± 1% H₂O and less than 0.01% NaCl, while the lower aqueous phase had less than 1% CHP. The presence of 5% H₂O in the organic phase (i.e. 56 water molecules per 20,000 daltons of protein) was selected to reflect estimates of water in protein interiors as a consequence of both the dynamic nature of protein structure (as, for example, demonstrated by hydrogen-exchange measurements (9)) and the occasional presence of water molecules inside proteins as seen by x-ray crystallography (10). Experiments were performed at an amino acid concentration of 1.00 mM where partition coefficients were observed to be independent of concentration providing experimental justification for the assumption of unity activity coefficients. Determination of partition coefficients by fluorescence detection with fluorescamine (11, 12) or directly by ultraviolet absorbance in the case of aromatic amino acids indicated a negligible effect of isotopic labeling upon the observed values of partition coefficients.

Table 1 reports the free energy of transfer from CHP to water (2 M NaCl) of the most commonly encountered amino acid side chains. Also included for comparison purposes are the corresponding values for the free energy of transfer from ethanol to water as summarized by Jones (13). A strong correlation (correlation coefficient, \( r = 0.79 \)) is observed between the two sets of data. The free energies of transfer from CHP to water also show a weaker correlation with a variety of other amino acid parameters such as observed accessibility in proteins (14) \( (r = 0.35, \text{"polarity" } (r = 0.47, \text{ refractivity } (16) \ (r = 0.24), \text{ and "bulk hydrophobic character" as estimated from the ethanol/water } \Delta G, \text{values of the amino acid side chains that surround a given type of side chain observed in 21 proteins of known tertiary structure } (r = 0.47) \) (17). Further weak correlations are found between the CHP values and octanol/water partition coefficients \( (r = 0.58) \) (18) and the hydrophobicity of amino acids as calculated from their hydrophobic fragmental constants \( (r = 0.54) \) (19). In general, it appears that the more polar side chains deviate most strongly from these simple correlations, most probably reflecting unspecified polar interactions of these side chains with the CHP phase.

Although a marked correlation exists between the ethanol/water and CHP/water transfer values, the results obtained from the two-phase system are on the average 1.2 ± 0.2 kcal/mol less than those obtained from the ethanol/water solubility measurements. The presence of a hydrogen atom on the CHP nitrogen to improve the model would be expected to further increase the differences in the two sets of values. Several possible explanations may account for these differences including (a) the difference in temperature between the two experiments, (b) the assumption of solution ideality in the solubility results, and (c) the nature of the organic phase intended to model protein interiors. The effect of (a) is found

### Table 1

| Amino acid | \( \Delta G^T_{\text{CHP} \rightarrow H_2O} \) kcal/mol | \( \Delta G^T_{\text{OH} \rightarrow H_2O} \) kcal/mol |
|-----------|----------------------------------|----------------------------------|
| Alanine   | -0.48 ± 0.06                      | 0.87                             |
| Arginine  | -0.06 ± 0.08                      | 0.85                             |
| Aspartic acid | -0.75 ± 0.09                    | 0.66                             |
| Asparagine | -0.87 ± 0.06                      | 0.09                             |
| Cysteine  | -0.71 ± 0.07                      | 1.52                             |
| Glutamic acid | -0.71 ± 0.07                    | 0.67                             |
| Glutamine | -0.32 ± 0.06                      | 0.90                             |
| Glycine   | 0                                | 0.10                             |
| Histidine | -0.51 ± 0.06                      | 0.87                             |
| Hydroxyproline | 0.24 ± 0.07                | NA*                             |
| Leucine   | 1.02 ± 0.09                      | 2.17                             |
| Isoleucine | 0.81 ± 0.07                      | 3.15                             |
| Lysine    | -0.09 ± 0.06                      | 1.64                             |
| Methionine | 0.81 ± 0.06                      | 1.67                             |
| Phenylalanine | 1.03 ± 0.06                 | 2.87                             |
| Proline   | 2.03 ± 0.06                      | 2.77                             |
| Serine    | 0.05 ± 0.04                      | 0.07                             |
| Threonine | -0.35 ± 0.07                      | 0.07                             |
| Tryptophan | 0.66 ± 0.06                      | 3.77                             |
| Tyrosine  | 1.24 ± 0.08                      | 2.67                             |
| Valine    | 0.56 ± 0.08                      | 1.87                             |

*NA, not available.

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Fig. 1. \( \text{N-Cyclohexyl-2-pyrrolidone} \)
to be small, while (b) is difficult to evaluate but potentially quite significant for very soluble amino acids (20). It is explanation (c), however, that probably accounts for most of this difference. Polar amino acids are potentially expected to undergo a variety of weak dipole-dipole and charge-dipole type interactions with the polar portion of the CHP molecule that would effectively decrease the apparent free energy of transfer from CHP to water. The presence of the 2 M NaCl in the aqueous phase used to enhance phase separation was observed to have only a small effect on partition coefficient values probably reflecting the low salt concentration at which hydration effects are complete.

The presence of 5% H₂O in the CHP phase could also be at least partially responsible for the differences between the CHP and ethanol-free energy transfer values. To test this possibility, the solubility of the individual amino acids was compared in pure CHP and CHP containing 5% H₂O. The free energies of transfer of amino acid side chains from CHP to CHP/5% H₂O were found to be -0.1 ± 0.1 kcal/mol for polar amino acid side chains and -0.5 ± 0.2 kcal/mol for apolar side chains (results not illustrated). Thus, the presence of water in the CHP phase has little effect on the free energy of transfer from CHP to water of polar amino acids, but it slightly increases the interaction of apolar side chains with the CHP phase. This result, which at first may seem a bit surprising, probably reflects a decrease in the polarity of the CHP phase as a consequence of the water present. Presumably, the approximately 1 water per 2 CHP molecules in the organic phase are hydrogen-bonded in an undetermined manner (the CHP carboxyl oxygen probably acts as a hydrogen bond acceptor with water) to CHP. This simple idea is supported by both the high affinity of water for CHP and small shifts in the infrared absorption bands of water dissolved in CHP. Most importantly, the differences between the values of the free energy of transfer of amino acid side chains from CHP and ethanol to water cannot be accounted for by the presence of CHP-associated water since removal of water from the organic phase should further increase the differences between the two sets of data.

Finally, the possible effect of CHP on the state of ionization of acidic and basic side chains should also be mentioned. The pH of the aqueous phase (6.3) ensures that ionizable side chains (with the exception of histidine) are primarily in their charged forms. The state of ionization of apolar side chains with the CHP phase is more difficult to ascertain. It is generally found that the presence of organic solvents weakens acids and bases with pK shifts as large as 0.5-1.5 occasionally encountered. Shifts of this magnitude should not substantially alter free energy transfer values. In support of this conclusion, variation of the aqueous phase pH by ±1 pH unit changes the ΔG values by less than 10%, a difference generally within the experimental error of the determinations. Furthermore, since the effect of CHP and ethanol on any contribution of ionization is probably similar, the difference between the CHP and ethanol free energy transfer values remains. Since the structure of CHP appears to more accurately represent the inside of a protein as outlined above, we suggest that the values obtained from the two-phase system more truly represent the actual free energies involved in transferring a side chain from an aqueous environment to a typical protein interior. In this regard, these results better account for the marginal stability of proteins in aqueous solutions (12 ± 5 kcal/mol) (21) and argue for less of a role for hydrophobic interactions in protein stabilization than is commonly assumed. This is consistent with recent suggestions (5, 22-24) of a more substantial contribution by electrostatic effects to the maintenance of protein structure than is generally realized.

The CHP/water two-phase system should provide a simple, relevant experimental system to further characterize the role of hydrophobic interactions in proteins. For example, we have found that between 30 and 60 °C (2 M NaCl), the partition coefficient of H₂O decreases by only 5%, suggesting that variation in partitioning temperature can be used to obtain estimates of both the standard enthalpy and entropy of transfer. If data of sufficient precision can be obtained, the constant pressure heat capacity of transfer may also be obtainable. Furthermore, since only low concentrations of partitioned solute are necessary, it should be possible to systematically vary the structure of defined peptides and determine ΔG values of these larger entities as well as examine the effect of point substitutions as a model for the thermodynamics of movement of portions of polypeptide chains into protein interiors.

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