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Review

Evaluation of methods for measuring amino acid hydrophobicities and interactions

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Abstract

The concept of hydrophobicity has been addressed by researchers in all aspects of science, particularly in the fields of biology and chemistry. Over the past several decades, the study of the hydrophobicity of biomolecules, particularly amino acids has resulted in the development of a variety of hydrophobicity scales. In this review, we discuss the various methods of measuring amino acid hydrophobicity and provide explanations for the wide range of rankings that exist among these published scales. A discussion of the literature on amino acid interactions is also presented. Only a surprisingly small number of papers exist in this rather important area of research; measuring pairwise amino acid interactions will aid in understanding structural aspects of proteins.

Keywords: Reviews; Hydrophobicity; Amino acids

Contents

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1. The significance

A problem that continues to evade researchers is a complete understanding of how proteins fold into their native state. The importance of this problem lies in the interactions of the individual amino acids that make up the tertiary structure. Four types of interactions are involved; hydrophobic, electrostatic, hydrogen bonding and van der Waals interactions with hydrophobic interactions believed to be the most dominant. Other interactions including amino–aromatic [1] and aromatic–aromatic [2] are believed to play a role as well in stabilizing protein structure. Kauzmann first stated that the hydrophobic interactions are believed to be the most significant [3], followed by Tanford in 1962 [4] and in the early 1990s reaffirmed by Dill [5]. The hydrophobic character of a particular amino acid is a significant property for understanding the structure of a protein because it gives insight into how a protein is going to fold into its native state. The topic has been discussed in the literature extensively and will be presented briefly in the next section.

2. Hydrophobicity and the hydrophobic effect

The concept of hydrophobicity has been examined carefully for many decades. Reasons for such interest are that it allows a better understanding of how amino acids interact within proteins as well as providing a way to predict structural properties of proteins (e.g., the ability of a protein to form an α-helix and recognizing β-strands on a protein). Dill has extensively addressed this topic [6] and recently provided an excellent review with emphasis on the partitioning of nonpolar solutes into water as well as the relevance of water structure in interpreting this concept [7]. Hydrophobicity is most commonly measured by the partitioning of a nonpolar solute between an oil phase and a water phase. As the solute is excluded from the water phase, an ordering of water molecules occurs around the cavity produced by the nonpolar solute. Hence, a decrease in entropy occurs during this process. Another thermodynamic phenomenon that occurs is a large increase in heat capacity. These two characteristic features define what has been termed the hydrophobic effect [8], which occurs at ambient temperature. Fig. 1 illustrates this phenomenon for the solute benzene from both a physical chemistry viewpoint [9] and chromatographically based upon van't Hoff analysis [10]. At 25 °C, a minimum in solubility is seen in the left plot and on the right van't Hoff plot, a maximum in retention is observed at the same temperature. Honig et al. have stated, in agreement with other

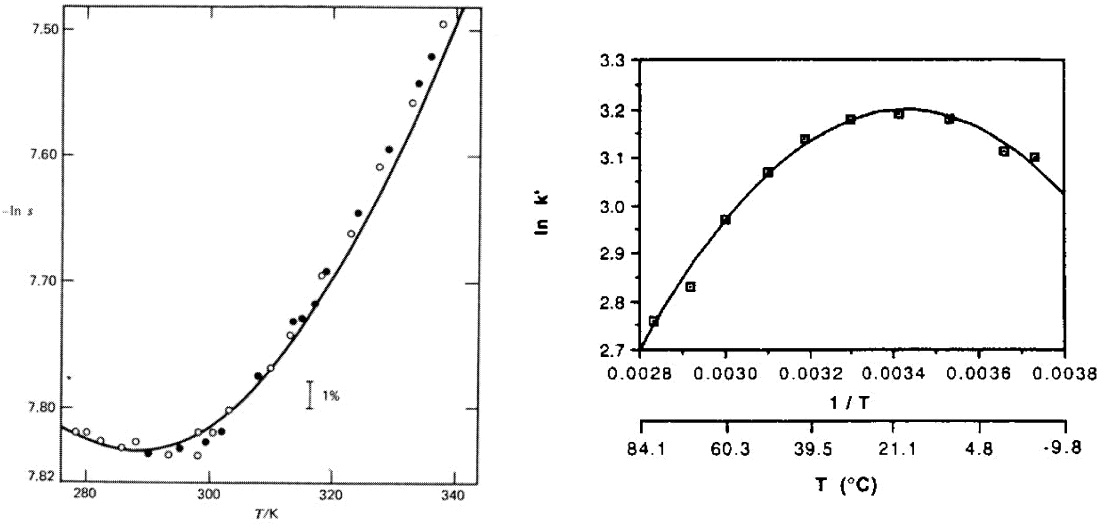

Fig. 1. Graphical representation of the hydrophobic effect for the molecule benzene. The left figure illustrates the minimum in solubility at 25 °C, while the right figure shows the maximum in retention of benzene at nearly the same temperature using liquid chromatography. Obtained with permission from Refs. [9] and [10].

researchers, that the hydrophobic effect is the major driving force in the protein folding process providing maximum stability for the protein [11].The following sections will address the different methods utilized to measure amino acid hydrophobicity.

3. Types of amino acid hydrophobicity scales

Kauzmann's suggestion that hydrophobic interactions are dominant in the protein folding process led to the development of these hydrophobicity scales. Reviews of this topic have been presented in order to provide clarity as well as track the various scales proposed [12–17]. In order to discuss the various scales, we will divide them based on the choice of method used to obtain the scale. Generally, two types of solutes are studied, amino acids, their side chain analogues or derivatives of the amino acids and secondly proteins and peptides. The majority of the scales are based on the latter. Amino acid derivatives are the next most popular because they better represent these molecules in a protein, normally derivatized at the amino terminus. The methods will be divided into five different categories: partitioning (particularly liquid–liquid); RPLC and chromatographic techniques; accessible surface area calculations; site-directed mutagenesis; and physical property measurements.

4. Partitioning methods

Partitioning between two immiscible liquid phases is the most common method of measuring hydrophobicity. A vast majority of these scales involve the use of different organic solvents. The solvents used for partitioning studies attempt to mimic the interior of the protein. A serious problem with many organic solvents though is that there is a slight miscibility with water, changing the character of both phases and making it difficult to obtain pure hydrophobicity values. The first major scale, developed by Nozaki and Tanford [18], used ethanol and dioxane as the organic solvents to model the protein interior, and proposed a hydrophobicity scale for nine amino acids. The solubility of each of these amino acids was initially determined in both organic solvents ranging from 0 to 100% organic solvent and from this measurement, the free energy of transfer of each amino acid was calculated. Their work remains as one of the most cited in the literature pertaining to establishment of hydrophobicity scales. Yunger and Cramer used octanol as an organic solvent to model the protein interior. Radiolabeled amino acids, which allow smaller values to be measured, were used to measure the free energy of transfer of 12 different amino acids [19]. Problems lie with impurities in radiolabeled amino acids, which can lead to large errors in the values that are obtained. Fauchere and Pliska [20], using *N*-acetyl-amino acid amides and octanol–water partitioning were one of the first to use both a complete set of amino acids and derivatized amino acids. The effects of pH were also studied to understand the partitioning of two derivatized amino acids, histidine and tyrosine. *N*-cyclohexyl-2-pyrrolidone was used by Lawson et al. [21], who measured the free energy of transfer of the 20 amino acids from the aqueous phase. They argued that this organic solvent is similar to the protein interior in general electrostatic potential as well as polar and apolar physical properties, including dielectric constant, viscosity, surface tension, heat of vaporization, and partial specific volume. *N*-Methylacetamide has also been used to mimic the interior of a protein [22]. This solvent has a higher dielectric constant and dipole moment compared to most organic solvents as well as both apolar regions and hydrogen bonding ability.

Partitioning methods have also been demonstrated using non-liquid phases such as vapor phases and micellar phases. Using micellar phases, two scales have been developed. Fendler et al. [23] used sodium dodecyl sulfate (SDS) micelles and measured the partitioning of 14 radiolabeled amino acids. Strong electrostatic interactions were believed to contribute to the measured partition coefficients. The second scale, developed by Leodidis and Hatton [24], was based on the free energy of transfer of the 20 common amino acids to AOT–isooctane microemulsions. They found the major factors driving partitioning to be solute–water and solute–interface interactions as well as the hydrophobic effect. AOT micelles were found to be more hydrophobic than octanol and were proposed as membrane mimetic. However, due to the hydrophobicity of the micelles, partition coefficients for the polar and charged amino acids were unable to be determined.

The utility of vapor phases was developed by Wolfenden et al. as a means to measure amino acid side chain affinity for water [25]. Theoretically, vapor phases form the simplest nonpolar solvent since the solute has no interactions with it [26]. Wolfenden's study measured the ''hydration potential'' and correlated this to the appearance of amino acids on the surface of proteins. Correlations with amino acids within the protein interior may be of more value, since these amino acids are more evolutionarily conserved. Contributions from hydrogen bonding and dispersive forces result in poor correlations with other scales. Also, the amphiphilic amino acids favor the aqueous phase due to the lack of competition for broken hydrogen bonds [27]. A second scale used these values along with aqueous–cyclohexane partitioning values of amino acid side chains [28]. From this, a cyclohexane–vapor partitioning scale was extrapolated. This scale provided a means for measuring the attraction by dispersion forces uncomplicated by hydrogen bonding. Cyclohexane has been shown to be an excellent choice as a model solvent for the protein interior because only van der Waals interactions exist with this solvent and the analyte [26]. Sharp et al. revised the hydrophobicity scales obtained from Fauchere and Pliska, as well as both scales from Wolfenden et al. by applying ideal gas equations and experimental molar volumes, to adjust for changes in volume entropy, resulting in a new derived scale.

Two partitioning scales used the hydrophobic fragmental constant method developed by Rekker [29]. Abraham and Leo [30] provided the first calculated amino acid hydrophobicities using the solute values of Hansch and Leo [31]. Some values were adjusted due to the zwitterionic nature of amino acids, including propagation of charge. In comparison with other scales, there was generally good agreement except for the residue proline. This disagreement was probably due to structurally dissimilar side chain and fragment comparisons. Black and Mould [32] developed another scale using a modification of Rekker's approach to measure amino acid hydrophobicities. Due to the wide-ranging polarities of amino acids, they used the smallest possible fragments rather than the typical fragment for their estimation.

Another novel partitioning scale was determined using an aqueous and polymer phase [33], with the polymer consisting of 12.5% Ficoll and 10.8% dextran. Twenty dinitrophenyl derivatized amino acids were used and the partition coefficients (K) were found to be a linear function of the equation:

$$\ln K = A + BI$$

where A is the relative hydrophobicity of each amino acid, and B and I are based on the ionic strength of the aqueous phase.

Problems with partitioning methods lie primarily in the inability to mimic the protein interior, which itself is difficult to assess [22,34]. The use of free amino acids is further complicated by the role of self solvation. Additionally, hydrogen bonds that are lost in the transfer to organic solvents are not reformed in organic solvents, but often are in the interior of the protein [35]. Liquid–vapor partition methods also have several disadvantages, including the incorporation of dispersion forces and disfavoring of the vapor phase by amphiphilic amino acids due to the lack of compensation for broken hydrogen bonds [27]. The relevance of liquid–vapor measurements to biochemical processes has also been questioned [36]. Liquid–micelle partitioning may not accurately represent hydrophobicity values due to other interactions. Partitioning methods question the choice of the oil or non-aqueous phase for whether or not that affects the free energy value obtained. Karplus [37] has addressed the relevance of partitioning values by comparing four commonly used hydrophobicity scales, illustrated in Fig. 2. The scales illustrate a wide variability in magnitude and sign for the amino acids containing polar side chains, while nonpolar amino acids do not show great deviation from scale-to-scale. If this is the case, Karplus argues the concept of hydrophobicity is then misused because researchers that are designing these new hydrophobicity scales are equating the free energy of transfer of the amino acid with hydrophobicity for all amino acids including the polar amino acids. As stated by Nozaki and Tanford [18], for solvent transfer energetics to be equated with hydrophobicity, two criteria must be met, namely the transfer values ($\Delta G^{\circ}_{\text{transfer}}$) must favor the oil phase and they must be largely independent of the oil phase used. Scales which include both nonpolar and polar amino acids can be termed ''hydrophobicity plus'' scales, which can still

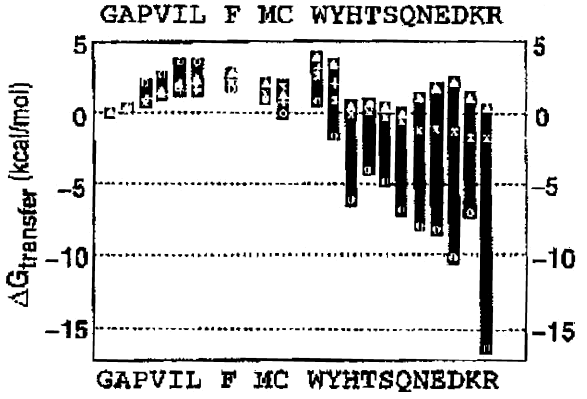

Fig. 2. Comparison of four commonly used oil partitioning scales for measure hydrophobicity for the 20 common amino acids. The one-letter abbreviations for each amino acid are provided. The four oil–water phases used were ethanol–dioxane (+), *N*-methylacetamide (△), octanol–water (*), water–cyclohexane (○). Obtained with permission from Ref. [37].

be beneficial but should be examined carefully. Obtaining meaningful free energy measurements from oil–water partitioning is difficult because of complexities of the oil phase [38,39].

5. Accessible surface area methods

With the advent of molecular modeling as well as increased libraries of protein structures, methods have been developed to estimate amino acid hydrophobicity based upon the degree of amino acid exposure to the solvent. These methods have almost exclusively been used for the prediction of protein folding. Typically, computer programs are used to ''roll'' a solvent molecule along the protein and determine the static accessibility of the individual amino acids to the solvent molecule.

Two main advantages exist with using accessible surface area measurements. First, unlike previous methods, this method does not involve modeling of solutes or the protein interior. Measurements are made on actual proteins and correlations are drawn between degree of exposure and hydrophobicity. Second, secondary as well as tertiary and even quaternary structures can be probed with this method.

One of the first scales based on this method of accessible surface area measurements was proposed by Lee and Richards [40]. They used the ''rolling'' solvent method and deemed any amino acid accessible if the solvent came within van der Waals contact. Synthetic peptides, Ala–X–Ala, were used to determine individual amino acid hydrophobicities. Additionally, three proteins, ribonuclease-*S*, lysozyme, and myoglobin were examined and it was found that polar amino acids are 3.5 times more accessible than non-polar amino acids. Chothia [41] compared these values with the partitioning measurements of Nozaki and Tanford [18], and showed two linear relationships, one made of the hydrophobic amino acids alanine, valine, leucine, and phenylalanine and one made up of the hydrophilic amino acids serine, threonine, histidine, methionine, and tyrosine. It was also reported in this study that 92% of buried polar amino acids form hydrogen bonds in the protein interior while the same solute–solvent hydrogen bonds are significantly weakened (1–2 kcal/mol) when the amino acids partition to ethanol.

Utilizing the computer method of Lee and Richards [40], Chothia [35] calculated the accessible surface area for individual residues of 12 proteins and for the extended chains, the secondary and tertiary structures of six other proteins. In this study, hydrophobic surfaces were defined as surfaces formed by non-polar atoms (carbon) or polar atoms (nitrogen, oxygen, and sulfur) with intramolecular hydrogen bonds. Hydrophilic surfaces were defined as those with polar atoms without hydrogen bonds. The formation of both α-helices and β-sheets add 2–3 kcal/mol in hydrophobic free energy and the surfaces buried between secondary structures are very hydrophobic. Chothia also established a relationship between molecular mass and accessible surface area. For the proteins studied, an approximate 9-fold increase in molecular mass increased the percentage of buried hydrophobic residues from 60 to 79% while the percentage of buried hydrophilic residues remained constant at approximately 75%. Hence, the proportion of the polar surface that was buried was independent of molecular mass.

Meirovitch et al. [42] provided two separate hydrophobicity scales from their study of 19 different proteins. Two parameters were examined, the average reduced distance from the center of mass and the average side chain orientation angle. Classifi-

cations from these two scales are compared resulting in glycine, alanine, and tyrosine to fall in an ambivalent category. They believed that this was due to competition between hydrophobic and hydrophilic groups on the same residue. Their values were compared to five other hydrophobicity scales with good agreement among three out of the five scales. One of the two scales which does not have agreeable values, Nozaki and Tanford's, is because single amino acids were used rather than an entire protein. Smaller number of protein samples may be a reason for discrepancy with Chothia's scale.

Wertz and Scheraga [43] examined the influence of water on protein structure using X-ray structures and a seven-step algorithm to determine which amino acid residues occupy the interior or exterior of 20 different proteins. Seven of the amino acids (alanine, arginine, cysteine, histidine, proline, serine, and tyrosine) had preferences not consistent with their typical assignment as nonpolar or polar. Part of the reason for this is the ambivalent groups on each of these amino acids.

Crystallography was used to study the surrounding environment of each amino acid residue of 14 different proteins [44]. Two terms were compared: hydrophobic index and bulk hydrophobic character, which is based on the surrounding hydrophobicity. Correlation between hydrophobic index and bulk hydrophobic character does not correlate as well as expected, with low values for tryptophan and tyrosine indicating the importance of one polar group on the preference for a hydrophobic environment. Bulk hydrophobic character values that are greater for valine than leucine or isoleucine demonstrate the ''ease of accommodation'' factor that bulk hydrophobic character possess.

Janin [45] developed an accessible surface area measurement using data from 22 proteins. The relationship between protein size and accessible surface area was reexamined and was related to the following equation:

$$A = 11.1M^{2/3}$$

where M is the molecular mass and A is the accessible surface area. For the proteins examined, 56% of the buried residues were leucine, isoleucine, valine, alanine, or glycine. Another 22% were phenylalanine, threonine, serine, or cysteine. The remaining 22% were made up of the other 11 amino acids, these include tryptophan and tyrosine which are often listed as the most hydrophobic.

Guy [46] utilized the hydrophobicity scales from the work previously mentioned to classify amino acid hydrophobicity as a function of layers in a protein rather than simply stating that they are buried or exposed. The amino acids lysine, arginine, tyrosine, and tryptophan tended to concentrate near the water–protein interface. Their data did not account for the size of the proteins as in Janin or Meirovitch's study. Results from this study correlate much better with partitioning experiments between organic solvents and water.

Accessible surface area measurements were continued using larger numbers of proteins. In 1987, Miller et al. [47] developed a scale using 46 monomeric proteins. An interior/surface distribution was developed and from this, the free energy of transfer was determined. Results show that the accessible surface area is composed of 58% non-polar amino acids, 33% polar amino acids and 9% charged amino acids. The values for the core amino acids are very similar to those calculated by Janin with 44% being valine, leucine, isoleucine, and phenylalanine, 21% alanine and glycine, 10% serine and threonine, and 25% the remaining 12 amino acids. Additionally, they reported the peptide termini to be very accessible.

One of the most frequently cited amino acid hydrophobicity scales combined accessible surface area measurements with water–vapor partitioning values [48]. A computer program was used to analyze the average hydrophobicity of a constantly moving segment of the protein. This method takes into account the hydrophobic properties of the 20 amino acids of an extensive library of proteins. There is good agreement between residue hydrophobicity spans, seven to 11 residues in length, and protein location with the most hydrophobic spans occurring in the interior of the protein. Furthermore, this method was able to determine the hydrophobic membrane-spanning segment of several membrane-spanning proteins.

Rose et al. [27] used accessible surface area measurements to develop two distinct scales. The first was based on the area lost upon transfer from a reference (unfolded) state to the native state. The

scale correlated well with most partitioning scales. The second scale was based upon a fractional accessibility defined as the mean accessible surface area divided by the standard state area. These two scales, although related, are not equivalent. Arginine, for example, makes a large contribution to the first scale due to its bulky size but a small contribution to the second scale due to the fact that its fractional access is high.

Disadvantages in accessible surface area methods lie in that they are dependent on both the definitions of polar and apolar atoms, as well as the limited database of protein structures. Accessible surface area methods also measure only the static accessibility, rather than accessibility for dynamic proteins in solution. In addition, most scales do not take into account the surface polarity before making the distinction between hydrophobic and hydrophilic [49]. It has been reported that accessible surface area measurements give insight into an average hydrophobicity of an amino acids type, but provide little information on individual residues [50].

6. Chromatographic methods

The most popular chromatographic method for measuring solute hydrophobicity has been reversed-phase liquid chromatography (RPLC). One of the reasons for the popularity lies in the belief that the nonpolar stationary phase mimics a biological membrane (Ref. [51] and references therein). Most methods use peptides as solutes and regression analysis to relate amino acid hydrophobicity to the retention of the peptide. The main advantage of using a peptide is that terminal charges do not exclude partitioning in RPLC. Most often peptides under about 15 amino acid residues in length are used to avoid the formation of secondary structures, which have a profound impact on retention. Occasionally, whole proteins are used and individual amino acid hydrophobicity is calculated in much the same manner as with peptides. Individual amino acids are rarely used and derivatization is then often necessary due to the inability of free amino acids to partition into a C_{18} bonded phase due to the charged termini. However, free amino acids have been used with other chromatographic techniques. Using amino acids is advantageous since they do not suffer from nearest neighbor effects and are not subject to location and frequency effects that can skew data obtained with peptides and proteins.

While a majority of the chromatographic amino acid hydrophobicity scales use RPLC, other chromatographic methods have also been used. In 1971, Aboderin [52] developed a scale using peptide retention on a hydrophilic gel with an aqueous mobile phase of 1-butanol and pyridine. As with many early studies, hydrophobicity was measured against a reference value, glycine. Aboderin reported unique hydrophobicity values for all 20 amino acids and reported a distinct break in the scale. Thin-layer chromatography has also been used to relate mobility values (R_F) of free amino acids to their hydrophobicity [53]. Gehas and Wetlaufer [54] used hydrophobic interaction chromatography (HIC) of dansyl derivatized amino acids in forming their hydrophobicity scale. In HIC, retention is based strictly on hydrophobic contact area and therefore provides a weaker interaction than RPLC. Retention is determined using the equation:

$$\text{Log } k' = \log k'_o + S\phi$$

where S is related to the solute hydrophobicity. Values from this study correlate well with partitioning values.

Meek [55] first introduced RPLC to measure amino acid hydrophobicity using an aqueous–acetonitrile gradient to examine 20 five peptides of varying length. Repetitive regression analysis was used to determine retention coefficients at both pH values of 7.4 and 2.1. At a pH of 7.4, all groups are charged including the termini and acidic and basic side chains. As the pH is lowered to 2.1, the acidic side chains and α-carboxyl termini lose their ionization through protonation.

Valko et al. [56] introduced a new chromatographic hydrophobicity index (ϕ_o) based on fast gradient HPLC measurements as an alternative for the octanol–water coefficient obtained from previous HPLC studies. Plass et al. [57] used this method to obtain ϕ_o values for 30 oligopeptide derivatives on five different columns. The tripeptide derivative of the type Z–Ala–Xaa–Val–OMe, where Xaa represents an amino acid, provided index values compar-

able with other hydrophobicity scales for three out of the five columns examined.

In 1984, the first popular scales were published which used amino acids rather than peptides [58]. PTH-derivatized amino acids were used as solutes and *S* values were measured to rank amino acid hydrophobicity. Guo et al. [59] developed a scale to attempt to normalize for the frequency and location of amino acids in peptides. Synthetic peptides were used with aqueous–acetonitrile mobile phases at both neutral and acidic pH values. The peptide used was Ac–Gly–X–X–$(Leu)_3$–$(Lys)_2$–amide where Ac is an acetyl group and X represents each of the 20 amino acids. The benefit of this scale is that since the peptides are synthesized, the frequency and location errors found in natural peptides are eliminated. Again, the most dramatic shifts in hydrophobicity measurements with pH occurred for acidic and basic amino acid side chains. Significant interactions with surface silanols were reported which affect each amino acid side chain differently. Interactions with surface silanols are one of the major drawbacks of RPLC, which is one of the reasons trifluoroacetic acid is often used as a mobile phase modifier. Scales were developed on C_{18} columns of different carbon loading showing an increase in the absolute value of amino acid hydrophobicity as a result of increased carbon load. Parker et al. [60] used Guo's scale with some success to predict antigenic portions of several proteins. A plot of surface hydrophobicity versus residue number was used to indicate protein surfaces hydrophobic enough to be antigenic.

RPLC amino acid hydrophobicity scales continued to be studied through the 1980s and 1990s. Jinno and Tanigawa [61] used tetrapeptides to determine retention coefficients and based their scale on the linear relationship between retention and hydrophobicity established by Sasagawa et al. [62]. Mant et al. [63] examined the retention of 19 proteins on stationary phases of various ligand lengths ranging from C_4 to C_{18}. They showed protein retention less than predicted by amino acid hydrophobicity values, probably due to secondary structure. Rothemund et al. [64] used both monomeric and polymeric C_{18} stationary phases and methanol–water mobile phases to examine the retention of synthesized tetrapeptides. They concluded that there is good linear correlation in mobile phases ranging from 0 to 40% methanol. Although hydrophobicity dominated the retention process, some π–π interactions were reported on the polymeric stationary phase resulting in higher hydrophobicity values for the three aromatic amino acids.

Wilce et al. [65] performed one of the most complete studies of RPLC characteristics affecting amino acid hydrophobicity scales. Using the retention data for 1738 peptides, three different nonpolar ligands (C_4, C_8, and C_{18}), and two different mobile phases (acetonitrile–water and 2-propanol–acetonitrile–water), they produced four new scales of hydrophobicity coefficients. Two of the four scales correlated well with two electronic characteristics: hydrogen bonding ability and positive charge. The third scale reflected inductive field strengths while the fourth reflected size and steric considerations. These scales were compared to 12 previously published scales of amino acid hydrophobicities. High correlations were observed between their scales and partitioning and accessible surface area experiments.

DeVido et al. [66] continued the use of RPLC to understand amino acid hydrophobicity in a study published in 1998. By using three C_{18} columns of differing bonding density, they demonstrated the degree of alkyl chain alignment can completely change the thermodynamic signature of an amino acid. They illustrated this phenomenon by van't Hoff analysis showing the temperature-dependent partitioning coefficients of the 20 amino acids. Since aquo-organic mobile phases were used, retention was extrapolated to pure water to ensure that hydrophobicity of the amino acids was being measured rather than solvophobicity. From their work, they determined the bonding coverage of the stationary phase determines the magnitude and even the sign of the heat capacity. Oil–water partitioning of nonpolar amino acids into grafted alkyl chains involves an enthalpically driven process compared to bulk oil, which is entropically driven. Partitioning into grafted alkyl phases of high surface densities involved small heat capacity changes while low-density phases had large heat capacity changes.

Contrary to all the RPLC amino acids hydrophobicity scales developed, Yoshida [67] published a *hydrophilicity* scale using normal-phase liquid chromatography, showing the retention of 121 peptides on an Amide-80 column. The contribution of each amino acid was determined from linear multiple

regression analysis and he coined the term hydrophilicity retention coefficient. These values provided a means for predicting retention of peptides of known amino acid content and sequence.

Silva et al. [68] examined the retention of the 20 underivatized amino acids on two different octadecyl columns and a phenyl column using buffered mobile phases at three different pH values. Partial least-squares and multiple linear regression of the amino acid retention data was performed against various molecular descriptors used to describe various properties of these molecules, including other hydrophobicity scales. Quantitative structure–activity (QSAR) and quantitative structure–retention relationships (QSRR) were obtained from these chemometric methods. They believed this statistical approach would be beneficial in assessing hydrophobic amino acid–membrane interactions.

Mant and Hodges [69] have recently examined the concept of amino acid hydrophobicity from the viewpoint of receptor–ligand interactions. Using the nonpolar stationary phase as the receptor, they examined retention of 18-residue amphipathic α-helical peptides on C_{18} and cyano columns to determine the effect of the receptor on measured hydrophobicity of the amino acid. They performed a second study [70] where two different 18-residue peptides were synthesized, one with Ala in its non-polar face and another with Leu in its non-polar face, to demonstrate the effects of varying hydrophobicity of the ligand. Mutants of each peptide were prepared in the center of the nonpolar face where each amino acid residue was replaced at the mutation point. The effect of salt in the mobile phase was also examined to observe its role in the hydrophobic nature of the amino acid. With the Ala-face mutants, the non-polar residues and three positively charged residues (Arg, His, Lys) show an increase in hydrophobicity in the presence of salt. The opposite trend is observed with the Leu-face mutants with significantly more hydrophilicity in the presence of salt.

Chromatographic methods are not unambiguous, however, due primarily to the dependence of the scales on the chromatographic parameters. The number and accessibility of surface silanols, the bonding density of stationary phase chains, the silica surface area and pore diameter, the choice and concentration of organic modifier, the choice and pH of any aqueous buffer, and even temperature can affect both absolute values of hydrophobicity, as well as relative rankings.

7. Site-directed mutagenesis

Site-directed mutagenesis is a biochemical method that has been used to measure amino acid hydrophobicity. Using DNA recombinant technology, one-to-one substitutions are made in the amino acid sequence of naturally occurring proteins. A main advantage of this method is that it is an actual measurement of protein stability based on insertion of a specific amino acid. The increased stability and activity of proteins that accompanies substitution encouraged research in this area. For example, the introduction of a disulfide linkage can increase protein stability by 1–5 kcal/mol (4–20 kJ/mol) provided destabilization or loss of activity of the protein does not occur [71]. Also the replacement of glycine with alanine adds 0.7–0.9 kcal/mol due to the higher propensity for helix formation.

Hecht et al. [72] made single amino acid replacements on the amino-termini domain of phage λ repressor along with making calorimetry measurements. The variety of substitutions made indicate that the hydrophobic core plays a more important role in the stabilization of the protein than the surface.

Yutani et al. [73] provided one of the most complete site-directed mutagenesis studies. Nineteen different amino acids were substituted at Trp 49 of the protein tryptophan synthase (α subunit), which is a buried residue. The free energy of unfolding was measured using a titration with guanadine hydrochloride, and they found increased stability with increasing hydrophobicity, up to a certain size limit, excluding some aromatic side chains. Another study by Matsumura et al. [50] replaced Ileu3 of the bacteriophage T4 lysozyme, a protein in an α-helix and 80% inaccessible to solvent [45]. Results show a linear relationship between hydrophobic contribution and protein stability. An increase in pH from 2 to 6 showed a decrease in stability for those amino acids that ionize upon the pH change. Importantly, correlations with liquid partitioning studies show a lack of dependence on the oil phase used for partitioning.

Correlations with accessible surface area studies show good agreement on average, but poor specific individual correlations.

Iso-1-cytochrome c was used as a sample protein to substitute a highly exposed amino acid residue at Lys73 [74]. Hydrophobic substitutions were made using methionine, phenylalanine, tryptophan, and tyrosine to ensure stablizing hydrophobic interactions in the unfolded state while not affecting the folded state free energy. Unfolding was accomplished using guanidine hydrochloride and examined with FT-IR spectroscopy. The results show protein stability was not greatly affected by the substitutions indicating that the protein does not unfold to a complete random state. Good correlation exists between this scale and liquid–liquid partitioning scales.

A recent study in the area of site-directed mutagenesis is the work of Takano and Yutani [75] where the change in stability of mutant proteins was used to produce a scale corresponding to side-chain contributions to protein stability. The change in stability of a mutant protein ($\Delta\Delta G$) can be expressed by summing the contributions from the hydrophobic effect, side-chain conformational entropy, hydrogen bonds, water molecules, secondary structure propensity, and cavity volume. Each of these parameters can be represented in terms of the conformational change of the mutation. The greatest difference is observed with polar amino acids, which provided a positive contribution to protein stability. Previous values from transfer experiments show a negative contribution by this class of amino acids.

One of the main drawbacks of site-directed mutagenesis methods is that no residue in a particular protein can be substituted with all 20 naturally occurring amino acids [73]. Other disadvantages include cost, perturbation of other interactions [76], and a lack of usefulness for purposes other than measuring protein stability.

8. Physical property methods

A variety of amino acid hydrophobicity scales exist based upon the measurement of a particular physical property, including surface tension, transition temperature, solvation energy, and partial molar heat capacity. Advantages of these methods lie in speed, ease of use, and flexibility in terms of solutes. Bull and Breese [77] in 1974 developed one of the most widely recognized scales by measuring surface tension values in sodium chloride solutions for the 20 naturally occurring amino acids. Using the number of moles of the amino acid adsorbed on the solution (T) and the thickness of the surface layer with the adsorbed amino acid (τ), they calculated the concentration ratio of amino acids at the surface to solution:

$$C_s/C = (T + \tau C \times 10^{-3})/(\tau C \times 10^{-3})$$

and from this the free energy of transfer from solution to the surface using the equation:

$$\Delta F = -RT \ln C_s/C$$

Surface tension measurements suffer from several important disadvantages. Hydrogen bonds that are broken and charged groups that are neutralized upon transfer from the aqueous solution into the protein remain intact at the solution–air interface [48].

Another physical property used for measuring amino acid hydrophobicity is the solvation free energy [49]. Solvation free energy is estimated as a product of an accessibility of an atom to the solvent and an atomic solvation parameter. The solvent accessibility is weighted by the polar character of each atom and the solvation parameter is extended by allowing calculation of individual atoms in residues. Results indicate that the solvation free energy lowers by an average of 1 kcal/residue (4.2 kJ/residue) upon folding.

Makhatadze and Privalov [78] measured the apparent heat capacity of various peptides and organic compounds using microcalorimetry over the temperature range of 5–125 °C. The amino acids were divided into three categories based upon their heat capacity (C_p) values, being classified as hydrophobic if increasing temperature lead to decreasing ΔC_p values. Threonine, tyrosine, and histidine could not be categorized based on this method.

Two studies were performed to measure the heat of reversible aggregational transition of poly (Val–Pro–Gly–X–Gly) where X is the amino acid of interest [79,80]. The heat of transition was measured by differential scanning calorimetry (DSC) and the initial study established a relationship between the relative hydrophobicity of the peptide and the inverse

of its temperature transition [79]. Copolypentapeptides were used in the second study with measurements again being done using DSC [80].

Several disadvantages exist with using the temperature transition to measure hydrophobicity. The charge and low solubility of some amino acids influence the hydrophobicity values obtained. Also the gradual melting of proteins can lead to values which are difficult to distinguish [77]. Finally, one must assume that hydrophobic interactions are entirely determined by the heat capacity or the change in heat capacity is constant and the change in entropy of individual residues can be extracted from the change in entropy of the protein [81].

9. Recent applications of amino acid hydrophobicity scales

Other recent studies in this area deal with using these hydrophobicity scales for predicting certain structural features of proteins. Palliser and Parry [82] (and references therein) used a wide variety of these hydropathy scales to demonstrate whether they can be used for locating β-strands on the surfaces of proteins. They examined approximately one hundred scales and found that they could use them for this purpose. Trinquier and Sanejouand [83] (and references therein) used the hydrophobicity scales to determine whether this property can better predict the preservation of the genetic code. They were able to group the amino acids into three separate clusters based on a scoring procedure they developed examining numerous hydrophobicity and other miscellaneous scales. The new ordering of the bases that was observed was uracil–guananine–cystosine–adnenine (UGCA) which they believe better reflected the conserved character of the genetic code compared to the UCAG ordering that is commonly seen. Reference to these two papers is strongly recommended to those who want to study applications of amino acid hydrophobicity scales.

10. Comparison of amino acid hydrophobicity scales

Examining the different methods in the previous sections for measuring amino acid hydrophobicity, it is reasonable to ask how the values from these methods correspond to a true measure of this significant property. To demonstrate this, we prepared histograms for all 20 of the naturally occurring amino acids comparing numerous published hydrophobicity scales.

The histograms illustrated in Fig. 3a–d show the frequency that a particular hydrophobicity ranking exists for each amino acid. As seen, there is a wide range of hydrophobicity values that exist for each amino acid. Some show a high hydrophobic ranking with one method while another method shows a high hydrophilic ranking for the same amino acid. With these discrepancies amongst the various rankings, it is very difficult to use these values for any sort of modeling of protein folding. What are the reasons for such deviations? Differences in these amino acid hydrophobicity scales can be attributed to many different factors that are difficult to separate. Most of the groups of amino acids rank consistently from scale to scale. Some though, change hydrophobic rank more than others. The aromatic amino acids are often found near the most hydrophobic but have been reported, in some scales, in the second half of the scale. This is probably due to their unusually large side chains. Also, while aromatic molecules tend to be rather hydrophobic, tryptophan and tyrosine also have fairly polar moieties. The amphiphilic character of these amino acids lead to varying ranking of hydrophobicity depending on the method and solute chosen. Another amino acid that shows unusual rankings is cysteine. This is due to the ability of cysteine to form disulfide linkages with other cysteine residues. For scales that use amino acids, cysteine appears more hydrophilic due to the lone sulfur atom in the side chain. However, when proteins and peptides are used and the disulfide bond can be formed, cysteine residues appear more hydrophobic.

The isomer pair of leucine and isoleucine provides some interesting results. Fig. 4 compares these two amino acids by breaking down their hydrophobic ranking as well as the method used to obtain this value. Using site-directed mutagenesis clearly shows isoleucine to be more hydrophobic than leucine. Only two partitioning methods have equal partitioning values for these amino acids. This is surprising

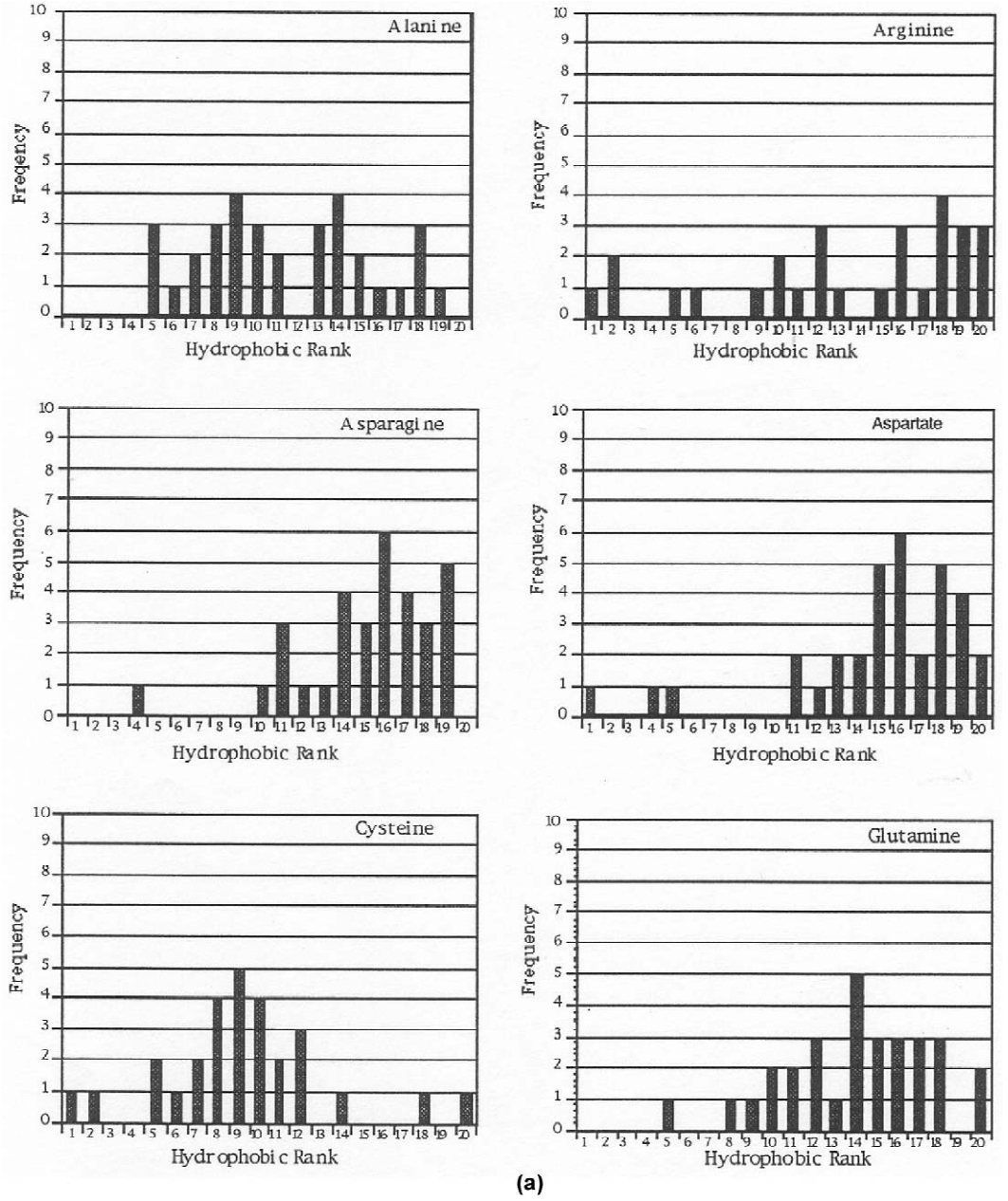

Fig. 3. Histograms illustrating the rank discrepancies in hydrophobicity scales for the 20 common amino acids. The *x*-axis provides a numerical ranking for hydrophobicity (1, most hydrophobic; 20, least hydrophobic) while the *y*-axis provides the number of hydrophobicity scales that provide that particular ranking.

since partitioning is not known to be shape selective. In the chromatographic methods, which are known to be shape selective, there is an almost even split to which amino acid is more hydrophobic.

An interesting observation can be made as well if one examines a particular hydrophobicity scale. The free energy difference between two amino acids is less than 1 kcal/mol. On average, the absolute transfer free energy of a methylene group is 2.5 kcal/mol. This makes it rather difficult to rank two

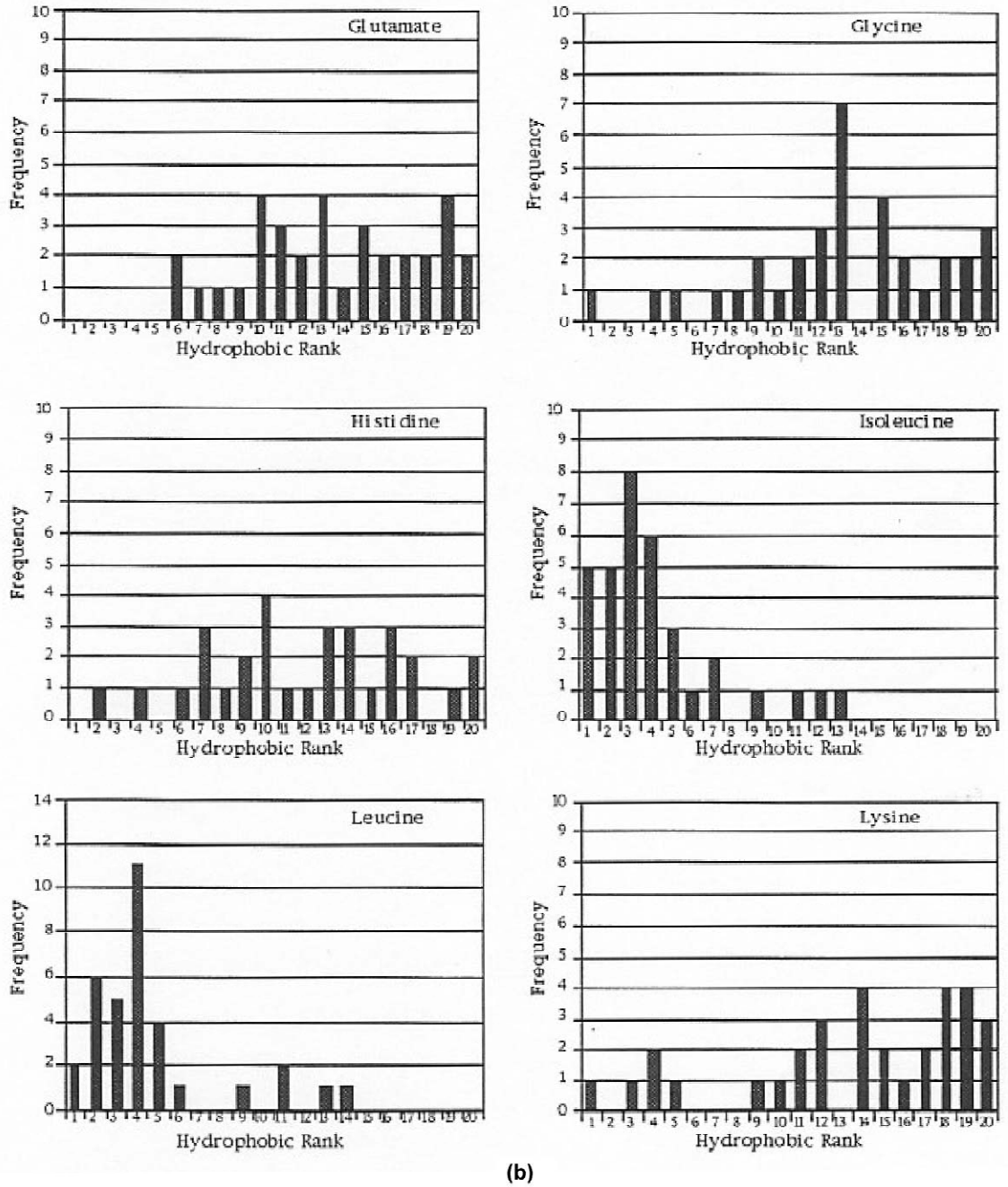

Fig. 3. (*continued*)

amino acids consecutively since the free energy difference is nearly indistinguishable.

Based on the information obtained from these scales, few amino acid hydrophobicity methods can be used reliably. Accessible surface area measurements are database limited and do not take into account the hydrophobicity of the protein surface. Chromatographic methods have shown much prom-

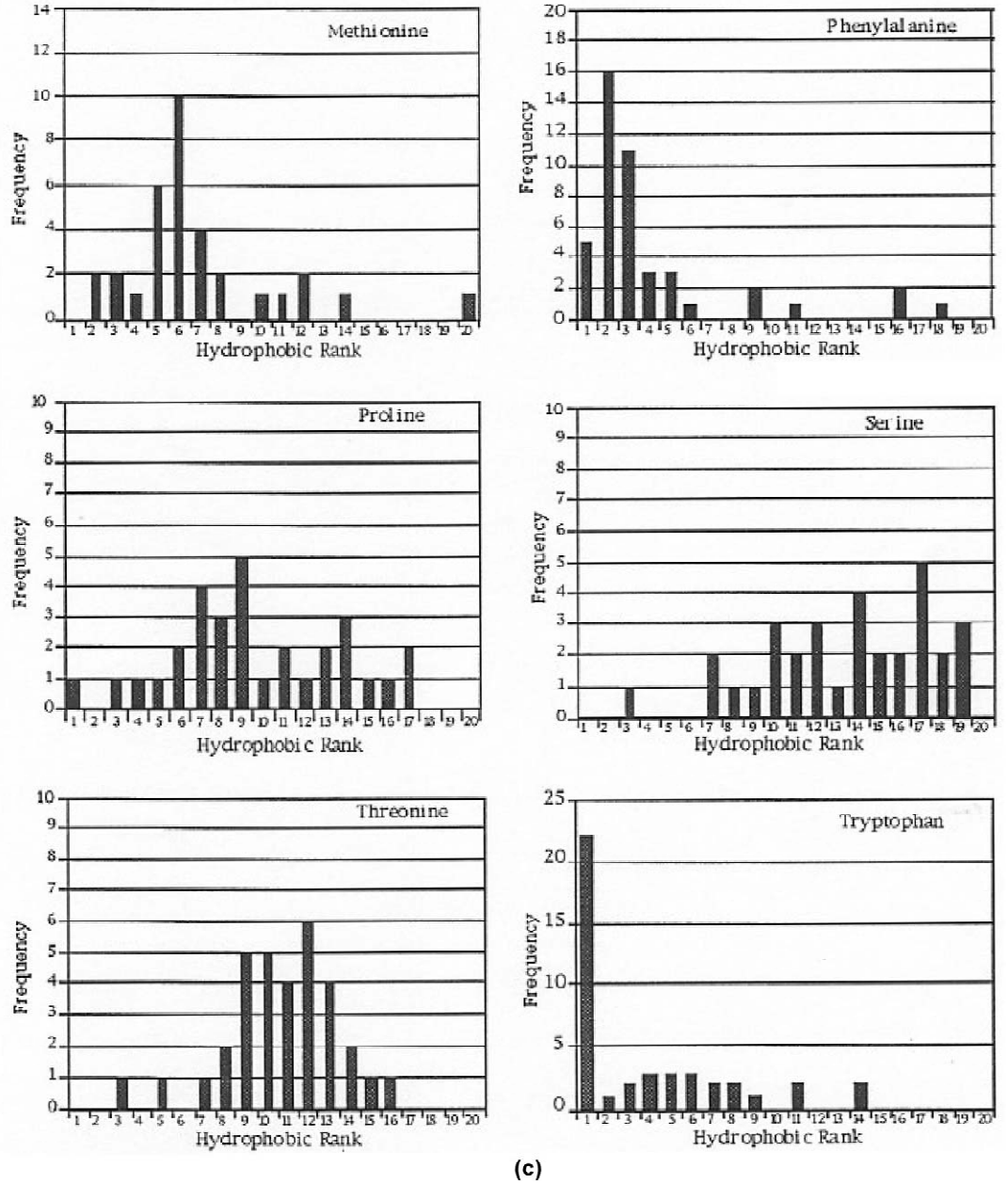

Fig. 3. (*continued*)

ise, particularly RPLC, but questions remain based on column choice as well as mobile phase considerations. The large number of reactive and unreactive silanols on the surface makes it likely solutes will interact with these moieties. Site-directed mutagenesis studies are highly dependent on the protein and residue chosen for the mutation. Physical property measurements are questioned as to relevancy. Also, the choice of solute is a key issue. No method has yet developed scales using both individual amino

Method	Leucine	Isoleucine
Chromatography	7	6
Partitioning (2)	5	4
Accessible surface area	3	5
Site-directed mutagenesis	0	4
Physical properties (1)	2	4
Total	**17**	**20**

Fig. 4. Comparison of the isomer pair leucine and isoleucine. Number in parentheses indicates the number of scales with the same value for that particular method. Column number indicates number of scales that have a higher value corresponding to the method.

acids and peptides or proteins, which would be beneficial as a comparison to demonstrate the relevancy of hydrophobicity values.

One of the most recent and quite complete studies in of these discrepancies in amino acid hydrophobicity scales is that of Trinquier and Sanejouand [83] discussed in the previous section. They combined the various hydrophobicity values from 144 scales and developed a scoring system based on the frequency that an amino acid is labeled hydrophobic or hydrophilic. Three clusters were produced as a result of this analysis:

hydrophobic: tryptophan, methionine, cysteine, phenylalanine, isoleucine, leucine, valine, glycine, arginine, serine;

hydrophilic: tyrosine, asparagine, lysine, aspartate, glutamate, histidine, glutamine;

ambivalent: alanine, threonine, proline.

Trinquier and Sanejouand state that these categories should be accepted with some degree of caution since it does not include a very large sample set. Such a system for categorizing hydrophobicity is likely the most rational approach.

11. Methods for studying amino acid interactions

In a recent mini-review, Cooper [84] addressed the issue of understanding the thermodynamics of interactions among biomolecules and stated there are models for examining these interactions but difficulty arises in distinguishing the various components such as protein–protein, protein–ligand, and nucleic acid–ligand. The advantages of calorimetric measurements over van't Hoff analysis is discussed as well as the issue of enthalpy–entropy compensation.

One approach commonly used to measure free energies is a model compound method known as BIPSE, an acronym for Break Into Pieces and Sum the Energies. There are three major steps in the BIPSE method: (1) dividing the interaction (i.e., partitioning, adsorption) into component substituents (i.e., amino acids); (2) using model compound experiments such as oil–water partitioning experiments to obtain the component interaction free energies; and finally (3) assuming the additivity of the free energies and summing the component terms to obtain a total free energy for the process. Mathematically this can be shown as:

$$\Delta G_{\text{total}} = \Delta G_{\text{solvation}} - T\Delta S_{\text{conformational}}$$

Although a large number of BIPSE models exist, there has been little success in obtaining quantitative values for free energies. One reason for this is that oil–water partitioning is used more in a correlative manner: free energies that correlate with those of oil–water partitioning are taken as indicative of hydrophobic driving forces [20]. Another problem with obtaining quantitative results from the BIPSE approach deals with the utility of oil–water partitioning experiments, which as mentioned previously have inherent flaws. A final problem with this approach is the assumption that free energies are additive, which one should carefully consider when utilizing these energy values for biomolecular modeling. Dill has addressed this issue of additivity and has determined the criteria for when this principle can be applied to biomolecules [85].

Another common method for measuring interaction energies is based on amino acid pairing propensities based on database potentials [86]. These potentials are calculated by considering the statistical frequency of pair-wise contact between a particular *i* and *j* residue pair. The potentials used for these experiments utilize a protein database based on the native structures of proteins,calculated as a Boltzmann distribution, which allows the determination of a contact energy for an *i–j* pair relative to a reference state. Problems lie with this method in such features as a choice of reference state, defining

contacts, correcting the contact potentials for the effects of chain connectivity, side-chain size, solvent contacts, and relative abundance of each residue. Also some questions arise about the appropriateness of using Boltzmann distribution laws to extract contact energies from contact frequencies in the database because the Protein Databank (PDB) is fixed, and a suitable temperature for considering these interactions is difficult to establish. They also do not adequately determine desolvation and solvation, hydrogen bonding, or steric interactions because they are not based on knowledge of the denatured states—they are parameterized using only native protein structures [87]. These reasons provide suitable evidence that statistical potentials are not physically meaningful quantities for the purposes of biomolecule folding. Ben-Naim [88] and Dill [89] have addressed the suitability of using these potentials.

Palecz [90] recently discussed the utility of calorimetry for measuring pairwise interactions of L-amino acids. He showed this method could measure these types of interactions as well as provide a more accurate way to measure hydrophobicity for all classes of amino acids.

12. Chromatography as a measurement tool for interactions

Chromatography has traditionally been used for the separation of mixtures. Rather than the chromatography column being used only as a separation medium, the experiment can be reversed and the column can be used as a tool for measuring interactions. The stationary phase can be considered as a ''receptor'' while the analytes injected on top of the column can be thought of as a ligand. Mant and Hodges have used this premise to measure interactions as well as to study hydrophobicities of amino acids. Ringo and Evans have used liquid chromatography as a tool for measuring chiral interactions [91]. Lin et al. [92] (and references therein) have extensively studied the area of peptides and proteins and how these molecules interact with a nonpolar stationary phase and have recently published an elegant review. The focus here will be on amino acid interactions and an overview of some of the major contributions in this area. As Dill stated [85], additivity is not readily applicable to biomolecules. Due to the discrepancies among hydrophobicity scales, it would also be difficult to simply add the values obtained from different hydrophobicity scales to obtain a value for a pairwise hydrophobic measurement. Another reason for emphasis on amino acid interactions is that a better understanding of how these biomolecules interact will lead to a clearer picture of how proteins fold into their native state.

One of the earlier studies in this area of amino acid interactions used thin-layer charge-transfer chromatography. Cserhati and Szogyi [93] used this technique to examine the interaction of a fungicide (1-phenyl-2-nitro-3-acetoxyprop-1-ene) with various amino acids and glutathione. Results showed preferential interaction with thiol-containing amino acids and glutathione. A second study [94] measured interactions with respect to the amino acid tryptophan, which was placed on a spot plate with the other amino acids being eluted with distilled water. From their study, they found arginine, asparagine, glutamate, methionine, phenylalanine, and threonine to interact with tryptophan while alanine, glycine, and serine showed no interaction. From regression analysis, they found the pK value of the amino acid side chain and the lipophilicity of the amino acid had the greatest effect on the interaction. The L and D forms of asparagine showed no significant difference on the interaction with tryptophan.

Another area of chromatography that has examined interactions of amino acids is hydrophobic interaction chromatography (HIC). HIC is primarily used for the analysis of large proteins and protein purification using a mildly nonpolar stationary phase compared to that used in reversed-phase LC. Vailaya and Horvath [95] performed a study using three different HIC stationary phases and a series of dansyl derivatized amino acids to measure hydrophobic interactions. Much work had been done to understand the role of salt concentration in HIC but until this study, the effects of temperature had not been investigated. Thermodynamic data from their study revealed significant effects from the heat capacity showing a large positive change in enthalpy and entropy at low temperatures and at high temperatures decreasing and approaching negative values. Calorimetric data corresponded very well with their

data showing the characteristic signature of the hydrophobic effect.

Peyrin et al. [96] measured the interactions of dansyl amino acids with a chiral stationary phase made of bovine serum albumin. Using van't Hoff analysis, they determined the retention of these amino acids at six different pH values ranging from 5.5 to 8. Based on the different shapes observed in the van't Hoff plots, they concluded that a phase transition occurred at neutral pH with the chiral stationary phase. Differential scanning calorimetry (DSC) verified this observation.

Basiuk and Gromovoy [97] examined amino acid adsorption on bare silica and octadecyl-derivatized silica by comparing the retention behavior of the 20 amino acids on these two supports. Van't Hoff analysis showed much better linearity on the octadecyl support compared to bare silica since retention on bare silica would be much weaker than on silica derivatized with octadecyl chains. Based on their thermodynamic data, they concluded that amino acid retention on bare silica is based on ionic interactions as compared to the octadecyl phase, where it is a combination of both ionic and hydrophobic interactions.

Pochapsky and Gopen [98] synthesized stationary phases with amino acid side chains bonded to the silica surface, which they termed mimic stationary phases (MSP), and used these to examine amino acid interactions. Two phases were synthesized, one with the bonded side chain of phenylalanine and the other with isoleucine. Nonpolar and polar amino acids were used as solutes and retention was measured on these stationary phases over a wide temperature range. Van't Hoff-type analysis was performed to obtain thermodynamic data corresponding to each pairwise interaction. Selectivity was calculated with respect to glycine, since it is the simplest amino acid with only hydrogen as its side group. The free energy of interaction was calculated using these plots to obtain the enthalpy and entropy. The curvature of the plots differed between aliphatic and aromatic amino acids, which they believed indicated a difference in retention mechanism. An adsorption model for retention was proposed based on their findings. In a second study [87], they utilized both the Ileu MSP and Phe MSP to try to distinguish amongst three types of interactions: aliphatic–aliphatic, aromatic–aromatic, and aromatic–aliphatic interactions. Thermodynamic data for these interactions were again based on van't Hoff analysis extended to estimate a value for the heat capacity (ΔC_p°). Aliphatic–aliphatic interactions showed the most pronounced changes in heat capacity by the large negative values, which correlate with what is observed in the hydrophobic effect. Aromatic–aliphatic interactions were the next strongest followed by the aromatic–aromatic interactions. The implications of these two studies are tremendous because it begins to pave the road to an understanding how amino acids can interact during the protein folding process.

13. The future

It is apparent from this review that a vast amount of work has been done in measuring amino acid hydrophobicities. A consensus on values and rankings still eludes us. A universal amino acid hydrophobicity scale would be ideal but perhaps far from reality as researchers continue to question the relevance of the measurement technique. Since the scope of this review was limited to amino acid hydrophobicities and interactions, we did not address the importance of peptides and the means by which they penetrate lipid bilayers which can provide hydrophobicity scales for peptides. Much work has been done in examining the interaction of transmembrane peptide segments with lipid bilayers, which is significant since lipid bilayers are the natural environment of such peptides. These studies are significant because it has been shown to provide hydrophobicity scales for membrane proteins as well as the composition of such proteins [99,100]. Rather than reaching a common scale for measuring hydrophobicity, we suggest continued work on measuring pairwise interactions of these amino acids. If we can obtain thermodynamic values corresponding to these interactions, it may be more beneficial than simply knowing hydrophobicities. Liquid chromatography has been used for these measurements [87,98]. Complementing this with calorimetry [90] will verify the values obtained from these measurements. We have recently attempted to measure pairwise interactions of amino acids using amino acid surfactants as

a pseudostationary phase (Biswas and Dorsey, unpublished research).

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