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Barbara A. Andrews[a]; Juan A. Asenjo[a]
[a] Centre for Biochemical Engineering and Biotechnology, Department of Chemical Engineering and Biotechnology, Institute for Cell Dynamics and Biotechnology; a Centre for Systems Biology, University of Chile, Santiago, Chile

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Theoretical and Experimental Evaluation of Hydrophobicity of Proteins to Predict their Partitioning Behavior in Aqueous Two Phase Systems: A Review

Barbara A. Andrews and Juan A. Asenjo

Centre for Biochemical Engineering and Biotechnology, Department of Chemical Engineering and Biotechnology, Institute for Cell Dynamics and Biotechnology; a Centre for Systems Biology, University of Chile, Santiago, Chile

Hydrophobicity is the main determinant in the partitioning of proteins in Aqueous Two-Phase Systems (ATPSs). There are a range of different methods available to evaluate hydrophobicity, including hydrophobic interaction chromatography, reverse phase chromatography, precipitation with ammonium sulphate, average surface hydrophobicity (3-D structure), aminoacid (AA) composition, hydrophobic imbalance, and a statistical description of the AA distribution. This paper reviews the present state of the art in the practical and theoretical evaluation and measurement of a protein's hydrophobicity and evaluates which are the best methodologies to estimate hydrophobicity to predict behaviour in ATPSs. The two methods that are more attractive, depending on the ATPS used, are those that consider the 3-D structure and the hydrophobicity of AA on the surface and the one that uses the parameter 1/m*, which is very simple to determine experimentally.

Keywords aqueous two-phase systems; hydrophobicity; proteins

INTRODUCTION

Several fundamental theories derived from classical polymer solution thermodynamics have been developed in an attempt to predict the partitioning behavior of proteins in aqueous two-phase systems (ATPS) (1–5). Most of these models are based on PEG/dextran or other biopolymer systems. PEG/salt systems are especially interesting for practical application in industry because of the economy involved in replacing the expensive dextran with more inexpensive salt. Predictive models for PEG/salt systems are more limited and/or empirical probably due to the complexity of these ATPSs.

The value of the partition coefficient, K (defined as the ratio of the protein concentration in the top phase to that of the bottom phase) relies on the physicochemical properties of the target protein and contaminants (e.g., hydrophobicity, charge, molecular weight) and their interactions with those of the chosen system (e.g., composition, ionic strength, addition of specific salt ions, pH). Depending on the manipulation of these system parameters the target protein and contaminants can be partitioned selectively (6–8). A simple model can correlate the physicochemical properties of proteins with their partition coefficient in ATPSs using the so-called modified group contribution approach (9–12):

$$K = K_{hphob} \cdot K_{el} \cdot K_{size} \cdot K_{sol} \cdot K_{aff} \quad (1)$$

where K_{hphob}, K_{el}, K_{size}, K_{sol}, and K_{aff} correspond to the contribution of the overall partition coefficient by hydrophobicity, electrostatic forces, size, solubility, and affinity, respectively. Not all of the properties are equally important and this evidently depends on the type of system used, e.g., hydrophobicity was shown to be very important in PEG/salt systems at high concentration of NaCl (8.8% w/w) (9,13,12). The effect of the protein concentration represented by the K_{sol} term has been evaluated previously (14).

Hydrophobic interactions are involved in various separation and purification techniques including precipitation, hydrophobic interaction chromatography (HIC), and reverse phase chromatography (RPC). Although HIC and RPC employ hydrophobic interaction between the protein and the matrix, previous work in our group on protein hydrophobicity in ATPS (9,15,13) has found no direct correlation between the hydrophobicity measured by RPC and HIC and the partition coefficient in ATPSs with a mixture of proteins, probably because these techniques measure surface hydrophobicity at the contact area between the protein and the nonpolar surface of a probe, e.g., the stationary phase. On the other hand, a good linear relationship was found between the hydrophobicity of the proteins measured as a function of their solubility and their

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Address correspondence to Barbara A. Andrews, Centre for Biochemical Engineering and Biotechnology, Department of Chemical Engineering and Biotechnology, Institute for Cell Dynamics and Biotechnology; a Centre for Systems Biology, University of Chile, Santiago, Chile. E-mail: bandrews@ing.uchile.cl

partition coefficient in systems of PEG/salt with a high concentration of NaCl. A correlation was proposed for predicting the partition coefficient of proteins in ATPS that takes into account the effect of hydrophobicity (13,16,12):

$$\text{Log K} = \text{R log}(\text{P}/\text{P}_0) \quad (2)$$

or

$$\text{Log K} = \text{R log P} - \text{R log P}_0 \quad (3)$$

where $\text{P} = (1/\text{m}^*)$ is the protein hydrophobicity in solution measured by precipitation and log P_o represents the intrinsic hydrophobicity of the given ATPS. Log $\text{P}_0 = \log \text{P}$ when $\text{K} = 1$; R represents the hydrophobic resolution which is the ability of the system to discriminate between proteins with different hydrophobicities.

Diamond and Hsu (1) studied the partitioning of dipeptides with different CH_2 chain lengths in PEG/Dextran systems and found that the longer the chain length and thus greater hydrophobicity, it gave higher values of the partition coefficient. K was also a function of tie-line length and this was directly proportional to resolution. Eiteman and Gainier (4) proposed a correlation similar to equations (2) and (3) and evaluated the effect of the hydrophobicity of different alcohols in their partitioning in PEG/phosphate ATPS. They also analyzed the effect of tie line length on the constant R (Eqs. 2 and 3), which had a linear dependence.

To quantify the protein surface hydrophobicity, the salt concentration at which the protein begins to precipitate in ammonium sulfate precipitation at a given protein concentration can be measured and is referred to as m^* (9,13,15,17,18). Hachem et al. (13) showed that for five proteins with a range of hydrophobicities, the hydrophobicity evaluated by the point m^* in ammonium sulfate precipitation correlated well with the partition behavior in PEG 8000/phosphate systems in the presence of relatively high concentrations of NaCl (4.8–19.6% w/w). This is a valid way to evaluate a protein's hydrophobicity when a fixed initial protein concentration is used (13). The correlation has also been evaluated with two chemically modified proteins: α-lactoglobulin and BSA (16). In both cases the protein was chemically modified to change its surface hydrophobicity.

In addition to hydrophobicity, charge may play a role in the partition of proteins in systems such as PEG/dextran and those with low NaCl concentration. In these systems the unequal distribution of cations and anions in the phases could cause the ions close to the interface to form an electrical double layer (19), which could influence the partition of charged particles, such as proteins, according to the sign and magnitude of their net charge (10,19). In PEG/salt systems the situation is even more complicated due to the presence of several different salts. When different amounts of NaCl are added to PEG/salt systems the ratio between the ions changes, which affects partition of proteins (10,19). Furthermore, a change in pH in PEG/salt systems and thereby of the charge of proteins can affect K (6,9,19,20).

Thus it is clear from previous work that the hydrophobicity of a protein plays an important role in determining its partition coefficient, K, in ATPS particularly in polymer-salt ATPSs. However, a protein's hydrophobicity, which is ultimately determined by the hydrophobicity of its amino acids, can be measured and evaluated by many different methods as has been already described. Cascone et al. (7) found that when adding important concentrations of NaCl to PEG/salt systems the partition coefficient of the protein thaumatin, could be directed to the more hydrophobic PEG phase. Schmidt et al. (6) showed that this effect could be much more dramatically observed for the rather hydrophobic protein α-amylase. A similar effect was observed for the hydrophobic protein tpA (21) and also, very interestingly, for the partition and purification of monoclonal antibodies which are also hydrophobic proteins (8). This pioneering finding has been exploited in a European project which has studied in detail the partitioning and important scale up and processing factors of the hydrophobic MAbs in ATPS (22–24). All these four proteins are quite hydrophobic, hence the effect observed of being able to dramatically increase the value of K by the addition of NaCl to PEG/salt systems clearly appears to be an effect of the protein's hydrophobicity.

This paper is a concise review of the present state of the art in the practical and theoretical evaluation and measurement of a protein's hydrophobicity and evaluates which are the best methodologies to estimate hydrophobicity to predict behavior in ATPSs.

Evaluation of Hydrophobicity and Partitioning in ATPS

The results discussed in the introduction, in which all proteins evaluated (including thaumatin α-amylase, tPA and monoclonal antibodies) are rather hydrophobic, have led to a more systematic evaluation of a range of proteins where the hydrophobicity was measured by Hydrophobic Interaction Chromatography (HIC), Reverse Phase Chromatography (RPC), and the protein's solubility measured as the point at which a standard solution of a protein starts precipitating in a typical ammonium sulphate precipitation curve (17,18). This can be expressed as the point m^* as shown in Fig. 1, where m^* represents the "hydrophilicity". The hydrophobicity of the protein can thus be represented by the value $1/m^*$. Asenjo et al. (9) and Hachem et al. (13) showed that a poor correlation was found between the value of K in ATPS and the behavior in both HIC and RPC but an excellent correlation with the parameter $1/m^*$ was found for the few proteins used in this study. The correlation was improved when NaCl was added to

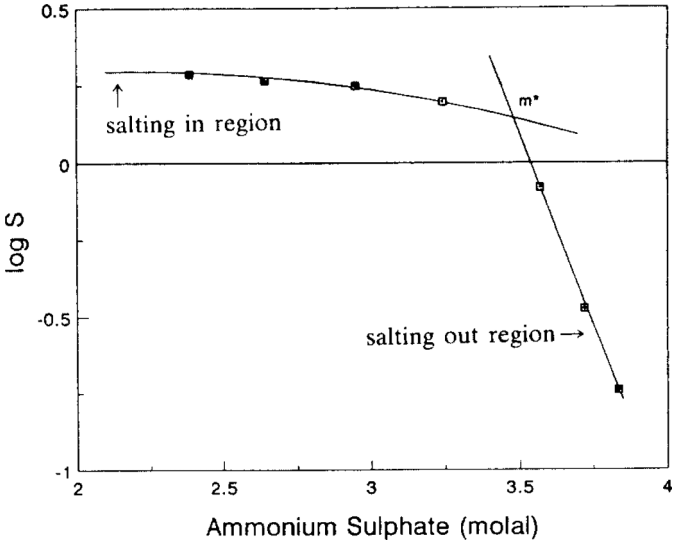

FIG. 1. The solubility/precipitation curve in ammonium sulphate of β-lactoglobulin A at 25°C. The intercept of the two fitted lines represents the solubility, m*. (From 9).

the PEG/phosphate systems used. NaCl concentrations of up to 17.6% were used but no effect was seen at concentrations above 9.6% as can be seen in Table 1. R corresponds to the resolution of the system in terms of the variation of partitioning as a function of the protein's hydrophobicity as has been shown in the Introduction in Eqs. (2) and (3).

A very detailed investigation of the effect of hydrophobicity using $1/m^*$ as the measure of the protein's hydrophobicity in solution in the partitioning of a range of proteins with different properties in 4 different ATPS (PEG/phosphate, PEG/sulphate, PEG/citrate and PEG/Dextran) with low and high concentrations of NaCl (0%, 0.6%, and 8.8%) was carried out (12). The main results are shown in Figs. 2 and 3 and Table 2. The hydrophobicity

TABLE 1

The calculated values of R (Resolution) and the intrinsic hydrophobicity, log P_0, of the ATPS at 20°C. (From 13). In Eqs. (2) and (3). log P_o represents the intrinsic hydrophobicity of the given ATPS. Log $P_0 = \log P$ when $K = 1$; R represents the hydrophobic resolution which is the ability of the system to discriminate between proteins with different hydrophobicities

Two phase system (wt%)	R	Log P_0
8% PEG, 12% PO_4	5.4	−0.23
8% PEG, 12% PO_4, 0.48% NaCl	8.3	−0.29
8% PEG, 12% PO_4, 4.8% NaCl	14.9	−0.38
8% PEG, 12% PO_4, 9.6% NaCl	22.4	−0.45
8% PEG, 12% PO_4, 17.6% NaCl	22.8	−0.45

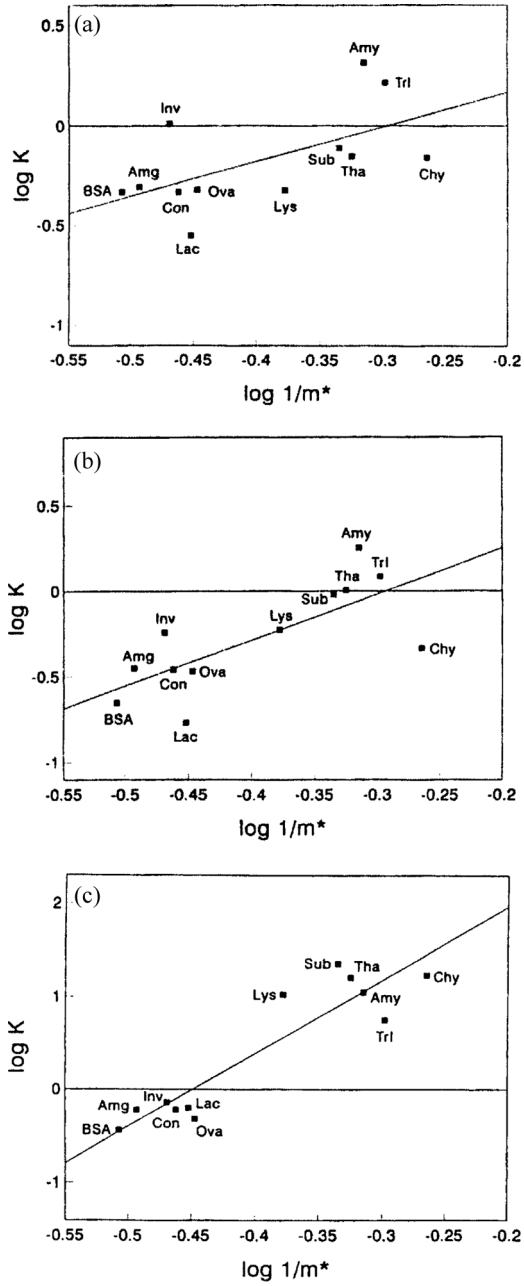

FIG. 2. The relationship between partition coefficient, log K, and hydrophobicity, log P, measured by ammonium sulphate precipitation ($\log P = \log 1/m^*$) in PEG/phosphate systems with a) 0%, b) 0.6% and c) 8.8% w/w NaCl at pH 7. (From 12).

values measured ($1/m^*$) correlated very well in all of the PEG/salt systems at high concentrations of NaCl ($r = 0.92 - 0.93$, where r is the correlation coefficient). In PEG/citrate systems the correlation was good at high and low concentrations of NaCl ($r = 0.81$ and 0.93 respectively). This system also showed a higher hydrophobic resolution. Clearly, in all these systems the resolution, R, of the partition coefficient as a function of the protein's

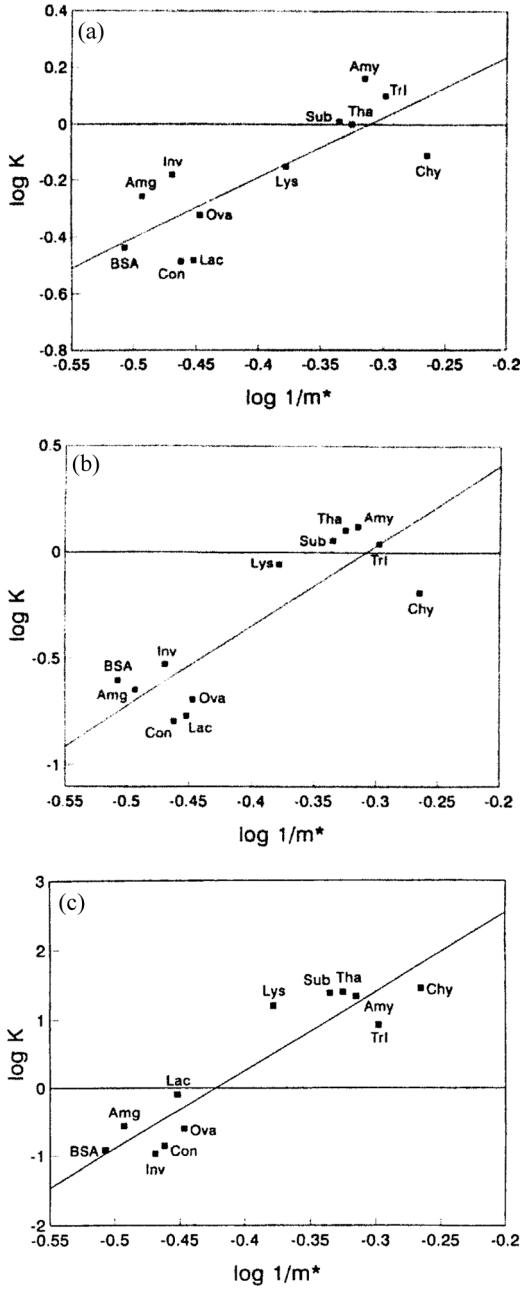

FIG. 3. The relationship between partition coefficient, log K, and hydrophobicity, log P, measured by ammonium sulphate precipitation ($\log P = \log 1/m^*$) in PEG/citrate systems with a) 0%, b) 0.6% and c) 8.8% w/w NaCl at pH 7. (From 12).

hydrophobicity increases dramatically (4–7 fold) in PEG/salt systems when the NaCl concentration in the system is increased from 0 to 8.8% w/w. In the PEG/Dextran system the R value increases 3-fold with a small increase in NaCl concentration (0–0.6%).

A very different study evaluated the surface hydrophobicity by estimating the hydrophobicity of the individual amino acids expressed on the surface of proteins with known 3D structure (25). The influence on partitioning of the surface exposed amino acid residues of eight monomeric proteins was studied. The surface exposed amino acids were evaluated using the computer software GRASP and the partitioning systems used were EO30PO70-dextran (copolymer of 30% ethylene oxide (EO), 70% propylene oxide (PO)). The EOPO copolymer is slightly more hydrophobic than PEG which results in a larger hydrophobicity difference between the two phases of an EOPO-dextran system compared to a PEG-dextran system. It was shown for the first time that the partitioning behavior of the proteins could be described by the differences in the surface exposed amino acids. Excellent correlation coefficients of 0.961 and 0.949 were found between partition coefficient and calculated hydrophobicity as can be seen in Fig. 4 which shows the partition coefficient of peptides with different numbers of amino acid residues. The slopes correspond to the numbers which give the relative values of the hydrophobicity.

Recent methods that have been developed to evaluate surface hydrophobicity of proteins such as the hydrophobic inbalance, HI, (26) and the statistical description of the surface amino acid distribution (27) which corresponds to a more rigorous description of the hydrophobic patches on the surface seem more appropriate to simulate the behavior of proteins in chromatography rather than in ATPS where the overall surface hydrophobicity is more important.

More recently mathematical and statistical models that use both the 3D structure and the amino acid composition

TABLE 2

Hydrophobic resolution (R), intrinsic hydrophobicity ($\log P_0$) and correlation coefficient (r). (From 12). In Eqs. (2) and (3). $\log P_0$ represents the intrinsic hydrophobicity of the given ATPS. $\text{Log } P_0 = \log P$ when $K = 1$; R represents the hydrophobic resolution which is the ability of the system to discriminate between proteins with different hydrophobicities

Systems	NaCl [%w/w]	R	Log P_0	r
PEG/phosphate	0	1.72	−0.30	0.59
	0.6	2.68	−0.29	0.74
	8.8	7.91	−0.45	0.92
PEG/sulfate	0	1.13	0.05	0.24
	0.6	1.98	−0.06	0.43
	8.8	8.51	−0.44	0.92
PEG/citrate	0	2.06	−0.31	0.81
	0.6	3.78	−0.31	0.86
	8.8	11.47	−0.42	0.93
PEG/dextran	0	0.88	−0.34	0.40
	0.6	2.76	−0.33	0.62
	8.8	2.49	−0.46	0.63

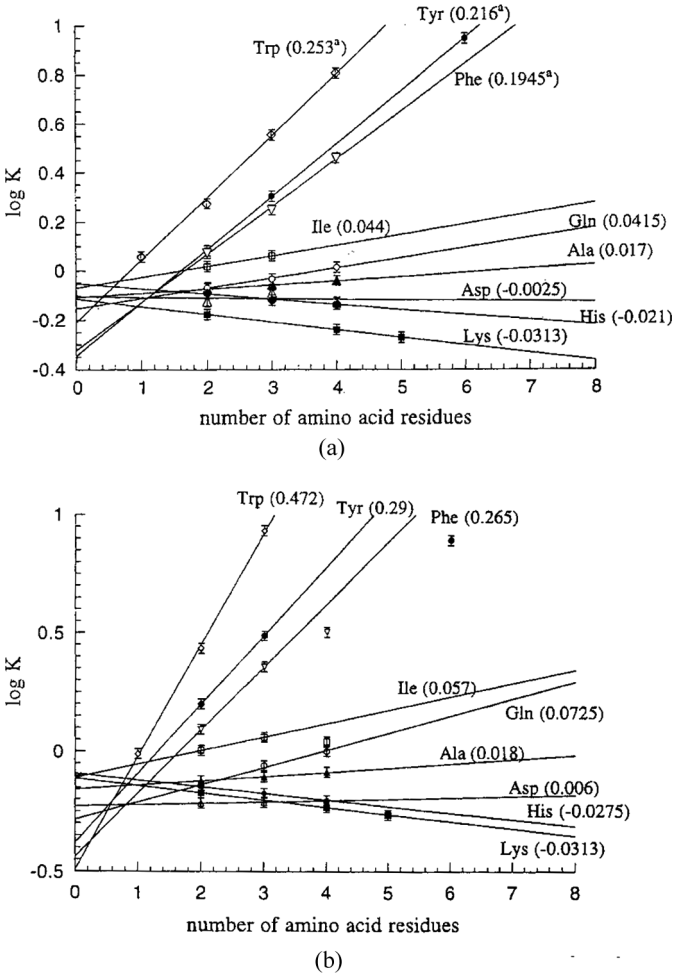

FIG. 4. Log K as a function of the number of amino acid residues a) 7.1% dextran/6.8% EO30PO70 and b) 9% dextran/9%EO30PO70. In brackets are the slope values which show the average contribution per residue to the log K for each peptide. (From 26).

of the proteins have been developed (28). Three mathematical models, ATPS I-III, based only on amino acid composition were evaluated. The best results were obtained with the ATPS I model which assumes that all of the amino acids are completely exposed. The correlation coefficients also improve as the NaCl concentration increases in the systems and therefore the effect of the protein hydrophobicity prevails over other effects such as charge or size. The ATPS I model exhibits the best correlation coefficients in PEG/dextran systems (0.88% NaCl). For the rest of the ATPS the correlation coefficients for the ATPS I model were higher than 0.67.

CONCLUSIONS

Hydrophobicity plays the key role in determining partition of proteins both in polymer/salt and polymer/polymer ATPSs. There are a range of different methods available that have been used to evaluate the hydrophobicity of proteins. These include: Hydrophobic Interaction Chromatography (HIC), Reverse Phase Chromatography (RPC), precipitation with ammonium sulphate (inverse of solubility), average surface hydrophobicity based on the 3-D structure, amino acid (AA) composition, hydrophobic imbalance (HI), and a statistical description of the AA distribution. Some of these consider the hydrophobicity of a protein, in solution, the "average" hydrophobicity and/or the "surface" hydrophobicity of a protein, as well as the "distribution" on the surface in hydrophobic "patches" (density distribution). The two methods that are more attractive, depending on the ATPS used (PEG/salt, PEG/polymer), are those that consider the 3-D structure and the hydrophobicity of AA on the surface (rather complex) and the one that uses the parameter $1/m^*$, which is the inverse of the protein solubility determined by precipitation and very simple to measure experimentally.

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