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Journal of Chromatography A, 668 (1994) 121–128

JOURNAL OF CHROMATOGRAPHY A

Polyethylene glycol–potassium phosphate aqueous two-phase systems
Insertion of short peptide units into a protein and its effects on partitioning

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Abstract

Two different tetrapeptides, AlaTrpTrpPro and AlaIleIlePro, were inserted near the C-terminus of the protein ZZT0. The Trp-rich peptide unit strongly increased both the partitioning of ZZT0 into the polyethylene glycol (PEG)-rich phase in a PEG–potassium phosphate aqueous two-phase system and its retention on PEG and propyl hydrophobic interaction chromatographic columns with potassium phosphate as eluent. Both the partitioning and the retention increased with increasing number of Trp-rich peptide units inserted into ZZT0. Insertion of Ile-rich tetrapeptide units affected the partitioning and retention to a much lesser extent. Partition data also indicated a folding of inserted Trp tetrapeptides units, probably to minimize their water contact.

1. Introduction

Polyethylene glycol (PEG)–salt-based aqueous two-phase systems are used in protein purification processes. The distribution of a protein in such systems can be described with the partition coefficient, $K_p = C_t/C_b$, where C_t and C_b are the protein concentrations in the top and bottom phases, respectively. During process optimization one has to compromise between concentration and yield of the target protein, the compromise being dependent on K_p. K_p is governed by several factors, some of which can be related to the system parameters and others to the target protein [1]. It is well established that an increase in the molecular mass of a phase-forming polymer, in this instance PEG, generally leads to a decrease in K_p [2]. This can be attributed to the so-called excluded volume mechanism, where a large volume occupied by PEG molecules squeezes proteins out of solution into the salt-rich bottom phase [3,4]. Charge effects also seem to be involved and an increased negative net charge (pI < pH of system) has been shown to favour the partitioning into the PEG-rich top phase [5]. In addition there are other system modifications that can be used for tuning the distribution of a protein, such as addition of other salts [6,7] or binding of affinity ligands to PEG [8].

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0021-9673/94/$07.00
SSDI 0021-9673(94)00024-4

The target protein behaviour is determined by the interaction of the surface exposed amino acid side-chains of the folded protein with solvent and solutes in the phase system. By changing or adding amino acids with charged, polar or non-polar residues to a protein, it should be possible to alter its partitioning.

Although a general picture of the features governing protein partitioning in PEG–salt systems can be given, the molecular mechanisms behind it are poorly understood. There is need for partition experiments performed with well characterized model peptides and proteins. We are using genetic engineering to modify a protein with respect to its content of short peptide units [9]. Several techniques will be used to probe the PEG–peptide and PEG–protein interactions. In this paper, we describe results obtained from partitioning in PEG–potassium phosphate aqueous two-phase systems. We also describe the interaction with two different solid surfaces, (i) PEG and (ii) propyl (C_3), measured as retention on packed-bed chromatographic columns.

2. Experimental

2.1. Chemicals

PEG 4000 (M_r 3500–4500) was obtained from Merck–Schuchardt, Germany. Yeast extract and casamino acids were obtained from Difco. IgG-Sepharose Fast Flow for affinity chromatography was purchased from Kabi Pharmacia. The tetrapeptide AlaTrpTrpPro was custom-made by the BM Unit, University of Lund. Indole-3-acrylic acid and dipeptide TrpTrp were purchased from Sigma. All other chemicals were of analytical-reagent grade.

2.2. Model proteins

The model proteins are shown in Fig. 1. Z is a hydrophilic synthetic IgG binding domain derived from the B domain of staphylococcal protein A (SpA) [10]. The three-dimensional solution structure of the recombinant B domain

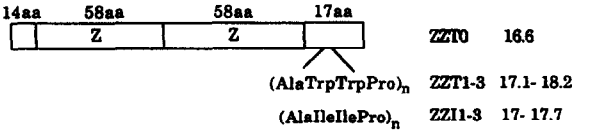

Fig. 1. Schematic structure of model proteins. ZZT0 is the control protein into which the two different tetrapeptide units, AlaTrpTrpPro and AlaIleIlePro, have been inserted. Proteins with up to three repeating peptide units inserted have been produced so far. Right-hand column: $M_r \times 10^{-3}$.

of SpA is composed of a bundle of three α-helices with most of the hydrophobic residues buried in the interior of the bundle [11]. The conformation of the C-terminal peptide stretch, into which the different peptide units are cloned, is not known. The design, cloning and cultivation of plasmid harbouring *Escherichia coli* RV308 and purification of ZZT0, ZZT1 and ZZT3 have been described previously [9]. ZZT2, ZZI1, ZZI2 and ZZI3 were produced following the same protocols. The IgG-binding property of Z was utilized for the purification of the different proteins and eluted fractions were freeze-dried and stored at −20°C until used. Relative amounts of full-length protein after the IgG step were in all instances except one ≥95% when determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. For ZZT2 it was *ca.* 60% (not shown).

2.3. Partition experiments

Aqueous two-phase partition experiments were performed with purified preparations of the proteins, the dipeptide TrpTrp and the tetrapeptide AlaTrpTrpPro. Two different protocols were used for the partition experiments. In both instances phase systems were prepared from 40% (w/w) stock solutions of PEG 4000 and potassium phosphate (dibasic/monobasic phosphate mole ratio = 1.42, corresponding to a pH of *ca.* 7). In the first instance phase systems were made up directly to their final total masses (5 or 10 g) in graduated centrifuge tubes by weighing in PEG and potassium phosphate from the stock

solutions and distilled water. Before the addition of the sample, which had been dissolved in distilled water to a desired concentration, the contents were thoroughly mixed. The phase systems were chosen as to obtain a top-to-bottom phase volume ratio of about 1:1, in order to provide more accurate sampling procedures. After a second mixing of the phases for 5 min, the phase systems were incubated at the desired temperature (indicated in the figure captions) for 5 min, centrifuged at 500 *g* for 4 min and incubated at the same temperature for a final 5 min. After reading the phase volume, samples from the top and bottom phases were carefully withdrawn with Pasteur pipettes for analysis. This procedure was followed in the partitioning of ZZT_n, ZZI_n and AlaTrpTrpPro.

In the second procedure, phase systems of the required compositions were made up in separation funnels (100–200 g total mass), mixed and left overnight for demixing of phases at a given temperature (indicated in the figure captions). Desired volumes (2–10 ml) of each of the equilibrated phases were removed and after addition of the solid sample and vortex mixing for 2 min the phase system was equilibrated overnight at the same temperature as previously. Finally, samples from the top and bottom phases were carefully withdrawn with Pasteur pipettes for analysis. This protocol was applied in the TrpTrp partition experiments.

Partition coefficients, K_p, were measured spectrophotometrically using absorbances at either 220 or 280 nm. Each top and bottom phase sample was referenced against an identical, but protein-free, top or bottom phase, respectively.

The phase system compositions (Fig. 2) used correspond to different tie-lines. Tie-lines describe the equilibrium in the phase system, *i.e.*, in this instance between PEG 4000-rich top phases and potassium phosphate-rich bottom phases, and with increasing length of the tie-line the difference in the composition of the phases increases. Although tie-lines have not yet been determined for the compositions in this phase system, the tie-line lengths for corresponding compositions can be assumed to increase in the order $TL_a \approx TL_b < TL_c < TL_d < TL_e < TL_f$.

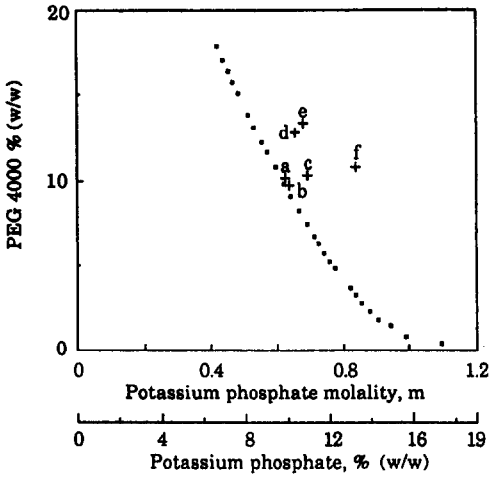

Fig. 2. The binodial (■) of a PEG 4000–potassium phosphate aqueous two-phase system (dibasic/monobasic phosphate mole ratio = 1.42) at 20°C [27]. Phase compositions indicated (+) are those referred to in this study used for partitioning of the dipeptide TrpTrp, the tetrapeptide AlaTrpTrpPro and ZZT_n and ZZI_n proteins. Phase compositions of PEG 4000 potassium phosphate are (a) 10.1:9.9, (b) 9.7:10.1, (c) 10.3:11.0, (d) 12.8:10.4, (e) 13.3:10.8 and (f) 10.8:13.3% (w/w).

2.4. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) was performed on two different column packings: (i) PEG HIC was run on a Hydropore-HIC Dynamax-TI bed, 5-μm packing, pore size 300 Å, 100 mm × 4.6 mm I.D., from Rainin Instrument; (ii) propyl (C_3) HIC was performed on a Bakerbond Wide-Pore HI-Propyl bed, 5 μm packing, pore size 300 Å, 50 mm × 4.4 mm I.D., from J.T. Baker. On both columns the ligands were covalently attached to a hydrophilic base matrix, which covers the wide-pore silica. Freeze-dried protein samples were dissolved in potassium phosphate (dibasic/monobasic phosphate mole ratio = 1.42) solutions of the same concentration as the elution buffer and spun on an Eppendorf centrifuge at 11 000 *g* for 5 min to remove solids. The injected sample volumes were 50 μl and the flow-rates were between 0.5 and 1.0 ml min^{-1}. Protein elution was followed at either 220 or 280 nm.

3. Results and discussion

The understanding of the partition behaviour of amino acids and short peptides is a starting point for understanding the partitioning of more complex structures such as peptides and proteins. Our concept based on genetic engineering of a model protein (*i.e.*, ZZT0, described under Experimental) allows us to study the interaction of PEG with short peptide units both as free molecules and as protein inserts. The reasons for selecting Trp as the first test amino acid were twofold. First, we know that *E. coli* β-galactosidase can be partitioned strongly into the PEG phase in a PEG 4000–potassium phosphate aqueous two-phase system [12,13], a property which has been utilized in process design [13]. What surface properties of β-galactosidase with a molecular mass of 460 000 prevent its exclusion from the PEG phase? Interestingly, β-galactosidase has an unusually high content of the amino acid Trp compared with the average *E. coli* protein, 3.8% in β-galactosidase [14] and 1% in average *E. coli* proteins [15]. Second, experiments with depeptides in PEG–phosphate aqueous two-phase systems (pH 7) have shown that Trp is the only amino acid to cause enhanced partitioning into the PEG-rich top phase [16]. Hence it may be that the partitioning of β-galactosidase reflects the fact that some of its 156 Trp residues are exposed at the protein surface.

3.1. Partitioning and HIC

Partition experiments with the ZZT_n proteins in PEG 4000–potassium phosphate systems (dibasic/monobasic phosphate mole ratio = 1.42) showed that with increasing number of Trp peptide units inserted the stronger was the distribution of the protein to the PEG-rich phase (Fig. 3) [9]. This was interpreted to be related to the exposure of Trp residues and their specific interaction with PEG. Thus, qualitatively, the importance of Trp as a partition enhancer was maintained when incorporated into a protein.

In another set of experiments, Ile, an aliphatic non-polar amino acid, was inserted into ZZT0 as a tetrapeptide unit. It should be stressed that the solubility of ZZT0 in the phase system was lowered more significantly by the insertion of Ile-rich peptides compared with the Trp-rich peptides, as clearly demonstrated by the non-quantitative total recoveries of protein ZZI2 (*ca.* 65%) and ZZI3 (*ca.* 25%) (Fig. 3). Keeping the solubility reasoning in mind, it is still possible to conclude when comparing the partitioning of ZZI_n ($n = 1, 2, 3$) and ZZT_n ($n = 1, 3$) proteins that the insertion of Ile residues into ZZT0 had less impact on directing its partitioning into the PEG-rich top phase (Fig. 3). This is in agreement with the partition behaviour of Ile when part of a short peptide (see Fig. 5) [16].

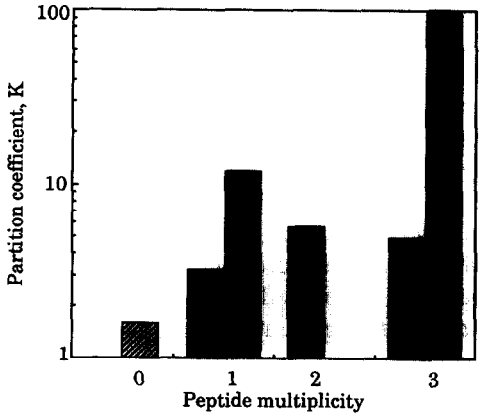

Fig. 3. Partition coefficients of (hatched box) ZZT0, (solid boxes) ZZI_n proteins and (screened boxes) ZZT_n proteins in a 13.3% (w/w) PEG 4000–10.8% (w/w) potassium phosphate (dibasic/monobasic phosphate mole ratio = 1.42) aqueous two-phase system (composition e, Fig. 2) at different tetrapeptide multiplicities, *n*. Partition experiments for ZZT_n and ZZI_n proteins were run at 20 and 22°C, respectively. Protein concentrations in the phase systems were approximately ZZT0 1, ZZT1 0.2, ZZT3 0.1, ZZI1 0.1, ZZI2 0.1 and ZZI3 0.1 mg ml^{-1}. Concentrations of ZZT_n and ZZI_n proteins in equilibrated phases were measured at 280 and 220 nm, respectively. Recoveries were about 100% for all proteins except ZZI2 and ZZI3, for which they were about 65% and 25%, respectively.

Another way to characterize the interaction of peptides and proteins with PEG in aqueous solutions is to use systems where PEG has been immobilized on a solid surface. This gives the possibility of varying the potassium phosphate concentration much more freely than in the

phase system where one has to consider the phase formation. The potassium phosphate composition at the critical point gives the highest and the lowest potassium phosphate concentrations possible in the PEG-rich top phase and the potassium phosphate-rich bottom phase, respectively. In the particular system used here (20–23°C), compositions a and b are close to the critical point as judged by their phase volume ratios. Therefore, the potassium phosphate concentration of the critical point is concluded to be about 0.6 molal (*ca.* 9.5%, w/w) (Fig. 2). Retention of ZZT3 on the commercial PEG HIC column occurred in a pure aqueous solution, whereas for the other proteins one had to use molal potassium phosphate concentrations above 0.5 to obtain similar retentions (Fig. 4A). The results obtained on the HIC column with the model proteins correlated qualitatively with their behaviour in the PEG–potassium phosphate aqueous two-phase system. This was expected as the separations with both techniques are promoted by water-structuring salts (see below).

The application of the more hydrophobic propyl-based HIC column led to a stronger retention in general for all the model proteins in comparison with the PEG column (Fig. 4B). Interestingly, the greatest impact on the retention behaviour on ZZT0 was still caused by Trp, whereas Ile affected the retention to a much smaller extent. Thus, on both the PEG and C_3 columns available Trp residues seem to play a much more pronounced role than Ile in the retention behaviour of proteins. Whereas the aliphatic side-chain of Ile is chemically inert owing to its non-polar character and absence of functional chemical groups, the side-chain of Trp is much more chemically reactive. The indole side-chain of Trp is polar and is the only amino acid side-chain capable of entering into charge-transfer interaction with electrophilic structures. The pyrrole nitrogen of the indole ring can act as a hydrogen donor in hydrogen bond interactions [17].

According to HIC theory, (i) the limiting slope of the plot of ln k' against the salt molality is proportional to the contact surface area between the protein and the column and (ii) the

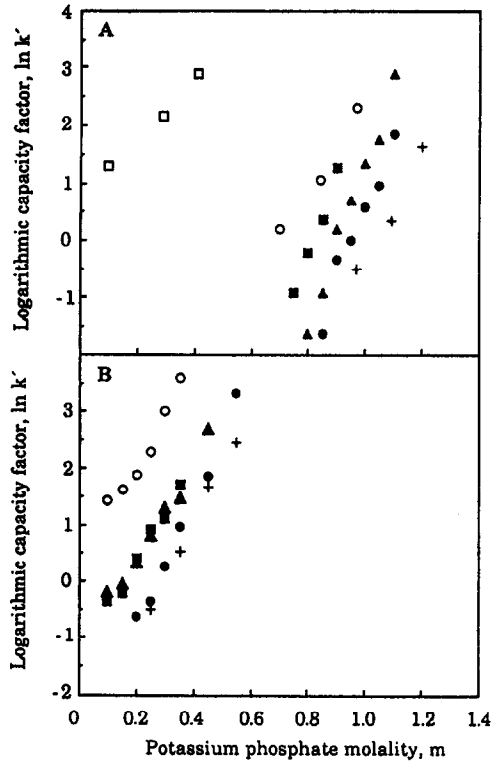

Fig. 4. Hydrophobic interaction chromatography of ZZT_n and ZZI_n proteins on (A) PEG- and (B) C_3 (propyl)-based columns. + = ZZT0; ○ = ZZT1; □ = ZZT3; ● = ZZI1; ▲ = ZZI2; ■ = ZZI3. The retention, expressed as the logarithmic capacity factor (ln k'), is plotted as a function of potassium phosphate concentration (dibasic/monobasic phosphate mole ratio = 1.42). The capacity factor is defined as $k' = (V_r/V_0) - 1$, where V_r and V_0 are the retention and void volumes, respectively. Approximate protein amounts loaded were: ZZT_n = 0.1–0.4 mg and ZZI_n = 0.05–0.1 mg for PEG HIC and ZZT_n = 0.05–0.1 mg and ZZI_n = 0.05 mg for C_3 HIC. Retention of ZZT_n proteins on the PEG column was detected at 280 nm. For all other runs 226 nm was used to follow protein elution. The void volumes were determined by measuring the retention of an injected aqueous pulse.

intercept on the ordinate of the same plot seems to characterize the strength of the interaction between the protein and the column [18,19]. If this is considered for the ZZT_n and ZZI_n proteins (Fig. 4), one recognizes that there is a much more significant shift in the position of the curves than in their slopes which could imply that the insertions of the two different peptide units into ZZT0 affected the strength of the

interactions more strongly than the contact surface area between the protein and either PEG or C_3. One exception to this could be protein ZZT3, where clearly both the limiting slope and the intercept are shifted.

3.2. Structural changes and interaction behaviour

An important issue, also when proteins are partitioned in PEG–potassium phosphate systems, is to understand the relationship between the conformation of a protein and its interaction with the surrounding environment. It is of importance for limiting target protein loss but also possibly as a tool for improving selectivity, *e.g.*, in the extraction. The structural effects of inserting various peptides into ZZT0 could be partly understood by comparing the partitioning of peptide units, the model protein ZZT0 and a protein in which a peptide unit has been inserted, ZZT1. A partition model for interacting molecules [20] can be applied:

$$K_{ab} = K_a K_b$$

where the individual partition coefficients for molecules a and b are used to predict the partition coefficient for the coupled/fused molecule ab. The equation is assumed to hold if the linking of molecules a and b does not change the structure of either of the two molecules involved. The model was tested on ZZT1 for different phase compositions by comparing measured K_{ZZT1} values with the calculated values, the latter obtained from K values for ZZT0 and TrpTrp. As TrpTrp, but so far, not AlaTrpTrpPro has been partitioned in exactly the same phase compositions as ZZT0 and ZZT1, this dipeptide was used for the comparison. That TrpTrp is a reasonable model for AlaTrpTrpPro is shown in Fig. 5. They have similar K values when partitioned in the PEG 4000–potassium phosphate systems and the K values of the two molecules follow a logical change with increasing tie-line length. Nevertheless, the model calculations revealed that $K_{ZZT1,\ measured} < K_{ZZT1,\ calculated}$ for all three

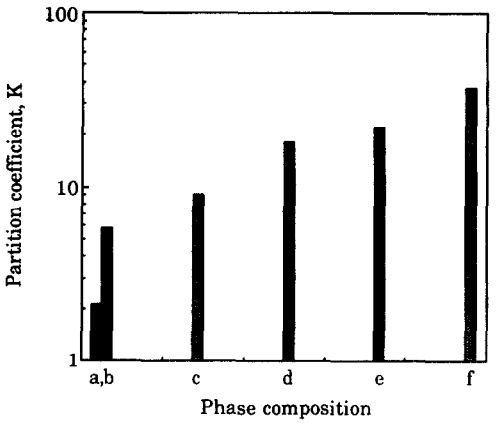

Fig. 5. Partitioning of the tetrapeptide AlaTrpTrpPro (screened boxes) in a PEG 4000–potassium phosphate (dibasic/monobasic phosphate mole ratio = 1.42) aqueous two-phase system at three different phase composition: a, c and f (indicated in Fig. 2). For comparison partition data for the dipeptide TrpTrp (solid boxes) at three different phase compositions, b, d and e, have been included (see Fig. 6) for TrpTrp details). The tie-lines (TL) corresponding to each phase composition increase in length in the order $TL_a \approx TL_b < TL_c < TL_d < TL_e < TL_f$. The partition temperature was 21°C. The total concentration of AlaTrpTrpPro in the phase systems was 0.4 mg ml^{-1} and the concentrations in equilibrated phases were measured at 280 nm. Recoveries were about 100%.

compositions tested (Fig. 6). A reasonable explanation is that the tetrapeptide unit folds together with the C-terminal peptide region into which it was inserted in such a way that it thereby becomes less accessible to interaction with the solvent. An additional effect of the tetrapeptide insertion could be a disturbance of the closest Z domain structure, which in turn could have an impact on the partitioning. A possible increase in the Z domain flexibility was indicated by an increase in the susceptibility to *in vivo* proteolysis with increasing number of AlaTrpTrpPro units inserted into ZZT0 [21].

The possibility of Trp playing an active role in the partitioning will be determined by its surface accessibility in each particular protein. Clearly the Trp residues in the ZZT_n proteins are still available for interaction, as revealed by the partitioning and chromatographic behaviours. Although a Trp residue, owing to its hydrophobic character, could be expected to prefer the

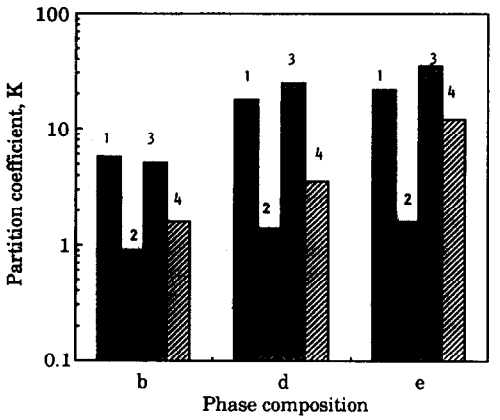

Fig. 6. Test of the model for interacting molecules, $K_{ZZT1} = K_{TrpTrp}K_{ZZT0}$, at three different phase compositions: b, d and e indicated in Fig. 2. The tie-lines (TL) corresponding to each phase composition increase in length in the order $TL_b < TL_d < TL_e$. Partition experiments for dipeptide and ZZT proteins were run at 22 and 20°C, respectively. Dipeptide and protein total concentrations in the phase systems were approximately TrpTrp 0.3, ZZT0 1 and ZZT1 0.2 mg ml^{-1}. Dipeptide and protein concentrations in equilibrated phases were measured at 280 nm. Recoveries were about 100% for all partitioned molecules. $1 = K_{TrpTrp}$; $2 = K_{ZZT0}$; $3 = K_{ZZT1}$ calculated; $4 = K_{ZZT1}$ measured.

interior of a protein, avoiding contact with the aqueous medium, it is also known that parts of it can be exposed on the surface of properly folded proteins [22]. Currently we are using an optical technique (total internal reflection fluorescence) to probe the accessibility of Trp residues in the C-terminal peptide stretch of ZZT0. The accessibility of the Ile residues in the C-terminal peptide stretch will be much more difficult to assess owing to a lack of suitable optical properties of Ile, although some information about the exposure of inserted Ile residues will be achieved when we apply the model for interacting molecules (see above) to AlaIleIlePro and ZZI_n proteins.

Finally, let us consider briefly the role that PEG and phosphate ions play as protein stabilizers [23]. Protein stabilizers seem to obey a general rule: co-solvents which, at high concentration, stabilize the native structure of proteins are preferentially excluded from the surface of a protein. On the one hand, phosphate ions (especially dibasic and tribasic), which are excluded from the protein surface through a mechanism referred to as the surface tension effect, stabilize folded protein structures under different types of physico-chemical conditions (*e.g.*, elevated temperatures, extreme pH). The stabilizing properties of salt ions (and salting-out effectiveness) increase with their effectiveness in increasing the surface tension of water. The formation of the PEG–phosphate system can be seen as salting-out of PEG by phosphate, which is related to the surface tension effect of phosphate on water. On the other hand, the interaction of PEG with proteins will depend to a great extent on the surrounding conditions. Under stabilizing conditions proteins are excluded from PEG through steric exclusion. However, under denaturing conditions, when a protein structure becomes more flexible or starts to unfold, PEG, owing to its hydrophobic character, can bind to unfolded proteins and folding intermediates. This property of PEG has been explored for the enhancement of protein refolding [24]. PEG is described as a strong hydrogen bond acceptor owing to the paired ether oxygen electrons, which provides for its water solubility [25]. It can form complexes with monomeric and polymeric electron acceptors (*e.g.*, hydrogen bond donors) [26]. A specific interaction between PEG and a protein UV 280-nm chromophore has been suggested to occur as an explanation for the large perturbations of the UV spectra of proteins in PEG solutions [22]. The chromophore could well be that of Trp.

4. Conclusion

Protein partitioning in a PEG–phosphate aqueous two-phase system could be described as a delicate balance between exclusion from the phosphate-rich phase by the surface tension effect, exclusion from the PEG-rich phase by the steric effect and/or a specific PEG–protein interaction, a limiting factor being the maximum solubility of the protein in the phase system.

A specific salt-promoted PEG–protein interaction via the Trp side-chain was indicated in this work. First, we have shown that Trp strongly

directed the partitioning of a model protein into the PEG-rich phase in a PEG 4000–potassium phosphate aqueous two-phase system and increased its retention on a PEG column with potassium phosphate as eluent. Both the partitioning and the retention increased with increasing number of Trp-rich peptide units inserted. Second, insertion of Ile-rich tetrapeptide units affected the partitioning and retention to a much smaller extent. The results also illustrated a relationship between PEG–phosphate partitioning and PEG HIC.

The model proteins showed a similar behaviour on C_3 and PEG columns, the only major difference being the stronger retention of each protein on the former.

Partition data also indicated a folding of inserted Trp tetrapeptides units, probably to minimize their contact with water. In future work to characterize PEG–salt interactions we would like to probe protein structure and structural changes more carefully and see how they are related to the interaction with PEG. In addition to methods described in this paper, we are now also using techniques such as ellipsometry, total internal reflection fluorescence spectrometry and microcalorimetry.

5. Acknowledgement

This investigation was supported by grants from Teknikvetenskapliga Forskningsrådet (Swedish Research Council for Engineering Sciences).

6. References

[1] J. Huddleston, A. Veide, K. Köhler, J. Flanagan, S.-O. Enfors and A. Lyddiatt, *Trends Biotechnol.*, 9 (1991) 381.
[2] P.-Å. Albertsson, *Partition of Cell Particles and Macromolecules*, Wiley-Interscience, New York, 3rd ed., 1986.
[3] K.C. Ingham, *Arch. Biochem. Biophys.*, 184 (1977) 59.
[4] T.F. Busby and K.C. Ingham, *Vox Sang.*, 39 (1980) 93.
[5] J.G. Huddleston, K.W. Ottomar, D.M. Ngonyani and A. Lyddiatt, *Enzyme Microb. Technol.*, 13 (1991) 24.
[6] H.L. Chiang and S.S. Wang, *Biotechnol. Techn.*, 2 (1988) 283.
[7] R.A. Hart and J.E. Bailey, *Enzyme Microb. Technol.*, 13, (1991) 788.
[8] S.D. Plunkett and F.H. Arnold, *Biotechnol. Techn.*, 4 (1990) 45.
[9] K. Köhler, C. Ljungquist, A. Kondo, A. Veide and B. Nilsson, *Bio/Technology* 9 (1991) 642.
[10] B. Nilsson and L. Abrahmsén, *Methods Enzymol.*, 185 (1990) 144.
[11] H. Gouda, H. Torigoe, A. Saito, M. Sato, Y. Arata and I. Shimada, *Biochemistry*, 31 (1992) 9665.
[12] L. von Bonsdorff-Lindeberg, *Licentiate Thesis*, Helsinki University of Technology, Esbo, 1989.
[13] A. Veide, T. Lindbäck and S.-O. Enfors, *Enzyme Microb. Technol.*, 6 (1984) 325.
[14] A. Kalnins, K. Otto, U. Rüther and B. Müller-Hill, *EMBO J.*, 2 (1983) 593.
[15] F.C. Neidhardt, in F.C. Neidhardt (Editor), Escherichia coli *and* Salmonella typhimurium. *Cellular and Molecular Biology*, Vol. 1, American Society of Microbiology, Washington, DC, 1987, p. 3.
[16] A.D. Diamond, K. Yu and J.T. Hsu, in M.R. Ladish, R.C. Wilson, C.C. Painton and S.E. Builder (Editors), *Protein Purification: from Molecular Mechanisms to Large-Scale Processes* (*American Chemical Society Symposium Series*, No. 427), American Chemical Society, Washington, DC, 1990, p. 52.
[17] T.E. Creighton, *Proteins, Structure and Molecular Properties*, Freeman, New York, 1984.
[18] J.L. Fausnaugh and F.E. Regnier, *J. Chromatogr.*, 359 (1986) 131.
[19] L. Szepesy and G. Rippel, *LC · GC Int.*, 5 (1992) 24.
[20] P.-Å. Albertsson, *Methods Biochem. Anal.*, 29 (1983) 1.
[21] S. Yang, A. Veide and S.-O. Enfors, *Eur. J. Biochem.*, submitted for publication.
[22] M. Laskowski, Jr., *Fed. Proc.*, 25 (1966) 20.
[23] S.N. Timasheff and T. Arakawa, in T.E. Creighton (Editor), *Protein Structure: a Practical Approach*, IRL Press, Oxford, 1990, Ch. 14, p. 331.
[24] J.L. Cleland, S.E. Builder, J.R. Swartz, M. Winkler, J.Y. Chang and D.I.C. Wang, *Bio/Technology*, 10 (1992) 1013.
[25] F.E. Bailey, Jr., and J.V. Koleske, *Poly(ethylene oxide)*, Academic Press, New York, 1976.
[26] F.W. Stone and J.J. Stratta, in H.F. Mark, G. Gaylord and N.M. Biales (Editors), *Encyclopedia of Polymer Science and Technology*, Vol. 6, Wiley-Interscience, New York, 1966, p. 103.
[27] K. Köhler, L. von Bonsdorff-Lindeberg and S.-O. Enfors, *Enzyme Microb. Technol.*, 11 (1989) 730.