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Effects of fused tryptophan rich peptides to a recombinant protein A domain on the partitioning in polyethylene glycol–dextran and Ucon–dextran aqueous two-phase systems

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Abstract

Genetic engineering has been used to construct fusion proteins with tryptophan containing peptides. The peptides and the fusion proteins have been partitioned in aqueous two-phase systems of poly(ethylene glycol) (PEG)–dextran and Ucon–dextran. The studied model protein was ZZT0, where Z is an engineered domain of domain B of staphylococcal protein A. The specially designed hydrophobic peptides, Ala-Trp-Trp-Pro (T1) and $(Ala\text{-}Trp\text{-}Trp\text{-}Pro)_2$ (T2), have been inserted into ZZT0, to give the peptide–protein fusions ZZT1 and ZZT2. In the experimental studies it was found that T1 and T2 preferred the PEG phase and even more the Ucon phase over the dextran phase. For T2 the partitioning was more one sided than for T1. For the fusion proteins, ZZT1 and ZZT2, the partitioning was enhanced into the PEG or Ucon rich phase as compared to ZZT0. The effects were lower than expected from independent contributions to the partition coefficient from the protein and the peptides. A heterogeneous lattice model was used to calculate theoretical peptide and protein partition coefficients. The calculations could reproduce the qualitative features of the experimental data. The model results suggest that a part of these experimentally observed effects is due to a depletion zone, i.e. a zone of reduced polymer concentration around the protein. The experimental results indicate a further reduction of the partition coefficient, beyond that predicted by the lattice calculations. A possible folding of the inserted peptide is discussed as a plausible mechanism for this further reduction in the partition coefficient.

Keywords: Partitioning; Aqueous two-phase systems; Thermoseparating polymer; Heterogeneous lattice model; Protein A

1. Introduction

For the purpose of separating, purifying or characterising biological macromolecules and cell particles, the method of partitioning in aqueous two-phase systems has found widespread use in various areas of biochemical research [1,2]. Also in the field of biotechnology there is an increasing interest in the technique for purification of recombinant proteins, since these systems may have an important use as means of non-denaturing extraction. Protein engineering techniques offer new possibilities to increase the effectiveness of extraction of recombinant proteins. As a way to enhance target protein selectivity in the partition systems, fusion of proteins or peptides to the target protein has been investigated

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[3–6]. The partition in a poly(ethylene glycol) (PEG)–salt system of a family of such fused proteins, ZZT*n*, where Z is an engineered domain of domain B of staphylococcal protein A [7] together with the tryptophan-containing peptides T*n* (=(Ala-Trp-Trp-Pro)$_n$) and the mother protein ZZT0 of which they are composed, has recently been examined, as well as the retention in hydrophobic interaction chromatography [6].

In this study we have examined the partition behaviour of the ZZ protein derivatives and tryptophan containing peptides in (i) PEG–dextran and (ii) Ucon–dextran aqueous two-phase systems. The Ucon polymer is a random ethylene oxide (EO)–propylene oxide (PO) copolymer of equal EO and PO content. PEG and Ucon are both thermoseparating polymers with cloud points (CP) of >100°C and approximately 50°C, respectively [8,9]. This property has been utilised for creating a recycling process for Ucon where, after a first separation step in a Ucon–dextran system, the phase rich in the thermoseparating polymer is recovered in a separate container and the temperature is raised above the CP. This leads to a new phase separation where the new top and bottom phases consist mainly of water and Ucon, respectively [10]. Proteins partition almost exclusively to the water phase in this second step and a separation of the protein and polymer is obtained. Key parameters for the outcome of this two step extraction procedure are (i) the yield of target protein in the primary phase system which has to be optimised, and (ii) the CP of the thermoseparating polymer which has to be as low as possible to avoid protein denaturation [11,12].

Genetic engineering gives the possibility to change protein properties in a systematic and planned way so the interactions dominating in the system can be altered. By studying the partition behaviour of ZZ protein derivatives in the described phase systems we aim to (i) gain increased understanding of the mechanism which govern protein partitioning, and make use of this knowledge to (ii) design peptide–protein fusion handles which can effectively enhance partitioning of a target protein to a thermoseparating EO–PO copolymer phase.

The experimental findings are discussed and interpreted in the light of theoretical calculations in a self-consistent mean-field lattice model of polymer solutions of the Scheutjens–Fleer type [13,14]. This type of model (extended to cover the case of a spherical lattice) has been used in a number of papers to study various general aspects of protein partition in polymer two-phase systems [15–17]. It has also been adapted to treat affinity partition [18,19] and partition of proteins with grafted polymers [20].

2. Experimental

2.1. Polymers and chemicals

The bottom phase polymer dextran T500, with a molecular mass 500 000, was obtained from Pharmacia Biotech (Uppsala, Sweden). The top phase polymers PEG 4000 and Ucon 50-HB-5100, having molecular masses of 4000, were obtained from Merck (Munich, Germany) and Union Carbide (New York, NY, USA), respectively. All chemicals were of analytical grade.

2.2. Peptides and proteins

The peptide T1 (=Ala-Trp-Trp-Pro) was synthesised by Bio Molecular Resource Facility, Lund University (Lund, Sweden) and T2 (=(Ala-Trp-Trp-Pro)$_2$) was synthesised by Scandinavian Peptide Synthesis (Köping, Sweden).

The procedures used to clone and cultivate the protein producing *Escherichia coli* RV 308 strain, as well as to purify the synthesised proteins, were as described elsewhere [3]. The proteins are shown schematically in Fig. 1. The structural element denoted Z is a synthetic immunoglobulin G (IgG) binding domain derived from the B domain of staphylococcal protein A [7] with a p*I* of about 4.8 [21]. The structure of the Z domain in solution is formed by a bundle of three α-helices [22]. The solution conformation of the stretch of 17 amino acid residues composing the C-terminal part of the ZZT0 protein is not known, neither is that of the 21 or 25 residues stretch of ZZT1 and ZZT2, with the tetrapeptide T1 or the octapeptide T2, respectively, inserted before the 8 last residues of the C-terminus of ZZT0 [6].

The IgG-binding property of Z was utilised for the purification of the different derivatives with chroma-

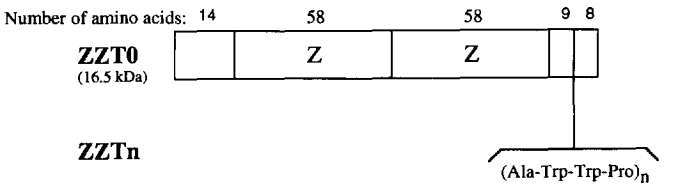

Fig. 1. The structure of the ZZT0 based model proteins. Z is a synthetic IgG-binding domain derived from staphylococcal protein A.

tography. The purity of isolated proteins (as judged by reduced sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was ≥95% in all cases except for ZZT2 where it was about 60%. Therefore, ZZT2 was further purified on a PEG-based column (Hydrophore-HIC Dynamax-T1 bed, 5 μm bead, pore size 300 Å, 100×4.6 mm I.D., from Rainin, Woburn, MA, USA) under isocratic conditions using a 0.5 molal potassium phosphate buffer, pH 7, followed by a desalting step on a gel filtration column (Pharmacia G25). All purified fractions were freeze-dried and stored at −20°C until further use.

2.3. Two-phase systems

For each system, the partitioning was studied at compositions representing two different tie lines, 6.0% PEG–5.7% dextran, 6.5% PEG–7.2% dextran, 4.4% Ucon–5.1% dextran and 4.6% Ucon–9.2% dextran, Fig. 2(a, b). The tie line length is defined as $[(C_{T,i}-C_{B,i})^2+(C_{T,j}-C_{B,j})^2]^{1/2}$, where C_T and C_B are the concentrations of the polymers i and j in top and bottom phases, respectively. The corresponding tie line lengths are shown in Table 1. At each of these points partition coefficients were determined for both the mother protein ZZT0 and the ZZT1 derivative, as well as for the free tetrapeptide T1. The partition coefficients of the ZZT2 protein derivative and the T2 octapeptide were only measured in the Ucon–dextran system. The ZZT2 protein had too low a solubility in the PEG–dextran system. To minimise the electrostatic contributions to the partition coefficients due to the net charge of the proteins, the systems were buffered to pH 5 by including 10 m*M* sodium acetate buffer (the p*I* of the Z-domains is 4.8). Stock solutions of 20% dextran, 40% PEG and 30% Ucon were prepared. The Ucon stock solution was ultra filtered (Filtron, Clinton, MA, USA) to remove impurities. Systems of a total

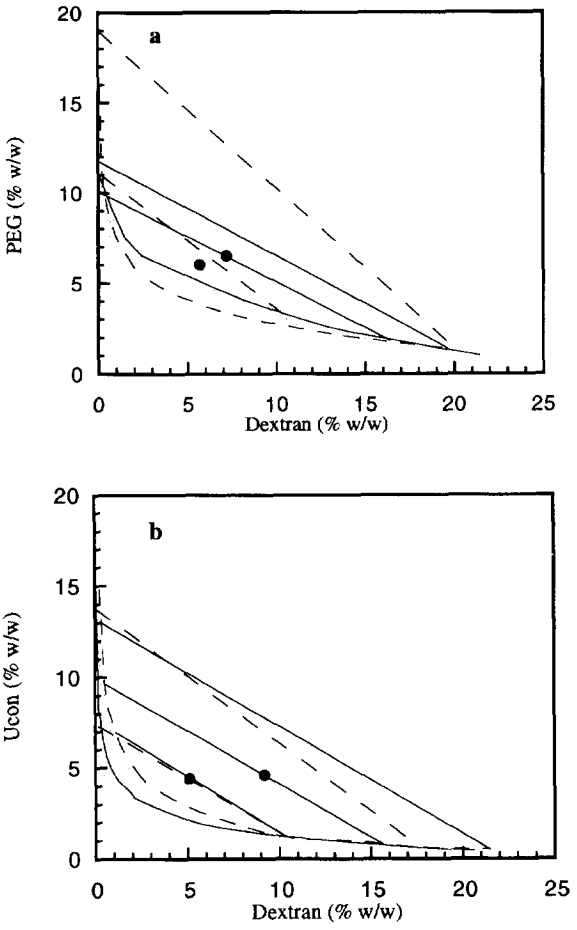

Fig. 2. (a) The experimental (—) and calculated (− −) phase diagrams of PEG 4000 and dextran T500 at 22°C. The following parameters were used: "PEG" 91 segments long, "dextran" 1000 segments long, $\chi_{(PEG-water)}=0.505$, $\chi_{(PEG-dx)}=0.11$, $\chi_{(dx-water)}=0.495$. (b) The experimental (—) and calculated (− −) phase diagrams of Ucon 50-HB-5100 and dextran T500 at 22°C. The following parameters were used: "Ucon" 78 segments long, "dextran" 1000 segments long, $\chi_{(Ucon-water)}=0.52$, $\chi_{(Ucon-dx)}=0.185$, $\chi_{(dx-water)}=0.495$. The calculated phase diagrams were designed to mimic the respective experimental phase diagram. The total compositions used for experimental partitioning are indicated by (●) on the tie lines.

Table 1
Experimentally measured and calculated partition coefficients (K) in the two systems

Substance	PEG–dextran		Ucon–dextran	
	Point 1 (TLL = 13.5%)	Point 2 (TLL = 18.0%)	Point 1 (TLL = 11.1%)	Point 2 (TLL = 17.5%)
Experimental data				
T1	1.2	1.4	1.4	1.8
T2	-	-	2.6	5.5
ZZT0	1.3	1.6	1.5	1.7
ZZT1	1.7	2.0	2.0	2.5
ZZT2	-	-	2.6	4.3
	(TLL = 13.5%)	(TLL = 18.3%)	(TLL = 11.0%)	(TLL = 17.6%)
Calculated data				
"T1"	1.4	1.5	1.5	1.9
"T2"	-	-	2.4	3.8
"ZZT0"	1.5	1.6	1.6	1.7
"ZZT1"	1.8	2.1	2.2	2.9
"ZZT1"[a]	1.7	2.0	2.1	2.7
"ZZT2"	-	-	2.9	4.8
"ZZT2"[a]	-	-	2.6	3.9

The experimental partition coefficients (upper part of the table), determined at $T = 22°C$ and pH 5.0, in 10 mM sodium acetate buffer, are mean values of two independent measurements. The lattice theory described in the text, designed roughly to mimic the PEG–dextran and Ucon–dextran systems, gives the calculated partition coefficients in the lower part of the table. The parameters used are those collected in Table 2, unless otherwise stated explicitly. Tie-line lengths (TLL) are indicated for each point.
[a] $RT\chi_{(P\ prot)} = -8.0$ kJ/mol.

weight of two grams were made up by weighing appropriate amounts of polymer stock solutions and H_2O in 10 ml graded and calibrated test tubes. The addition of peptides and proteins from pre-made stock solutions was based on volume to give final concentrations of 0.4 mg/ml for ZZT0 and ZZT1 and 0.2 mg/ml for ZZT2. The peptides T1 and T2 were used at final concentrations of 0.05 mg/ml.

The partition of a substance is described by the partition coefficient K, which is defined as $K = C_T/C_B$ where C_T is the concentration of the substance in the top phase and C_B is the concentration of the substance in the bottom phase. All partition coefficients are average values from two repeated experiments. The systems were equilibrated at room temperature and were left for 30 min and then centrifuged for three min at 3700 rpm. The top and bottom phases were separated and diluted 2–20 times. The phases were analysed for their protein or peptide content. The recombinant proteins ZZT0, ZZT1 and ZZT2 were analysed with Coomassie Brilliant Blue G at 620 and 465 nm [23] and with the respective protein as standard. The peptides T1 and T2 were analysed by subtracting the absorbance at 310 nm from the absorbance at 280 nm.

3. Description of the model

We make a consistent model of the protein partition in polymer two-phase systems by proceeding in two steps. In the first one, the compositions of the two phases are calculated by using the traditional many-component Flory–Huggins lattice theory for homogeneous polymer solutions [24–26] (Fig. 2a,b) whereas in the second step the partition of the protein between the two phases is calculated employing a heterogeneous lattice theory to evaluate the free energy change of introducing the protein in each of the two polymer solutions; the difference between the two free energy changes then gives the logarithm of the partition coefficient (Fig. 3) [17,19]. Below we give a brief description of the model for the heterogeneous system adapted to our purpose. For more formal derivations, we refer to some of the

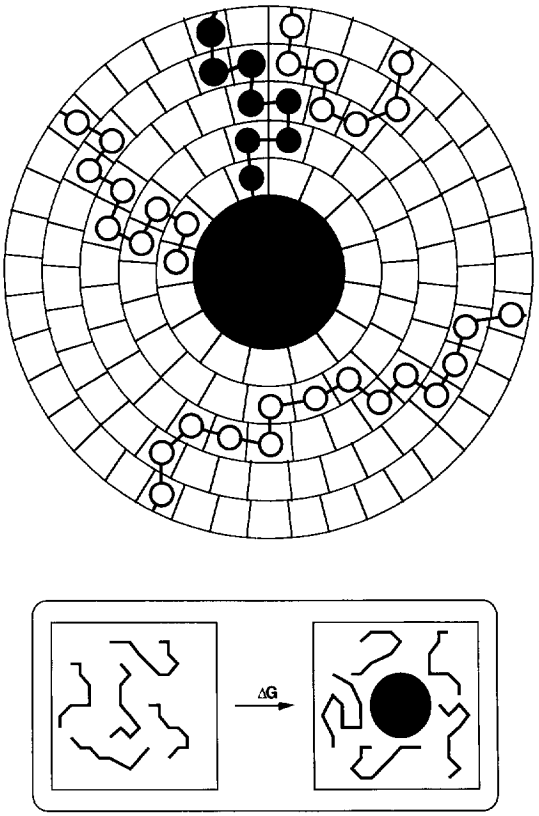

Fig. 3. (Top) Schematic two-dimensional illustration of the spherical lattice used, with the sphere representing the protein in the middle. Parts of three phase polymer chains are shown (unfilled circles), as well as a part of the C-terminal grafted peptide sequence (shaded circles). Unfilled lattice cells contain solvent. (Bottom) Schematic picture of the process for which the free energy change is determined in the heterogeneous lattice calculation.

detailed accounts published in the literature on polymers at interfaces [13,14,25–28].

The self-consistent description of the thermodynamics of polymer solutions near a surface, as initiated by Scheutjens and Fleer [13,14], is an extension of the Flory–Huggins lattice theory of homogeneous solutions. We consider a polymer solution in the close vicinity of a protein which is simply modelled as a hard sphere having a homogeneous surface. The space close to the surface is divided into layers parallel to the surface, each layer further divided into lattice sites, see Fig. 3. Within each layer the volume fractions of each species are constant, implying a mean-field approximation, while there may exist concentration gradients in the direction perpendicular to the surface. The components present in the solution will be solvent: two types of "free" polymers, polymer 1 and polymer 2; and in the present case with these recombinant proteins, the grafted polymer, which represents the polypeptide tail.

An expression for the (Helmholtz) free energy (A) of the polymer solution at the interface (relative to the reference state of separated and amorphous components) in terms of the system composition is next formulated. It contains two terms, according to

$$\beta(A - A^*) = \beta(U - U^*) - \ln(\Omega/\Omega^*) \quad (1)$$

where $\ln(\Omega/\Omega^*)$ is the mixing entropy (divided by k) from the configurational degeneration and U the total interaction energy relative to separated components with $\beta = 1/kT$, k = Boltzmann's constant, and T the absolute temperature. Our systems are at constant volume and pressure so $\Delta A = \Delta G$ and ΔG will give $\ln K$.

Let us consider first the configurational term. The presence of the surface gives a reference direction in the system, and the lattice layers can thus be numbered along this direction, the layer numbers being a measure of the distance from the surface. Various conformations of the polymer chains can then be distinguished according to the different ordering of their segments with respect to the layer numbers. That is, a conformation in this one-dimensional heterogeneous model is defined as a sequence of layer numbers, one number for each segment of the polymer chain (of course the connectivity requirement must be satisfied; consecutive segments can have layer numbers differing at most one unit). Since the energy of a conformation depends in this model only on these layer numbers (random mixing within the layers), the number of ways to realise a conformation (in the above sense), i.e., its degeneration, gives its contribution to the entropy. When the self-exclusion on a mean-field level is taken into account, and the contributions from all conformations of all the components are included, the total conformational degeneration relative to the reference state (of separated and amorphous components), constituting the mixing entropy of the system (divided by k), is given simply by

$$\ln \frac{\Omega}{\Omega^*} = -\sum_x \sum_c n_{xc} \ln \frac{n_{xc} r_x}{\omega_{xc}} \quad (2)$$

where n_{xc} denotes the number of component x (i.e. polymer 1, polymer 2, grafted tail, or water) in conformation c, r_x the total number of segments in component x and the factor ω_{xc} is the probability of a "walk" according to the conformation c on an empty lattice. In the case of a homogeneous solution, this expression reduces to the familiar Flory–Huggins expression $\ln(\Omega/\Omega^*) = -\Sigma_x (n_x \ln \phi_x)$, as expected, where n_x is the number of component x and ϕ_x the volume fraction of component x.

The interaction energy is obtained by summing all the nearest-neighbour interactions between the segments, but taking into account that the volume fractions of the segments may vary between the layers. The resulting expression becomes

$$\beta U = \frac{1}{2} \sum_{i=0}^{M} L_i \sum_{A}^{s} \sum_{A'}^{s} \phi_{Ai} \chi_{AA'} \langle \phi_{A'i} \rangle \quad (3)$$

where $<...>$ denotes an average over the actual and adjacent layers, and the volume fractions ϕ_{Ai} are given by n_{Ai}/L_i, n_{Ai} being the number of segments of species A (i.e., monomer of polymer 1, monomer of polymer 2, water, or protein) located in layer i with L_i sites. The notation Σ^s implies that the sum includes the surface species as well, i.e., the interaction with the protein, and $\chi_{AA'}$ denotes the Flory–Huggins interaction parameter between segments of type (species) A and A' [29].

The equilibrium distribution of the conformations of the components is such that it minimises the free energy under the constraint of filled volume and also meets the requirement that the first chain segments of the grafted component should all reside in the first layer. From these criteria a set of implicit and non-linear equations in the layer volume fractions can be derived which are solved numerically [13,14,25,27].

4. Results and discussion

4.1. Experimental partition results

All the experimentally determined partition coefficients are collected in Table 1. In all systems the top phases were the PEG rich or Ucon rich ones and the bottom phases dextran rich ones. It is possible to compare the two systems, PEG–dextran versus Ucon–dextran, because the chosen tie line lengths between the systems are alike, see Table 1. The peptide T1 partitioned preferentially into the PEG phase in the PEG–dextran system and in the Ucon–dextran system the peptide partitioned into the Ucon phase with somewhat higher partition coefficients. The preference for the Ucon phase over the dextran phase was even more pronounced for the T2 octapeptide, which had partition coefficients 2–3 times as high as those of the tetrapeptide T1. From these data it is also evident that the incorporation of the tetrapeptide T1 sequence into the C-terminal stretch of the ZZT0 protein conveys an increased partition into both the PEG phase and the Ucon phase to the resultant ZZT1 recombinant protein. In the same way the insertion of the octapeptide T2 enhanced the partition of the ZZT2 protein to the Ucon phase, and the change in partition coefficient is appreciably larger than for ZZT1.

Fig. 4 shows the logarithm of the partition coefficient as a function of tie line length. It is a well founded assumption that at the critical point, i.e., the point of transition from one to two phases, the partition coefficient of a soluble molecule is equal to one [1,30,33]. This has been used in Fig. 4, and a linear relationship between log K and tie line length was obtained. The slopes can be used for comparing the partitioning in the two systems of the peptides and recombinant proteins. Fig. 4 shows that the Ucon system gives a more one-sided partitioning than the PEG system for T1, ZZT0 and ZZT1. The fusion of tryptophans has been shown to direct the partitioning of proteins to the PEG phase in PEG–salt systems [6]. In the Ucon–water system formed above the CP of Ucon, tryptophan is most strongly partitioned to the Ucon phase compared with other amino acids [31]. Studies in PEG–hydroxypropyl starch and Ucon–hydroxypropyl starch systems have indicated that proteins which have tryptophans exposed on the surface have higher affinity for the EO–PO copolymer than for PEG [12]. Our results in this study are in agreement with these previous results.

The determination of the partition coefficients of all three species, i.e., mother protein, free peptide and fused protein, at the same points in the phase

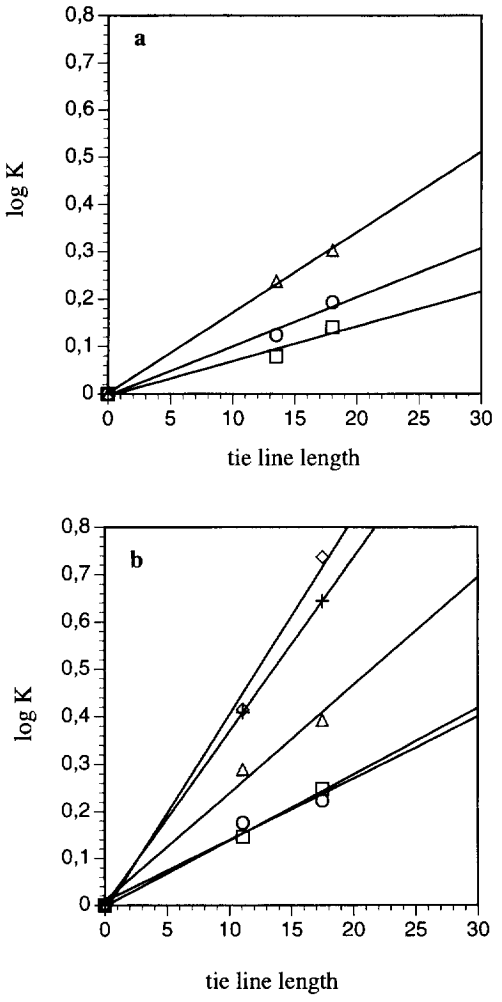

Fig. 4. The logarithm of the partition coefficient as a function of tie line length for T1 (□), ZZT0 (○), ZZT1 (△), T2 (◇) and ZZT2 (+) in (a) the PEG–dextran system and (b) the Ucon–dextran system.

diagram makes it possible to directly compare the magnitude of this increase in the protein partition coefficient with the partition coefficient of the free T1 or T2 peptide. In the case of independent contributions we should have $K_{ZZT1} = K_{ZZT0}K_{T1}$ [1,32,33]. However, the data in Table 1 show that the ZZT1 protein partitioned less into the phase to which the T1 peptide partitions than would be expected from independent contributions from the ZZT0 and T1 moieties to the partition of the "complex" ZZT1. The exception is the partition behaviour at the short tie line point of the PEG–dextran system, where actually $K_{ZZT1} > K_{ZZT0}K_{T1}$. However the partition coefficients of ZZT1 in the PEG–dextran system are uncertain because there were difficulties with precipitation of the protein in that system. The hypothesis about a reduced contribution to the partition coefficient from the peptide unit when inserted into the ZZT0 protein which seems to be motivated by the ZZT1 results, is borne out further by a comparison of the T2 and ZZT0 partition coefficients in the Ucon–dextran system versus the ones of ZZT2. As is seen in Table 1, K_{ZZT2} is clearly smaller than the product of K_{ZZT0} and K_{T2}, and in these cases the reduction is much more pronounced than for the tetrapeptide.

These recombinant proteins have earlier been investigated with respect to their partition in PEG–salt systems [6,34] and in combination with elution behaviour in hydrophobic interaction chromatography with PEG and propyl groups as hydrophobic agents [6]. A marked effect of the Trp-containing peptide tail was noted both in the partition coefficients of the recombinant proteins and in their retention volumes on the hydrophobic columns. Also the free tetrapeptide showed a clear preference for the PEG-phase, a fact that was seen, by a comparison with the partition of the Trp–Trp dipeptide, to mainly be due to the two Trp residues which it contains. The reduction of K_{ZZT1} in the PEG–salt system from the value expected from the independent contribution case $K_{ZZT1} = K_{ZZT0}K_{(Trp\text{-}Trp)}$ (assumed to approximately equal $K_{ZZT0}K_{T1}$) was tentatively attributed [6] to a folding of the T1 tetrapeptide unit together with the C-terminal peptide region of ZZT0 into which it is inserted in such a way that it thereby becomes less accessible to interaction with the solvent. Below we will discuss this matter for the present case of ternary two-polymer systems in the light of model calculations in the heterogeneous lattice theory of polymer solutions.

4.2. Model calculations

As was remarked in the description of the model above, the model calculations proceed in two steps. First the composition of the phases is calculated by a

direct numerical minimisation of the classical Flory–Huggins expressions for the free energy of mixing in (homogeneous) polymer solutions with a suitable set of interaction parameters (chosen so as to give a reasonable fit between the calculated and the experimental phase diagrams). The phase diagrams calculated in that way to represent the experimental PEG–dextran and Ucon–dextran ones are given in Fig. 2 together with the experimentally determined phase diagrams. The same procedure of minimising the (multi-component) Flory–Huggins free energy expression was also used to calculate the partition of the T1 tetrapeptide and the T2 octapeptide (modelled as a homotetramer, P_4, and a homooctamer, P_8, respectively). The calculated partition coefficients depend essentially on the (unknown) interaction parameters between P and the other species in the system, and hence these parameters were adjusted to give reasonable correspondence with the experimental values.

Secondly, the partition coefficient for the protein is obtained from the difference between the free energy of the protein in each of the two phases in equilibrium, calculated using the heterogeneous lattice model presented above. To that end the ZZT0 protein is modelled as an impenetrable sphere with a flexible polymer "tail" grafted to its surface, representing the 17 amino acid residue peptide stretch bound to the C-terminus of the native Z-protein domain (the similar N-terminal peptide stretch is ignored in this context, since it is not modified when the proteins ZZT1 and ZZT2 are derived by the incorporation of the T1, T2 peptide respectively into the ZZT0 protein). The reason to model the C-terminal stretch differently is that it is a wholly artificial amino acid sequence, which may be assumed not to fold as tightly as the Z-domain, but rather to adopt a considerably more open and flexible conformation. The models of the ZZT1 and ZZT2 proteins are then easily derived from that of ZZT0 by making the grafted polymer a triblock copolymer, $S_9P_4S_8$ or $S_9P_8S_8$, where P_4 and P_8 represent the T1 and T2 peptides while S_9 and S_8 are the two parts of the "tail" of ZZT0, into which they are inserted.

The partition coefficients calculated from this heterogeneous model depend on the interaction parameters between the (protein) surface and the species in the solution, and since these are not known from independent sources the same situation is at hand for the computation of the partition of the ZZT0 protein as for the peptides above. Thus, values of the surface interaction parameters were selected so as to roughly fit the calculated partition coefficients of (the model of) ZZT0 to the measured ones. No attempt was made to rigorously obtain the best possible fits by using some error-function since we are not primarily interested in the parameter values per se, but rather the model predictions about the relation between the partition coefficients of the "free" constituents T1, T2 and ZZT0 on one hand and that of their "complexes" ZZT1 and ZZT2 on the other. To that end it is sufficient to have parameters giving qualitatively right trends and magnitudes of the partition coefficients of T1, T2 and ZZT0.

Having assigned values to all the necessary model parameters by fitting to the experimental partition data of T1, T2 and ZZT0, we can finally calculate predicted partition coefficients of the ZZT1 and ZZT2 proteins from the thermodynamic model above. This is so because in the model we explicitly describe the part of the ZZT1 (ZZT2) structure where the T1 (T2) peptide is inserted as forming a block copolymer, where the interaction parameters of the constituting homopolymer blocks could all be determined by the fitting procedures already accounted for.

The parameter values used in the calculations are collected in Table 2, while the resultant calculated partition coefficients are presented in Table 1. Regarding these results, we note in the first place that the incorporation of the P_4 tetramer or the P_8 octamer into the S_{17} tail of "ZZT0" does indeed give rise to an increased partition coefficient of both "ZZT1" and "ZZT2". Thus, the preferential partition of the P_4 and P_8 sequences towards the top phase is conveyed to "ZZT1" and "ZZT2" respectively, and these oligomers "steer" the partition towards the phase to which they preferentially partition themselves in the model in the same way as they do experimentally. Furthermore, this increase in the partition coefficient is of the same order of magnitude as the partition coefficient of the free P_4 or P_8 oligomers, also in accordance with the experimental

Table 2
Parameter values used in the model calculations, unless stated explicitly: $T=298$ K; protein radius, 4 lattice units; 1 (fused peptide)protein. The tails were S_{17} for ZZT0, $S_9P_4S_8$ for ZZT1 and $S_9P_8S_8$ for ZZT2, where S_{17} is the C-terminal of ZZT0, P_4 and P_8 the inserted tetra- and octapeptides (Fig. 1). The degrees of polymerisation were: PEG 91 segments, Ucon 78 segments and dextran 1000 segments. All interaction parameters with the C-terminal amino acids (S) in the mother protein have been put to zero

	$RT\chi_{AA'}$ (kJ/mol)				
	Dextran	PEG	Ucon	Prot	P
Water	1.23	1.25	1.29	0	0
Dextran		0.27	0.46	−1.15	1.0
PEG				−1.05	−0.5
Ucon				−0.95	−2.0
Prot					0

findings. We will now look at the quantitative relation between the partition coefficients K_{ZZT1}, K_{ZZT2} and the products $K_{ZZT0}K_{T1}$, $K_{ZZT0}K_{T2}$. We will concentrate on the case with long tie lines, since the trends are clearer due to less influence of experimental uncertainties. Beginning with the tetramer case, we find for the Ucon–dextran system that $K_{ZZT1} < K_{ZZT0}K_{T1}$, and the value of K_{ZZT1} is 9% below the independent-contribution value ($K_{ZZT0}K_{T1}$), while the reduction of the corresponding experimental values is 19%. In the case of the PEG–dextran system, the theoretical reduction is 13% and the experimental one 9%.

Turning to the results for the partition of the ZZT2 protein in the Ucon–dextran system, we find that the model predicts a reduction of the independent-contribution value of 25%. However, in this case the experimentally measured reduction is as high as 54%. Clearly, here the discrepancy is much larger than in the case of ZZT1. The deviation from the independent-contribution value found in all cases in the model calculations (as in the majority of the experimental results) is a reduction of the partition, i.e., the ZZT1 and ZZT2 proteins are found to partition less unevenly. In this kind of model such a reduction comes about as a natural consequence of the depletion zone that is predicted [15–20] to be created around large impenetrable particles (such as protein molecules) in solutions of flexible polymers. In this zone in the vicinity of the protein the local concentration of phase polymers is lower than far away from the protein. In the present case, where the peptide is bound to the (C-) terminus of the ZZT0 protein and hence has to stay close to the impenetrable ZZ-part of the protein, it gives a contribution to the partition coefficient that corresponds to the free peptide partition at a point in the phase diagram with lower polymer concentration, i.e., closer to the critical point. The contribution from the bound peptide is accordingly smaller than the free peptide partition coefficient between the actual (bulk) phase compositions.

In view of this picture, a possible interpretation of the larger reduction would be to assume an attraction between the segments of the inserted peptide and the protein surface. Then the peptide segments should move closer to the surface, i.e., more into the depletion zone, and their contribution to the partition coefficient should hence decrease. This is a reasonable assumption because of the hydrophobicity of the tryptophan residues. Thus, we performed a series of model calculations of ZZT1 and ZZT2 partition coefficients for different values of $RT\chi_{(P-prot)}$, which is the parameter that measures the strength of the peptide–protein attraction in the model. In Table 1 the results for the value $RT\chi_{(P-prot)} = -8.0$ kJ/mol are given. By doing this we obtained calculated partition coefficients for ZZT2 more in accordance with the experimental ones, whereas the calculated values for ZZT1 were only changed to a small degree.

Hence, we have shown that the reduced contribution from the inserted peptide can be captured by the effect of the depletion zone around the protein as obtained in the heterogeneous lattice model used above. However, from the present analysis we cannot exclude that some other mechanism might be operating. One plausible such mechanism could be some kind of local folding of the inserted peptide sequence which thereby reduces the contact with the solution. Since folding is a co-operative process, it would be expected to require some minimum sequence length of the inserted peptide to start occurring in appreciable amounts. This could explain why this effect is stronger for ZZT2 relative to ZZT1 with its shorter peptide sequence. Further study by more direct

methods is needed to get a clearer picture of this matter.

5. Conclusions

We have found that the incorporation of the Trp-containing peptides T1 and T2 into the C-terminal stretch of a synthetic recombinant protein (ZZT0) conveys an increasing partition into the PEG and Ucon phases of PEG–dextran and Ucon–dextran polymer two-phase systems. The increase in partition coefficient is however less than would be expected from independent contributions from the peptide and the unmodified protein to the total partition coefficient. A possible way of explaining this fact is offered by comparison with results of theoretical calculations in a heterogeneous lattice model of polymer solutions. According to this model the reduced contribution from the inserted peptide is found to be due to the existence of a depletion zone around the protein, where the phase polymer concentration is lowered. Thus, the incorporated peptide experiences phase compositions which are closer to the critical point, between which it has a lower partition coefficient, and hence gives a lower contribution to the protein partition coefficient. The experimental results indicate a further reduction of the partition coefficient, beyond that predicted by the lattice calculations. A possible folding of the inserted peptide is discussed as a plausible mechanism for this further reduction in the partition coefficient.

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