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Aqueous two-phase systems containing self-associating block copolymers
Partitioning of hydrophilic and hydrophobic biomolecules

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Abstract

A series of proteins and one membrane-bound peptide have been partitioned in aqueous two-phase systems consisting of micelle-forming block copolymers from the family of Pluronic block copolymers as one polymer component and dextran T500 as the other component. The Pluronic molecule is a triblock copolymer of the type PEO–PPO–PEO, where PEO and PPO are poly(ethylene oxide) and poly(propylene oxide), respectively. Two different Pluronic copolymers were used, P105 and F68, and the phase diagrams were determined at 30°C for these polymer systems. Since the temperature is an important parameter in Pluronic systems (the block copolymers form micellar-like aggregates at higher temperatures) the partitioning experiments were performed at 5 and 30°C, to explore the effect of temperature-triggered micellization on the partitioning behaviour. The temperatures correspond to the unimeric (single Pluronic chain) and the micellar states of the P105 polymer at the concentrations used. The degree of micellization in the F68 system was lower than that in the P105 system, as revealed by the phase behaviour. A membrane-bound peptide, gramicidin D, and five different proteins were partitioned in the above systems. The proteins were lysozyme, bovine serum albumin, cytochrome *c*, bacteriorhodopsin and the engineered B domain of staphylococcal protein A, named Z. The Z domain was modified with tryptophan-rich peptide chains in the C-terminal end. It was found that effects of salt dominated over the temperature effect for the water-soluble proteins lysozyme, bovine serum albumin and cytochrome *c*. A strong temperature effect was observed in the partitioning of the integral membrane protein bacteriorhodopsin, where partitioning towards the more hydrophobic Pluronic phase was higher at 30°C than at 5°C. The membrane-bound peptide gramicidin D partitioned exclusively to the Pluronic phase at both temperatures. The following trends were observed in the partitioning of the Z protein. (i) At the higher temperature, insertion of tryptophan-rich peptides increased the partitioning to the Pluronic phase. (ii) At the lower temperature, lower values of K were observed for ZT2 than for ZT1.

Keywords: Aqueous two-phase systems; Temperature effects; Partitioning; Proteins; Peptides

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1. Introduction

The tendency for biomolecules to partition unevenly in aqueous two-phase systems has been studied extensively since its discovery in the 1950s by Albertsson [1]. Different aqueous two-phase systems can be used for partitioning; polymer/polymer, salt/polymer and surfactant/polymer systems are examples of systems studied [2]. Most attention has been focused on the poly(ethylene oxide) (PEO)/dextran system, dextran being a polysaccharide. Several factors can influence the outcome of the partitioning such as: length of the tie-line, type of salt in the system, pH, temperature and the molecular size of the phase-forming polymers [2]. For example, an increase in the length of the tie-line results in a more extreme partitioning to one of the phases due to the greater difference in hydrophobicity (or hydrophilicity) between the phases [3]. The properties of the partitioned biomolecule, in terms of surface hydrophobicity and charge, also affect the partitioning. The fusion of hydrophobic tails on a protein enhances partitioning to the more hydrophobic phase [4,5].

The phase behaviour of the aqueous PEO–PPO–PEO/dextran T500 system has recently been presented [6]. The PEO–PPO–PEO polymer is a block copolymer (where PPO is poly(propylene oxide)) often referred to as Pluronic (registered trademark of BASF). This type of block copolymer self-associates upon increasing temperature and/or concentration into micellar-like aggregates consisting of a core dominated by PO units, surrounded by a corona of EO chains [7–9]. The temperature at which the copolymer molecules start to self-associate is referred to as the critical micellization temperature (CMT) and the corresponding concentration, the critical micellization concentration (CMC). The most interesting feature found was the strong effect on the phase diagram of an increase in temperature from 30 to 35°C. More precisely, the phase separation was segregative at both temperatures, but at the higher temperature the top phase became more concentrated with respect to the Pluronic polymer, due to the significant redistribution of water. Physically, this phenomenon was attributed to the onset of micellization. So, when viewing one micelle as a pseudo-particle, the osmotic pressure of water changed (upon micellization) such that water partitioned more to the dextran phase.

The effect of temperature on partitioning was examined in a subsequent study where hydrophobic amino acids and oligopeptides were partitioned in the Pluronic P105/dextran T500/water system [10]. The partitioned solutes, phenylalanine, tryptophan and oligopeptides of these amino acids, partitioned predominately to the Pluronic-rich phase. Temperature was found to have a profound effect on the partitioning of the oligopeptides. Partitioning to the Pluronic-rich phase increased at the higher temperature, and it was concluded that this was mainly the result of the redistribution of water, and not the temperature-triggered creation of micelles.

Block copolymers have been used in partitioning studies in recent years [11–14]. However, little attention has been paid to the effect of temperature-induced micellization on the partition behaviour in such polymer solutions. Surfactant-based systems have been used to extract hydrophobic membrane proteins. Hydrophobic proteins were found to partition, to a large extent, to the surfactant-rich phase [15,16].

To further explore the potential of block copolymers in partitioning applications, we carried out partitioning studies on a wide range of different proteins (hydrophilic as well as hydrophobic membrane proteins) in the Pluronic P105/dextran and Pluronic F68/dextran T500 systems. The hydrophilic proteins (lysozyme, bovine serum albumin and cytochrome *c*) used in this study have been studied extensively as model proteins in partitioning experiments [2,17–19]. As hydrophobic biomolecules, the integral membrane-bound protein bacteriorhodopsin and the membrane-spanning peptide gramicidin were chosen. Finally, a synthetic domain of staphylococcal protein A modified with short tryptophan (Trp)-rich tails, was studied [20,21]. The experiments were performed at two different temperatures, 5 and 30°C, to study the effect of block copolymer self-aggregation on partitioning.

2. Methods and materials

2.1. Materials

The block copolymers P105 and F68 were a gift

from BASF, Parsippany, NJ, USA. P105 and F68 with molecular weights of 6500 and 8400 (according to the manufacturer [22]), respectively, have the following nominal compositions, P105: $(EO)_{37}(PO)_{56}(EO)_{37}$ and F68: $(EO)_{76}(PO)_{29}(EO)_{76}$. Dextran T500, with a molecular weight of approximately 500 000, was purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. The polymers were used as received.

Albumin (bovine, ≥96%), cytochrome *c* (from horse heart, 99%), gramicidin D (from *Bacillus brevis*) and bacteriorhodopsin (from *Halobacterium halobium*) were purchased from Sigma, St. Louis, MO, USA, and used without any further purification. Gramicidin D is a mixture of gramicidin A, B and C [23], all linear peptides with 15 residues. These differ in amino acid sequence at two positions [24]. Lysozyme (from hen egg-white) was obtained from Boehringer Mannheim, Germany, and used as received.

A synthetic IgG-binding domain, Z, derived from the B domain of staphylococcal protein A was used [21]. The Z protein was modified with peptide tails rich in Trp residues, which were inserted near the C-terminus (see Fig. 1). The preparation procedure for ZT0, ZT1 and ZT2 is, in principle, the same as for ZZTX (which contains an extra Z domain) and has been described elsewhere [4,25]. The structure of the Z domain in solution consists of a bundle of three α-helices [20], while the structure of the flanking peptide tails is not known. Molecular mass, isoelectric pH (p*I*) and net charge at pH 7.0 for the proteins used are given in Table 1.

The two non-ionic surfactants used, Triton X-100 [octylphenol (ethylene oxide)$_{9.5}$] and $C_{12}E_6$, were purchased from Sigma and Nikko Chemicals, Tokyo, Japan, respectively. All salts and chemicals were of analytical grade. Millipore water was used in all preparations.

Table 1
Some properties of the proteins used in this study

Protein	Molecular mass	p*I*	Net charge at pH 7.0
BSA	69 000 [37]	5.0 [37]	−18 [34]
Lysozyme	13 900 [38]	11.0 [39]	+7 [40]
Cytochrome *c*	13 000 [38]	9.4 [37]	+6 [36]
Bacteriorhodopsin	26 000 [41]	–	–
Gramicidin	2000[a]	–	–
ZT0	10 000[b]	–	–

[a] Calculated from the structure of [Val]–Gramicidin A [24].
[b] Obtained by reduction of the molecular mass for ZZT0 [4] by the approximate weight of one Z unit.

2.2. Phase diagram determination

Phase diagrams were determined for P105/dextran T500/water and F68/dextran T500/water at 30°C. The prepared samples were left to equilibrate for approximately 1 h in a Haake water bath (accuracy ±0.1°C). The phases were carefully separated with a Pasteur pipette and suitably diluted.

The concentrations of the polymers in the two phases were determined in two steps [1]. First, the dextran concentrations in both phases were determined by polarimetry. The Pluronic polymers showed no optical activity. Second, the concentration of the Pluronic polymer was determined by measuring the total refractive index of the solutions and then subtracting the contribution by dextran. Stan-

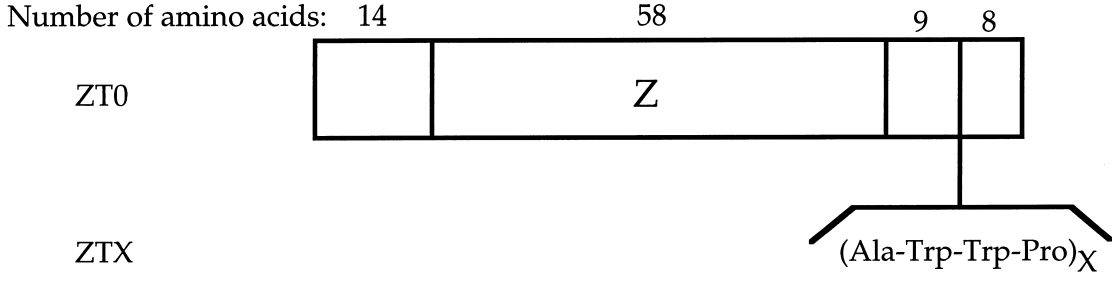

Fig. 1. Schematic structure of the ZT0-based proteins. Z is a synthetic IgG-binding domain derived from staphylococcal protein A. The T peptides are inserted before the last eight residues at the COOH terminus.

dard curves were established for aqueous solutions of Pluronic polymers and dextran T500. The polarimetric measurements were performed using a digital polarimeter (model DIP-360) from Jasco International, Japan, equipped with water-thermostated sample cells connected to a Haake F3 water circulator. The refractive index measurements were performed with a 2142 differential refractometer from LKB, Bromma, Sweden.

2.3. Partitioning experiments

The partitioning experiments were performed at 5 and 30°C using the P105/dextran system (and the F68/dextran system for ZTX). The polymer concentrations were, in the P105/dextran system 6.4% (w/w) Pluronic P105 and 5.6% (w/w) dextran T500, and in the F68/dextran system 5% (w/w) F68 and 7% (w/w) dextran T500.

For lysozyme, bovine serum albumin and cytochrome *c* the phase system (total mass: 5 g) consisted of the following:, 0.5 mg protein per ml of the system, 10 m*M* of pH regulating substance (sodium phosphate (NaP), pH 7.0; β-alanine, pH 6.9; sodium acetate, pH 5.0 or glutamic acid, pH 3.1). $NaClO_4$ was included in some systems at a concentration of 100 m*M*.

ZTX was partitioned in both the P105/dextran T500 and the F68/dextran T500 systems containing 5 m*M* NaP. The procedure employed with ZTX was as follows. ZTX was dissolved in 25 m*M* NaP solution, pH 7 and centrifuged for 10 min at ~1000 rpm. The solution was mixed with polymers and water to a concentration of 0.4–0.5 mg ZTX per ml of system.

Partitioning experiments with bacteriorhodopsin were performed in the P105/dextran system. The hydrophobic protein bacteriorhodopsin is a membrane-bound, light-driven proton pump [26]. The addition of detergent was necessary to solubilize the bacteriorhodopsin. The non-ionic surfactant $C_{12}E_6$ was found to be suitable and a stock solution was prepared as follows. About 2 mg bacteriorhodopsin was dissolved in a 2 ml solution of 50 m*M* $C_{12}E_6$ and NaP (pH 7.0). One ml of this solution was mixed with the phase-forming components to a total weight of 3 g, with the following concentrations: bacteriorhodopsin 0.33 mg/ml system; 16 m*M* $C_{12}E_6$ and 16 m*M* NaP. The systems were then allowed to phase separate.

Gramicidin is a cation channel, 15-residue-long peptide, which spans membranes as a dimer [24]. Gramicidin was partitioned in the P105/dextran system. To solubilize gramicidin, Triton X-100 was used together with NaCl. The following stock solution was prepared: 2 mg gramicidin per ml; 63 m*M* Triton X-100; 125 m*M* NaCl, and incubated for 30 min at 37°C to obtain a clear solution. After mixing the stock solution, polymers and water to a final mass of 3 g, the concentrations before phase separation were, 0.5 mg gramicidin per ml of the system; 33 m*M* NaCl; 16 m*M* Triton X-100.

Samples separated at 5°C were equilibrated for at least 2 h in a cold room or in a refrigerator whereas 1 h in a water bath was sufficient for the 30°C samples. Duplicate and reference systems (without the biomolecule) were prepared in all experiments.

The results of the partitioning experiments are presented as partition coefficient, *K*, defined as,

$$K = c^t/c^b \quad (1)$$

where c^t and c^b are the concentrations of the partitioned solute in the top and bottom phases, respectively.

2.4. Protein and enzyme assay

Two protein reagents were used to analyse the protein concentrations: Coomassie Brilliant blue G-250 (from ICN Biomedicals, Aurora, OH, USA) and a reagent kit from Sigma containing Bicinchoninic acid solution and copper (II) sulphate. The concentrations of lysozyme, albumin and cytochrome *c* were determined using Coomassie Brilliant Blue G-250 by measuring the absorbance at 595 and 465 nm [27]. The gramicidin, ZTX and bacteriorhodopsin concentrations were determined with a reagent mixture of bicinchoninic acid and copper (II) sulphate as described by Smith et al. [28], absorbance being detected at 562 nm. This method was more compatible with the surfactant-containing systems. The concentrations of Triton X-100 in the P105/dextran T500 systems were determined by measuring the absorbance at 277 nm.

3. Results and discussion

3.1. Phase diagrams

The phase diagrams determined for the F68/dextran/water and P105/dextran/water systems at 30°C are shown in Fig. 2. The systems phase separated into two isotropic phases, the top and bottom phases being enriched in Pluronic and dextran, respectively. As can be seen in Fig. 2, the shape of the binodals and slopes of the tie-lines at 30°C, differ significantly between the two polymeric systems. The phase behaviour at 5°C for the above systems has been presented previously [6]. The effect of an increase in temperature on the tie-lines was considerable in the P105 system, but less pronounced in the system with Pluronic F68 (see Figs. 2 and 4 in Ref. [6]). This is attributed to the difference in the ability of the Pluronic polymers to form micelles [6]. In the Pluronic P105 system, the copolymers self-associated and formed micellar-like structures in the top phase at 30°C while at 5°C the Pluronic polymers were predominantly found in their unimeric state (a single polymer chain). It is unclear to what extent F68 had associated at 30°C, but the degree of aggregation was definitely lower than that in the P105 system, as reflected by CMT data [29] and phase behaviour [6]. One composition was chosen from each phase diagram in this study and these are presented, together with the final phase compositions in Table 2.

3.2. The effect of temperature on partitioning

Upon increasing the temperature from 5 to 30°C in systems containing Pluronic P105, two main courses of events can influence the outcome of partitioning. (i) Considerable redistribution of water from the Pluronic to the dextran phase leading to an increase in the Pluronic concentration in the top phase and a decrease in the dextran concentration in the bottom phase. The partition coefficient of a solute with a strong affinity for the Pluronic polymer (for instance hydrophobic peptides of tryptophan [10]), will increase due to the 'dehydration' of the Pluronic phase and 'hydration' of the dextran-rich phase. (ii) The temperature will exceed the CMT of P105, and copolymer self-aggregation will occur. This will create a new, more hydrophobic 'phase' in the top phase, namely the interior of the micelle which consists of the more hydrophobic PO units. This new pseudo-phase may attract hydrophobic and/or amphiphilic solutes so as to enhance partitioning to the Pluronic phase beyond that which would be expected

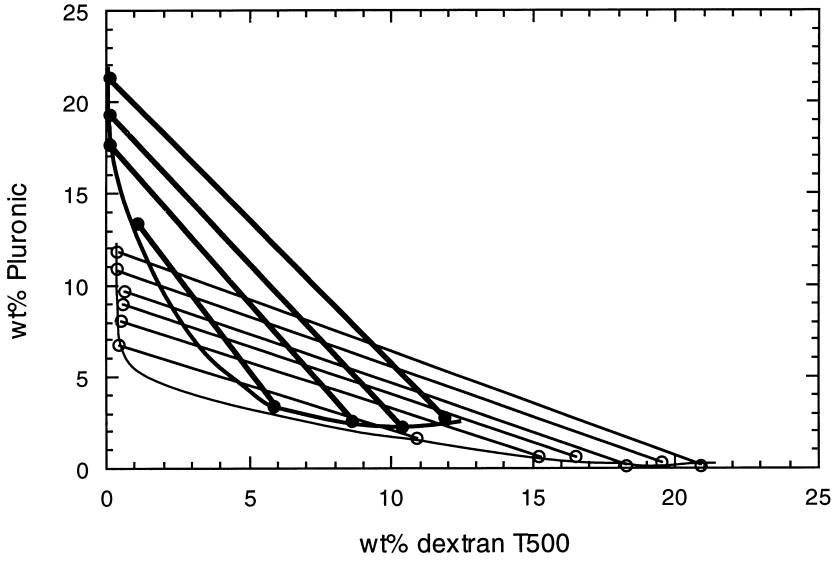

Fig. 2. Phase diagram for the P105/dextran T500/water (●) and F68/dextran T500/water (○) systems at 30°C. Concentrations in %w/w.

Table 2
Compositions of the phases in the Pluronic/Dextran T500/water systems at 5 and 30°C. All concentrations are given in %(w/w)

Temperature	Total system		Top phase		Bottom phase	
	P105	Dextran	P105	Dextran	P105	Dextran
5°C[a]	6.4	5.6	9.0	0.5	1.0	17.0
30°C	6.4	5.6	16	0.5	3.0	7.5
	F68	Dextran	F68	Dextran	F68	Dextran
5°C[a]	5.0	7.0	8.0	0.5	0.5	17.0
30°C	5.0	7.0	8.5	0.5	0.5	15.5

[a] Data from Ref. [6].

from the changes in Pluronic concentration in the phase [see point (i) above].

One possible way to separate these effects for a biomolecule with an affinity for the Pluronic polymer is to plot the natural logarithm of K ($\ln K$) against the difference in P105 concentration between the top and bottom phase, ΔP105 [$=$%(w/w) $P105_{top} -$ %(w/w) $P105_{bottom}$]. If a linear dependence is obtained, and the line passes through the point $\ln K = 0$, $\Delta P105 = 0$, this would provide some evidence that no micellization effects have occurred [10]. However, if an exponential-like behaviour is obtained from such a plot, this would indicate that the partitioning behaviour was due to the interaction between the partitioned molecule and the polymer micelle.

The reason for using the F68 polymer was to investigate the effect of the temperature itself, since F68 has a lower ability to form micelles. This is also shown in the small changes in phase volumes and phase compositions upon increasing the temperature (see Table 2).

3.3. The effect of salts on partitioning

The choice of salt is very important in partitioning experiments. If the partitioned proteins exhibit a positive or negative net charge [pH$\neq$isoelectric pH (pI)] the addition of salts and/or electrolytes to the system will affect the partitioning of the proteins. A common explanation is that the cations and anions of the salt have different affinities for the separated phases, resulting in an uneven distribution (within the requirement of electroneutrality in the phases) of the salt, resulting, in turn, in an electrostatic potential difference over the interface [1,30–32]. Different salts cause different potential differences, meaning that it is possible to direct a charged particle to either of the two phases by choosing a suitable ion pair [1]. For example, in a PEO/dextran two-phase system containing the salt Na_3PO_4, the PO_4^{3-} ion has an affinity for the more hydrophilic dextran phase, which will induce a negative potential on the 'dextran side' of the interface. Such a potential difference will direct particles of positive charge to the dextran phase. The anion ClO_4^-, on the other hand, has a tendency to partition to the more hydrophobic PEO phase, and the opposite behaviour, is observed with a negative potential on the PEO side of the interface. This phenomenon has been utilised in cross-partitioning experiments to determine the isoelectric points of proteins [1]. The affinity of ions for different phases agrees well with the Hofmeister scale, also known as the lyotropic series [33]. The anions used in this work can be arranged in the following order, with regard to hydrophobicity; $ClO_4^- > Cl^- > CH_3COO^- > HPO_4^{2-}/H_2PO_4^-$. The sodium ion was used as the counterion in all cases. The partition coefficients of the salts are very close to unity, but the considerable impact of the salt on the partitioning behaviour is maintained if the salt concentration is much higher than the partitioned solute concentration. The electrostatic potential can be reduced by using zwitterionic amino acids at their pI as pH-regulating agents. The partitioning behaviour should then depend more on other parameters mentioned previously.

3.4. Hydrophilic proteins: albumin, lysozyme and cytochrome c

These proteins were partitioned in the P105/dextran system at 5 and 30°C at the compositions given in Table 2. Buffer, salt and pH were varied in the experiments with these three proteins. The partitioning results are shown in Figs. 3–5, where the ln *K* values are plotted against the type of salt.

3.4.1. BSA

At pH 7.0 and 6.9, the protein, with p*I* = 5.0, had a negative net charge of −18 [34]. Assuming that $NaClO_4$ creates a negative potential on the Pluronic side of the interface, this would lead to lower values of *K* with this salt present. The values of *K* of BSA in the $NaClO_4$ system were lower than in solutions with only NaP present (Fig. 3). NaP and $NaClO_4$ have the opposite effect on the sign of the potential at the interface, and therefore it is reasonable that the *K* values in systems with β-alanine as the dominating salt (where the electrostatic effects should be smaller than with a traditional ion pair) were in between the *K* values for systems with these two salts. When the pH equals p*I* (i.e. at 5.0 in NaAc), the effect of an electrostatic potential should (ideally) be zero, and it can be seen in Fig. 3 that the values of *K* (in NaAc) were approximately the same as in the β-alanine case. At pH 3.1 the protein was positively charged, and it can be seen that the addition of $NaClO_4$ increased the *K* values drastically. For example, in the system at 5°C (pH 3.1), the addition of $NaClO_4$ changed the *K*-value from 0.1 to 7.7, an increase of almost two orders of magnitude.

In systems with pH = 3.1, the values of *K* were higher at 5° than at 30°C. The temperature effect was pronounced in the system with only glutamic acid but less pronounced in the $NaClO_4$ system (at pH 3.1). The opposite effect of temperature was observed for BSA in the remainder of the salt systems. In these, a small increase in the values of *K* was observed when the temperature was increased. This

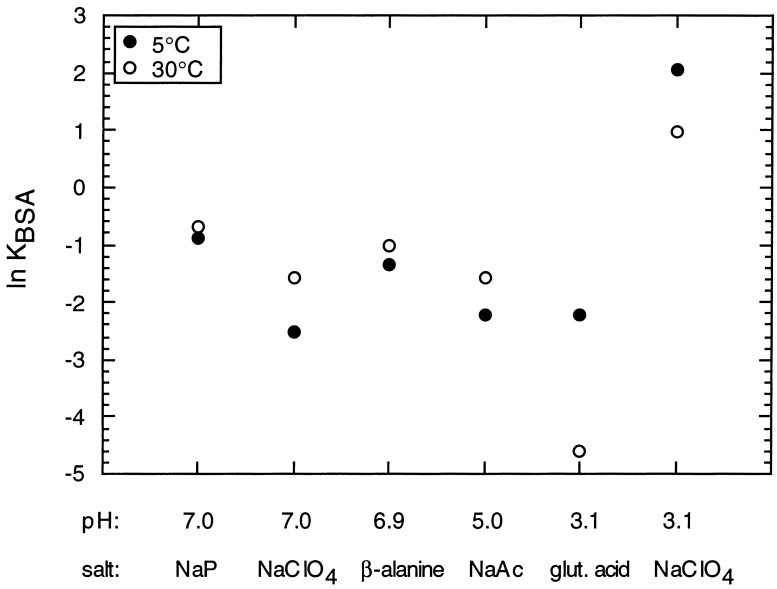

Fig. 3. Partitioning of BSA in the Pluronic P105/dextran T500 system. The natural logarithm of the partitioning coefficients of BSA is plotted against the type of salt/buffer at 5 (●) and 30°C (○). Concentrations of the salts: [sodium phosphate buffer (NaP)] = [β-alanine] = [glutamic acid] = 10 m*M*, [$NaClO_4$] = [sodium acetate (NaAc)] = 100 mM. Typical uncertainty in *K* obtained from double samples were approximately 5%.

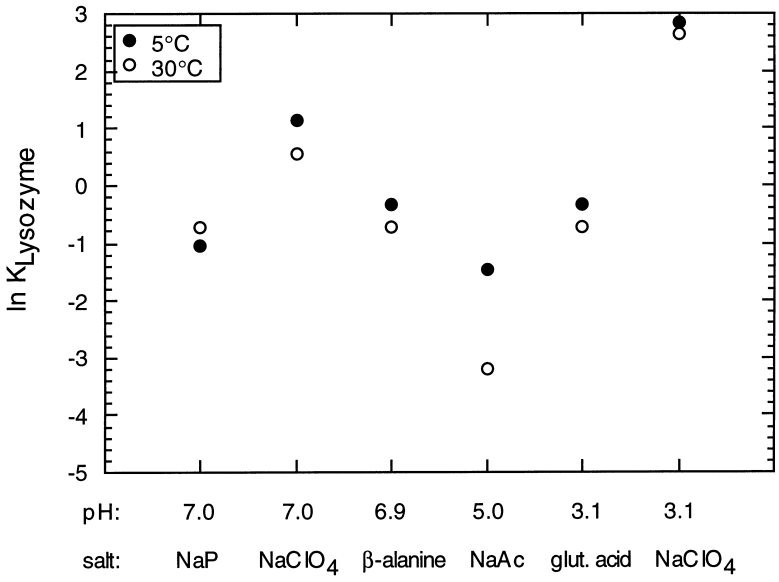

Fig. 4. Partitioning of lysozyme in the Pluronic P105/dextran T500 system. The natural logarithm of the partitioning coefficients of lysozyme is plotted against the type of salt/buffer at 5 (●) and 30°C (○). Concentrations of the salts: see Fig. 3. Typical uncertainty in *K* obtained from double samples was approximately 7%.

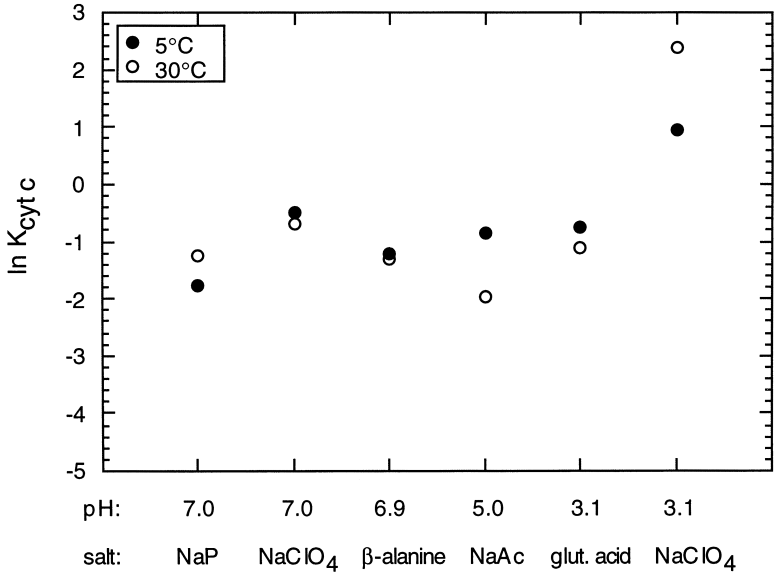

Fig. 5. Partitioning of cytochrome *c* in the Pluronic P105/dextran T500 system. The natural logarithm of the partitioning coefficients of cytochrome *c* is plotted against the type of salt/buffer at 5 (●) and 30°C (○). Concentrations of the salts: as in Fig. 3. Typical uncertainty in *K* obtained from double samples was approximately 5%.

is surprising, since one would expect a hydrophilic protein like BSA to favour the dextran phase in which the water content increases upon temperature increase.

3.4.2. Lysozyme

Lysozyme has a p*I* of 11.0, and was therefore positively charged in all experiments (net charge of +7 at pH 7.0). This was reflected at pH=7.0 (Fig. 4), where the addition of $NaClO_4$ resulted in a redistribution of lysozyme from the bottom dextran phase to the Pluronic phase. The values of *K* in the system with β-alanine were between those obtained with NaP and $NaClO_4$. At pH=5.0, with NaAc as salt, lysozyme was strongly partitioned to the bottom phase, especially at 30°C. This seems reasonable since the positive net charge was increased to approximately 10 at pH 5.0 [35]. The net charge was further increased at pH 3.1 in the glutamic acid system, but the zwitterionic amino acid does not produce as large a potential as the NaAc salt, which was reflected in the higher *K* values for lysozyme in glutamic acid systems than in NaAc systems (see Fig. 4). Not surprisingly, the hydrophobic salt $NaClO_4$ resulted in higher values of *K* (around 10) at pH 3.1. The effect of temperature on the partitioning of lysozyme was moderate, except in the NaAc system, where the values of *K* decreased with increasing temperature.

3.4.3. Cytochrome c

This protein was positively charged in all experiments (p*I*=9.4, net charge of +6 at pH 7.0 [36]). Fig. 5 shows the same trends as for lysozyme. However, the partitioning of cytochrome was less extreme and the *K* values were generally found in a closer ln *K* interval than in the case of lysozyme.

Again the effect of a temperature increase was evident in the NaAc system, and the addition of $NaClO_4$ increased the *K* values, specially at pH 3.1. Moreover, at pH 3.1 with $NaClO_4$ present, a higher *K* value was obtained at the higher temperature than at the lower temperature (5°C). A possible explanation of this behaviour may be that the presence of $NaClO_4$ together with acidic conditions (pH 3.1) induces exposure of hydrophobic residues due to conformational changes in the protein.

3.5. Comparison with a random copolymer system

The Ucon polymer is a random copolymer and has, to our knowledge, no distinct self-associating properties as do the Pluronic molecule. Ucon 50HB 5100 has the same PO/EO ratio (=1) as Pluronic P105. The partitioning of hydrophilic proteins in a Ucon 50HB 5100/Reppal PES 200 system has been determined earlier [17]. The bottom phase polymer was hydroxypropyl starch (Reppal) and not dextran as was used in this work. The partition coefficients in the Ucon 50HB 5100/Reppal PES 200 system [17] were, in most cases, very close to the values obtained in this study (Figs. 3–5), although some differences can be observed. For BSA, the *K* values were higher in the Ucon systems, with $NaClO_4$ and NaAc present. However, this comparison indicates that the structural design (block contra copolymer) and micelle formation have minor effects on the partitioning behaviour of these hydrophilic proteins. Thus, when partitioning hydrophilic proteins the Pluronic system seems to be very similar to the Ucon system.

3.6. Partitioning of recombinant proteins with a tryptophan tag

Partitioning of the recombinant proteins ZT0, ZT1 and ZT2 was performed in the two polymer systems, P105/dextran and F68/dextran at different temperatures, 5 and 30°C. The partitioning results are shown in Fig. 6, where ln *K* is plotted against the number of fused peptides, *T*, to the Z protein.

In the P105 case (Fig. 6a), higher values of *K* were obtained for ZT0 and ZT1 at 5°C compared to at 30°C, but partitioning of ZT2 at 30°C resulted in higher *K* values than at 5°C. It should be stressed that in the low-temperature experiments (5°C) the value of *K* for ZT2 was lower than that for ZT1, which is surprising since ZT2 is more hydrophobic than ZT1 (Fig. 6a). Fig. 6a also shows clearly that the partitioning of the Z protein is changed from the dextran phase to the P105 phase at 30°C due to the insertion of Trp-rich tags (ZT0→ZT2).

Turning to the F68/dextran system (Fig. 6b), the same trends were found, including the decrease in partition coefficient for ZT2 compared with ZT1 at 5°C. However, in the F68 system, the highest *K* values were obtained in 30°C systems for every

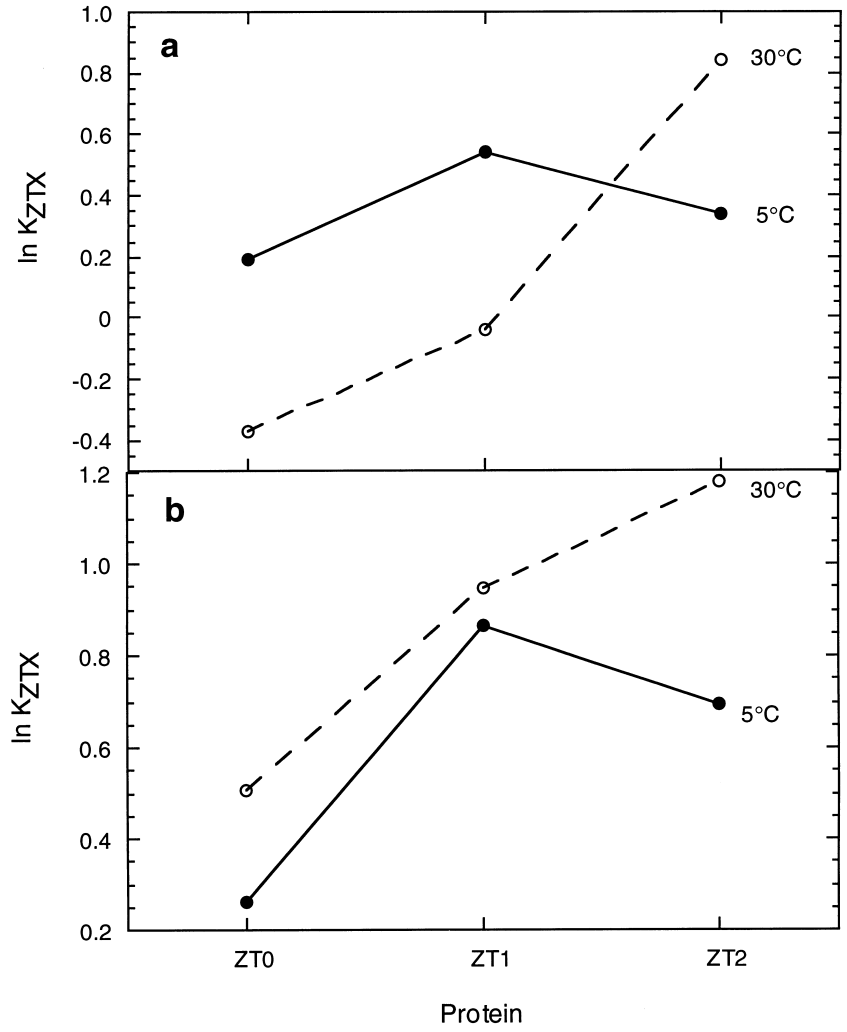

Fig. 6. Partitioning of ZT0, ZT1 and ZT2 in the P105/dextran T500 (a) and F68/dextran T500 system (b), at 5 (●) and 30°C (○). The natural logarithm of the partitioning coefficients is plotted against the protein modification (see Section 3.6). All systems were in 5 m*M* NaP (pH 7.0). Typical uncertainty in *K* obtained from double samples was approximately 4%.

protein. Generally, the values of *K* were higher in the F68 system than in the P105 containing system for all ZTX. The reason for this behaviour is unclear.

A similar protein series has been studied previously in PEO/salt [4], PEO/dextran and Ucon/dextran systems [5], namely the ZZTX proteins, which contain two Z domains but are otherwise identical to ZTX. The results of these earlier investigations showed that the *K* value for ZZTX increased with the number of inserted peptides, *T*, in both Ucon/dextran and PEO/dextran systems [5], which is in contrast to the results obtained here at 5°C and shown in Fig. 6. Furthermore, it was also shown, in a P105/dextran system, that the logarithm of *K* for tryptophan-containing oligopeptides increased linearly with the number of tryptophan units in the peptide [10]. A plausible explanation of the unexpected decrease in the values of *K* at 5°C may be that the ZT2 either self-associates into interprotein complexes or that the fused hydrophobic tail, to some extent, folds intramoleculary. Both these courses of events should result in a more hydrophilic ZT2 protein since

the hydrophobic residues of the peptide become more or less shielded from the surrounding solution. This explanation does not hold for the trends observed at 30°C, where ln *K* showed a more or less linear dependence on the number of *T* units (Fig. 6). Instead, at 30°C, the micelle (P105 case) or the unimer (F68 case) may interact favourably with the fused T2 peptide which prevents the peptide aggregating and/or folding.

3.7. Membrane-bound peptide and protein

3.7.1. Gramicidin

The partitioning of the membrane spanning-peptide gramicidin in P105/dextran (at the compositions given in Table 2) resulted in *complete* partitioning of the peptide to the P105-rich phase at both 5 and 30°C. The partitioning at both temperatures resulted in almost no difference in peptide concentration in the bottom phase between the reference system (without peptide) and the system with gramicidin. Therefore, no reliable values of *K* can be presented. Gramicidin was dissolved in Triton X-100 solution before the protein was added to the phase-separating polymer system, and therefore the partitioning behaviour of Triton X-100 was examined in protein-free systems. Partitioning coefficients for Triton X-100 were found to be approximately 22 and 60 at 5° and 30°C, respectively. Thus, the effect of the Triton X-100 partitioning to the P105 phase on the gramicidin partitioning is difficult to separate from the intrinsic partition of gramicidin. However, the system seems to be suitable for the extraction of gramicidin.

3.7.2. Bacteriorhodopsin

It was not possible to dissolve this hydrophobic protein directly in a Pluronic solution. (Pluronic copolymers L64, F68 and P105 were tested.) The non-ionic surfactant $C_{12}E_6$ was, however, found to be suitable for solubilization. Bacteriorhodopsin was partitioned in the P105/dextran system at 5 and 30°C and the results are shown in Fig. 7, where ln *K* of bacteriorhodopsin is plotted against ΔP105. The partitioning coefficient was increased from 1.1 to 9.7 when the temperature was increased from 5° to 30°C. Hence, a strong effect was obtained, which is likely to be the result of the formation of micelles in the top phase at 30°C. Since bacteriorhodopsin is membrane-bound, it is reasonable to assume that a P105 micelle would enhance partitioning to the P105-rich phase. However, the low-molecular-weight surfactant

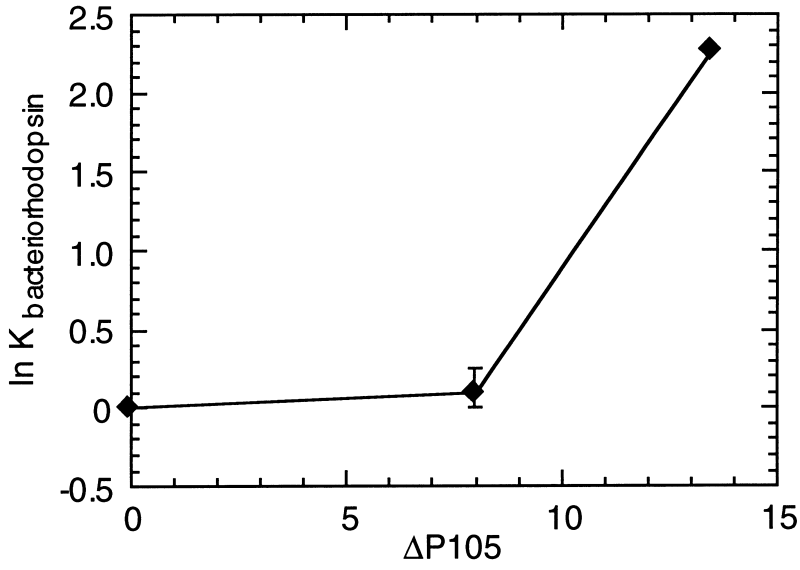

Fig. 7. The natural logarithm of the partitioning coefficient of bacteriorhodopsin plotted against ΔP105 (see Section 3.7.2) in 16 m*M* $C_{12}E_6$ and NaP. The values of ΔP105 were 8 and 13.5% (w/w) at 5 and 30°C, respectively. The error bar indicates the uncertainty obtained from double samples. The uncertainty in *K* at 30°C was approximately 1%.

$C_{12}E_6$, will also act in the same direction, and therefore the increase in the values of *K* for bacteriorhodopsin upon temperature increase will be the result of the increased partitioning of $C_{12}E_6$, which will form mixed micelles with the Pluronic associates. The $C_{12}E_6$/bacteriorhodopsin 'complex' will thus be partitioned to the Pluronic phase. The partitioning coefficient of $C_{12}E_6$ was not determined in the P105/dextran system. However, partitioning data on Triton X-100 are available (see above) and since these two surfactants are similar, it is reasonable to assume that $C_{12}E_6$ also partitions to the Pluronic top phase.

4. Conclusions

The partitioning of various proteins was performed in aqueous two-phase systems. The Pluronic triblock copolymers P105 and F68, and dextran T500 were used as phase-forming components. Pluronic copolymers self-associate upon temperature increase. The partitioning experiments were performed at two temperatures, 5 and 30°C which, at least in the Pluronic P105 case, correspond to the unimeric (single Pluronic chain) and micellar states of the polymer at the concentrations used.

Three hydrophilic proteins (bovine serum albumin, lysozyme and cytochrome *c*), two hydrophobic membrane-bound biomolecules (bacteriorhodopsin and gramicidin) and a specially prepared protein (Z) with fused hydrophobic peptides (T), were used in this study.

The temperature effects were moderate in partitioning experiments with the hydrophilic proteins, bovine serum albumin, lysozyme and cytochrome *c*, although some effects were observed. In the studies with these proteins, different salts and pH regimes were used. Partitioning results were compared with corresponding results obtained from a Ucon/Reppal system and we conclude that, in the case of hydrophilic proteins, Pluronic systems are similar to the random copolymer analogues.

The engineered proteins, ZT0, ZT1 and ZT2 with tryptophan-containing tags, showed interesting partitioning behaviour. At 30°C the values of *K* increased with increasing number of hydrophobic peptides fused onto the Z domain, but at 5°C, a decrease in the values of *K* was observed upon going from ZT1 to ZT2. This trend was found in both polymeric systems (P105/dextran and F68/dextran). This decrease might be attributed to a shielding of the tag, by either aggregation of ZT2 or folding of the T2 tail.

The effect of temperature on the partitioning was strong for the membrane-bound integral protein. The surfactant $C_{12}E_6$ was added to the system to solubilize bacteriorhodopsin. A marked increase in *K* upon temperature increase was observed. This indicates that the micellization of Pluronic P105 plays an important role in the partitioning of bacteriorhodopsin. Mixed micelles of $C_{12}E_6$ and Pluronic P105 were probably obtained.

Gramicidin partitioned totally to the Pluronic-rich phase at both temperatures. This was probably the result of the high affinity for Triton X-100 surfactant (which was added to the system to solubilize gramicidin) to the Pluronic phase.

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