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AFFINITY PARTITIONING OF PROTEINS IN AQUEOUS TWO-PHASE SYSTEMS CONTAINING POLYMER-BOUND FATTY ACIDS

I. EFFECT OF POLYETHYLENE GLYCOL PALMITATE ON THE PARTITION OF HUMAN SERUM ALBUMIN AND α-LACTALBUMIN

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SUMMARY

The partition of serum albumin between the two aqueous phases of a polyethylene glycol–dextran–water two-phase system is strongly influenced by replacing a small fraction of the polyethylene glycol by its palmitate ester. The partition of albumin is compared with the partition of polymer-bound palmitate using polyethylene glycol [1-^{14}C]palmitate. The data indicate that a maximum ("saturation") effect on the partition of albumin corresponds to a binding of fewer than two palmitate groups per albumin molecule. The effect on α-lactalbumin corresponds to the maximum binding of 0.5 palmitate group per protein molecule. These data do not fit the most favoured available model for affinity partitioning. The deviation can be attributed to the observation that the palmitate groups probably form micelle-type aggregates in the polyethylene glycol-rich phase at the concentrations used for albumin extraction.

INTRODUCTION

Soluble proteins can partition between the two liquid phases of water–dextran–polyethylene glycol two-phase systems owing to the high concentrations of water in both phases. This fact has been utilized both for separation of proteins and for studies of their isoelectric points and tendency for mutual association[1–6]. An important contribution to this method has been the observation that proteins (*e.g.* albumins) with exposed hydrophobic surfaces selectively partition towards the upper phase when the system contains palmitic acid groups covalently bound, via an ester linkage, to polyethylene glycol (the polymer enriched in this phase)[7–9]. The hydrophobicity of a number of proteins could be estimated[10] by comparing the change in the partition coefficient of the proteins produced by introduction of polymer-bound palmitoyl groups.

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This type of liquid–liquid partition, where partition of a protein is selectively influenced by a specific ligand coupled to one of the phase-forming polymers, has been termed "affinity partitioning". It has been analysed theoretically by Flanagan and Barondes[11] and their model fits well to the experimental data on affinity partitioning of a myeloma protein using 3,5-dinitrophenyl as ligand. However, the effect of polymer-bound palmitate on the partitioning of albumin results in deviations from the above model[12,13], and the possible existence of additional equilibria, *e.g.* interactions between the palmitoyl groups, must be considered. The present work aims at a broader understanding of the factors determining the affinity partitioning of proteins, especially when hydrophobic ligands are used. There is a growing need for such an understanding because polyethylene glycol palmitate is increasingly used for isolation and studies of membrane-bound particles[14–18], for which no well-characterized model particle is known even though some progress in this field has been achieved by studying the partition of liposomes[19,20].

MATERIALS AND METHODS

Chemicals

Dextran 500 (M_r = 500,000) was purchased from Pharmacia. Polyethylene glycol was obtained from Union Carbide, New York, U.S.A. (M_r = 6000–7500). Human serum albumin, fatty acid free, was from Miles, and α-lactalbumin and α-chymotrypsin were purchased from Sigma. Polyethylene glycol palmitate was synthesized as described earlier[7] and contained 0.12 mmol palmitate per gram of polymer (40% substitution). All other chemicals were of analytical reagent grade.

Labelled polyethylene glycol palmitate

[1-^{14}C]Palmitic acid, (0.216 mCi/mg) was purchased from The Radiochemical Centre, Amersham, U.K. It was diluted with 170 times its weight of unlabelled palmitic acid and then converted into palmitoyl chloride by maintaining a toluene solution containing traces of pyridine and excess thionyl chloride at 100°C for 30 min. The solvent and remaining thionyl chloride were removed by evaporation under vacuum at 80°C. The acyl chloride residue was treated with polyethylene glycol (50 g per gram of palmitic acid) dissolved in toluene. The mixture was dried by azeotropic distillation and the solution was refluxed for 1 h after addition of dry pyridine (0.05 ml per gram of polymer). The solution was filtered and the polymer precipitated at 3°C. It was further purified by three-fold crystallization from non-aqueous methanol at 3°C. The final product contained 18 μCi per gram polymer.

Two-phase systems

The systems were prepared from aqueous solutions of polyethylene glycol (40%, w/w) and dextran (20%, w/w) as described previously[1,21]. The concentration of the dextran solution was determined polarimetrically at 20°C, using the specific rotation[1] $[\alpha]_D^{20}$ = 199° ml g^{-1} dm^{-1}. After thermostatting, the systems were gently mixed and left to settle at the same temperature (23°C) for 30 min. Before sampling the systems were centrifuged for 4 min at 250 *g* to ensure phase separation. No change in the temperature, as might occur during centrifugation at higher speeds, could be detected.

Partition of albumins

To determine the partitioning of a protein between the two phases, equal volumes were withdrawn from the phases and each was diluted five-fold with water. The absorbance was measured at 280 nm using a Hitachi SP-II spectrophotometer. Correspondingly diluted phases from systems not containing protein were used as blanks. The partition coefficient, K, of a protein was calculated as the ratio between the absorbances in upper and lower phase.

Partition of polyethylene glycol [1-^{14}C]palmitate

This was measured by withdrawing 100–500 μl of each phase and mixing it with Pico-Fluor scintillation cocktail liquid (Packard, U.S.A.). The samples were counted (for 5 min with automatic corrections for background and quenching) in a Packard liquid scintillation counter (Model PLD).

Viscosity

The viscosities of the phases and the polymer solutions, respectively, were determined with reverse-flow U-tube viscosimeters. The densities were obtained by use of U-shaped micropycnometers.

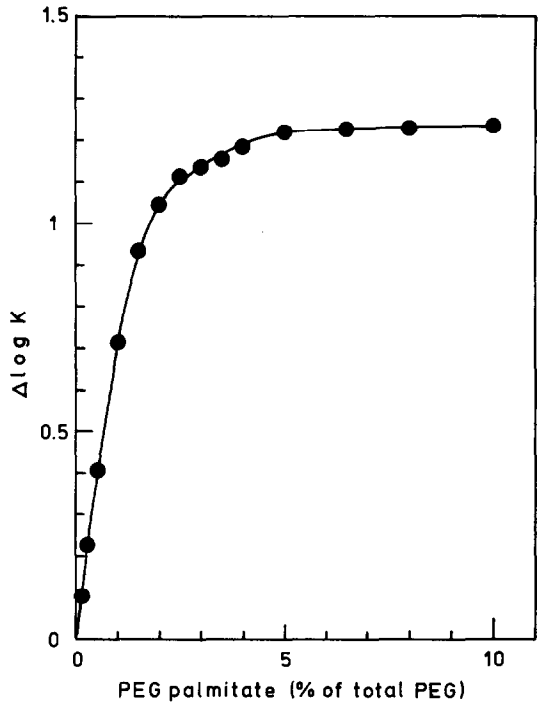

Fig. 1. Increase of log K (Δlog K) of human serum albumin as a function of PEG palmitate concentration expressed as a percentage of total PEG. System composition: 4% (w/w) dextran, 4% (w/w) PEG (including PEG palmitate), 10mM potassium phosphate buffer (pH 7.1), 100 mM potassium chloride, and 2 g/l albumin. Temperature, 23°C.

RESULTS

A typical extraction curve depicting the effect of increasing concentration of polyethylene glycol palmitate (PEG palmitate) on the logarithmic partition coefficient, log K, of albumin is shown in Fig. 1. This curve resembles a binding isotherm in that log K increases with the concentration of the ligand in the phase system until it attains a plateau ("saturation") value, log K_{max}.

The partition of human serum albumin at various concentrations of the phase-forming polymers, dextran and polyethylene glycol (PEG), is shown in Fig. 2. If the concentration of either polymer, expressed as a percentage of the total weight of the system, is below 3.6%, only one phase is obtained.

The logarithmic partition coefficient (log K) for albumin decreases monotonically with increasing polymer concentrations when the systems do not contain any

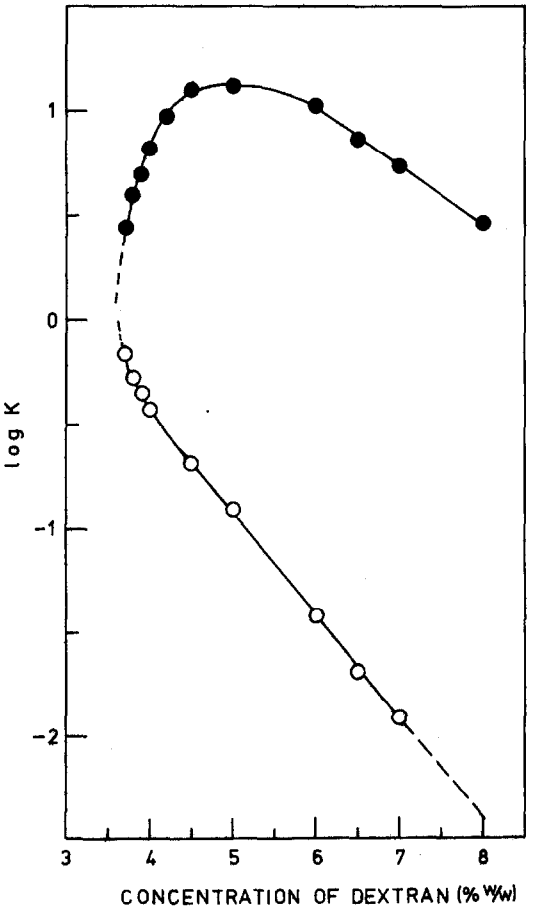

Fig. 2. Effect of polymer concentrations on the partitioning of human serum albumin without (○) and with (●) PEG palmitate (10% of total PEG). System composition: equal concentrations (in % w/w) of dextran and PEG (including PEG palmitate), 10 mM potassium phosphate buffer (pH 7.1), 100 mM potassium chloride, and 2 g/l albumin. Temperature, 23°C.

PEG palmitate. When part of the PEG (10%) is replaced with PEG palmitate the partition coefficient shows a large increase as the total concentrations of PEG and dextran are both raised from 3.6 to 4.5% (w/w). At higher polymer concentrations the log K value first levels off and then decreases. The concentration of PEG palmitate is so high that any further increase does not effect the partition of albumin, *i.e.* log K = log K_{max} (*cf.* Fig. 1 and refs. 8 and 9). According to the theory[11] the increase in log K of albumin, Δlog K, due to introduction of palmitoyl groups, should be given by eqn. 1 at saturation (Δlog K_{max})

$$\Delta \log K_{max} = n \cdot \log K(\text{PEG palmitate}) + \log \frac{K(\text{ass, U})}{K(\text{ass, L})} \quad (1)$$

where n is the number of binding sites for palmitate per albumin molecule, log K(PEG palmitate) is the logarithmic partition coefficient of PEG palmitate, and K(ass, U) and K(ass, L) are the association constants for the albumin–PEG palmitate complex in the upper and lower phases, respectively. Consequently, if the two association constants are assumed to be equal, then the ratio Δlog K_{max}/log K(PEG palmitate) should be equal to n.

To investigate the partition of PEG palmitate the palmitoyl group was labelled with ^{14}C. Its partitioning in systems of the same composition as in Fig. 2 is shown in Fig. 3. The systems contained an excess of palmitoyl groups with respect to the serum albumin (molar ratio 153–326). The ratio Δlog K_{max}/log K(PEG palmitate) is nearly constant with values in the range 0.8–1 (Fig. 3).

The partitioning of PEG palmitate alone (in the absence of albumin) is strongly dependent on its concentration (Fig. 4). At low concentrations it partitions as PEG

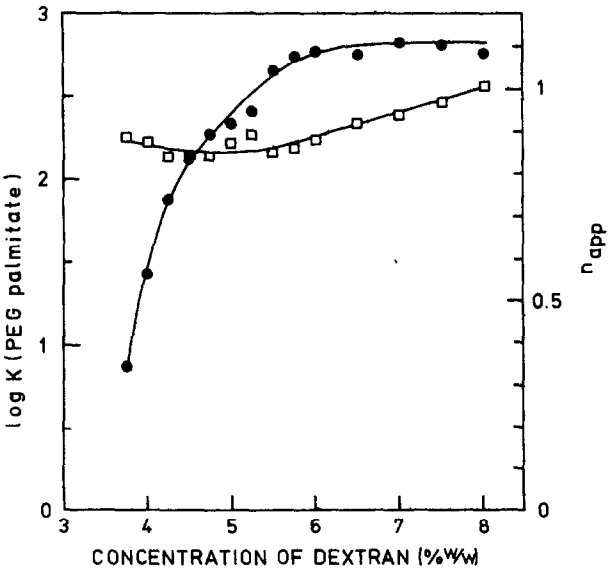

Fig. 3. Partitioning of PEG palmitate, 10% of total PEG, as a function of the concentration of phase-forming polymers (dextran and PEG) (●). System as in Fig. 2 but without albumin. The ratio log K_{max}(albumin)/log K(PEG palmitate) = n_{app} (□) has been calculated with data taken from Fig. 2.

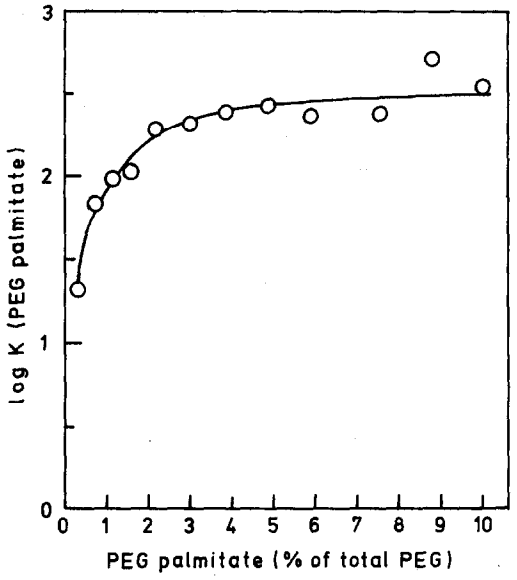

Fig. 4. Partitioning of PEG palmitate and its dependence on concentration (in per cent of total PEG). System composition: 6% (w/w) dextran, 6% (w/w) total PEG, 10 m*M* potassium phosphate buffer (pH 7.1), and 100 m*M* potassium chloride. Temperature, 23°C.

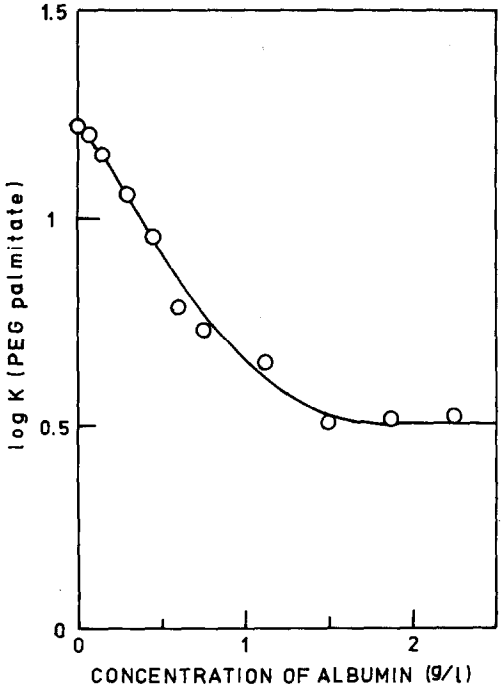

Fig. 5. Partitioning of PEG palmitate in low concentration (4.3 μM palmitate) in the presence of human serum albumin (0–2.25 g/l, 0–33 μM). System composition otherwise as in Fig. 4.

(log K = 1.3), but the partition coefficient increases ten-fold as the concentration of PEG palmitate increases.

The partitioning of PEG palmitate (at a low, constant concentration, 4.3 μM PEG-bound palmitate) in the presence of increasing amounts of albumin has been investigated (Fig. 5). The partition coefficient of the ligand–PEG decreases to a plateau value of log K = 0.5 at a high concentration of protein. Because the molar ratio albumin: PEG palmitate is 7.7 at the highest concentration of protein it can be assumed that not more than one polymer-bound ligand molecule is bound per albumin molecule. The partition coefficient obtained by measuring the partitioning of [^{14}C]palmitate at this point represents the partitioning of the albumin–ligand (1:1) complex. Because log K for free albumin in this system is −1.4, the binding of one PEG palmitate cause a shift in log K of 1.9 units. In this system log K = 1.3 for PEG as well as for PEG palmitate. The ratio $\Delta\log K_{max}/\log K$(PEG palmitate) is therefore 1.4 at low concentrations of PEG palmitate if one assumes that each PEG palmitate is bound to a single albumin molecule.

Another protein that can bind PEG palmitate, α-lactalbumin, shows a constant value for $\Delta\log K_{max}$ (0.5), which is independent of the concentrations of dextran and

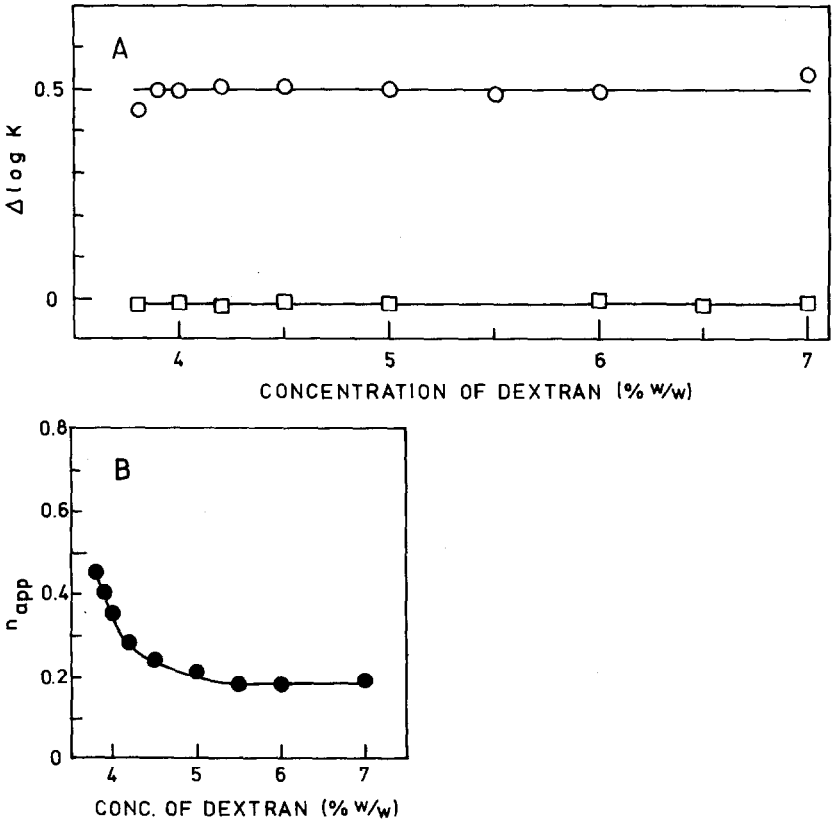

Fig. 6. (A) Partitioning of α-lactalbumin (○) and α-chymotrypsin (□), 2 g/l, at various concentrations of phase-forming polymers. System composition as in Fig. 2. (B) Variation of n_{app} = $\Delta\log K$(lactalbumin)/log K(PEG palmitate) with concentrations of the polymers.

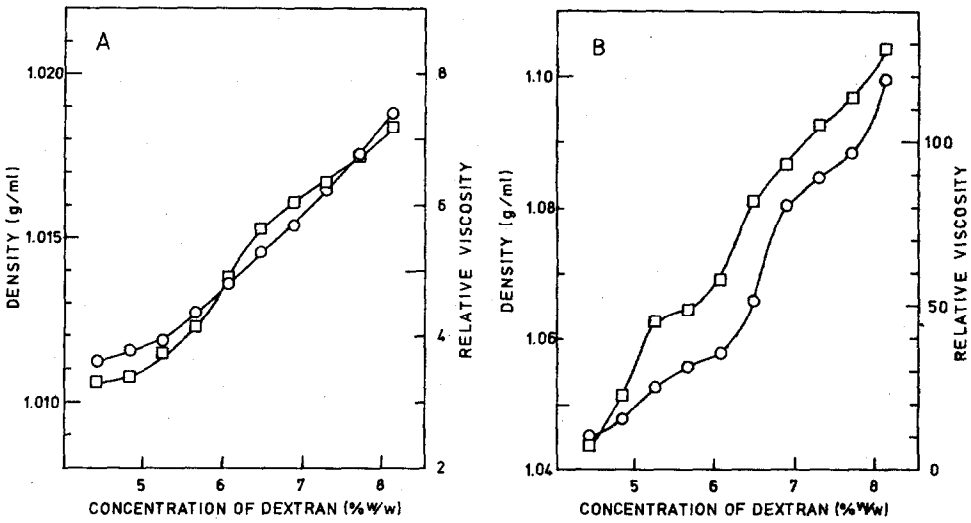

Fig. 7. Viscosity (□) and density (○) of the two phases in systems containing various concentrations of dextran and PEG (no salts added) at 23°C. The viscosity is given relative to water. (A) Upper phase; (B) lower phase.

PEG (Fig. 6A). A non-binding protein, α-chymotrypsin (Fig. 6A), shows a constant and near zero value for $\Delta \log K$ (< 0.04). The apparent n value for α-lactalbumin therefore decreases with increasing concentrations of PEG and dextran at high concentrations of PEG palmitate (Fig. 6B).

When the polymer concentrations of the two-phase systems are changed, both the densities and viscosities of the phases show irregular changes (Fig. 7). The abrupt changes indicate that the liquid structure of the phases passes through several different stages.

DISCUSSION

The results presented show that the influence of PEG palmitate on the partitioning of proteins is more complex than previously thought[12,13] on the basis of the model for affinity partitioning[11]. Human serum albumin has at least five binding sites with various degrees of association strength with fatty acids[22]. The apparent number of binding sites, n_{app}, calculated from the data presented here, using the theory of Flanagan and Barondes[11] and assuming the same binding constants in both the upper and lower phases, is less than 2.

For the case where the binding constants in the two phases are equal, the theory of Flanagan and Barondes[11] reduces in effect to the concept that the change in the solvation of the protein, due to the binding of PEG-bound ligand to it, constitutes the change in the free energy ($\Delta \log K$) of transfer from the lower to the upper phase and that each bound ligand makes an equal contribution ($\log K_L$) to this free energy. Several factors may cause the value of n_{app} for serum albumin to be lower than expected: (a) the solvation layer created by the binding of, on average, 1.5 PEG palmitate per albumin may not change significantly following the binding of more

PEG palmitate to albumin; (b) the ligand molecules already bound to the protein sterically hinder the binding of further ligands; (c) the palmitoyl groups are to a large extent trapped in a pseudogel structure and therefore do not participate in the assumed equilibrium; (d) the association constants in the two phases differ significantly.

The fourth factor (d) is probably not a determining factor. The only experimental results available so far[11,23] indicate that the association constants in the two phases are practically equal in phase systems with a composition not far from the critical composition, a condition fulfilled by at least some of the systems used in this work.

If we compare the physical dimensions of serum albumin and the PEG used here, we find that the protein, idealized as an ellipsoid is *ca.* 4.5 × 15 nm assuming the axial ratio 4.9[24], while the linear polymer chain is *ca.* 60 nm. Because it must be assumed that the polymer tail, except for the fact that it is attached to the protein molecule at one end, is moving freely over the protein surface it can cover a considerable area. One to two bound PEG molecules should be enough to create a PEG "atmosphere" around the albumin molecule. The binding of more PEG palmitate molecules might therefore be hindered or may not enhance the affinity of the protein for the upper phase. This is in line with the factors (a) and (b).

The fact that the viscosity changes as the concentrations of the polymer changes points to structural changes in the liquid (or semi-liquid) state of the phases, caused probably by the self-association of the polymers to yield supramolecular structures. These structures may act both as a protein-excluding network and as a semistable matrix with ligands well spaced for multiple attachment to a protein.

The fact that PEG palmitate at relatively high concentration has a partition coefficient ten times larger than that of PEG points to a strong ligand–ligand interaction in the upper phase. The critical micelle concentration (CMC) for PEG palmitate can be evaluated from Fig. 4. It is in the range 1–2% (of total PEG which in turn is 6% of the total system) PEG palmitate, or 0.6–1.2 g PEG palmitate per kilogram of the system. This can be compared with the CMC for PEG palmitate of the same degree of substitution measured directly by Axelsson[13], who found a value of 0.6 g per kilogram of solution. Because the PEG palmitate contains 0.12 mmol palmitate per gram (the degree of substitution is 40%), the CMC is 72–144 μM. This is less than the value of 2.8 mM found for free palmitic acid and more than the values for palmityl-PEG with short PEG chains[25]. From the latter an extrapolated value of the CMC for palmityl-PEG with M_r = 6000, 10–20 μM, is obtained. It should be remembered that palmitate dimerized appreciably at concentrations much below the CMC[26].

The observed differences in the affinity partitioning behaviour of serum albumin and α-lactalbumin in palmitate-containing systems can also be attributed to the formation of PEG palmitate "micelles", if the self-association of the palmitate is stronger than its affinity for α-lactalbumin. In this case, as pointed out by Tanford[27], only a part of the binding isotherm can be experimentally observed. This would explain why the calculated value of $\Delta \log K_{max}$ for α-lactalbumin is 0.5 (n_{app} = 0.2–0.5) and also why, in contrast to the value for serum albumin, this value remains constant at all ligand-to-protein ratios used in this work. In order to observe higher values of n for α-lactalbumin, one would have to work at concentrations of PEG below its CMC and correspondingly low concentrations of α-lactalbumin. Such work using [^{125}I]-labelled proteins is under way.

CONCLUSIONS

When proteins such as human serum albumin are partitioned in aqueous two-phase systems containing PEG palmitate, the strong tendency of this PEG derivative to form structures by self-association results in an affinity partitioning effect much less than expected from the known affinity of these proteins for palmitic acid.

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