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A new micellar aqueous two-phase partitioning system (ATPS) for the separation of proteins

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Abstract

Partitioning of six typical globular proteins with molecular weights ranging from 12.6 to 250 kDa was investigated using an aqueous two-phase system formed by heating a solution containing the individual proteins and *n*-dodecyldimethylphosphine oxide (APO12) above the cloud point of the nonionic surfactant (approximately 40 °C). The partition coefficient, K_p, was much greater at 55 than 45 °C and depended on both APO12 and protein concentrations. The value of K_p for bovine β-lactoglobulin (β-L) varied from 2 to 60, and was larger for 1.0 mg/mL solutions than for ovalbumin (2× greater), bovine serum albumin (3× greater) and lysozyme (12× greater). Catalase and cytochrome *c* were apparently denatured in the presence of 20 mg/mL of APO12 and were not investigated. Large values of K_p for β-L resulted when the pH of APO12 mixtures containing phospholipids and either a cationic or anionic surfactant in molar ratios of 10:0.5:1.0 was partitioned above or below the isoelectric point of the protein, respectively. The affinity of the proteins for the APO12 micelle was responsible for partitioning of the proteins into the upper phase. Finally, DSC studies with β-L showed that the denaturing action of *n*-decyldimethylphosphine oxide (APO10) below 61 °C and APO12 at 22 °C was reversed by dilution or dialysis, respectively.

Keywords: Two-phase partitioning; Micelles; Nonionic surfactant; Proteins; ATPS

1. Introduction

Several aqueous two-phase partitioning systems (ATPS) have been developed for the separation of cell particles and macromolecules under mild conditions, including viruses, proteins and nucleic acids, by making use of their unequal distribution between two liquid phases [1]. Factors that effect partitioning include the molecular weight of the polymers forming the two liquid phases, polymer concentration, surface properties including charges of the components of the mixture, temperature, solvent polarity and supporting electrolyte.

Proteins and viruses have been separated with a mixed micellar system of *n*-decyltetraethylene oxide ($C_{10}E_4$) containing either anionic sodium dodecyl sulfate (SDS) or cationic alkyltrimethylammonium bromide [2]. A thermo-separating cationic-modified ethylene oxide polymer–SDS system attracts or repels proteins of opposite or like charge, respectively, depending on the pH of the solution [3]. Positive or negative charged mixed micelles were shown to strongly affect the partition coefficients of five typical globular proteins [4] and a pH responsive copolymer system has been used to achieve high separation ratios from a mixture containing cytochrome *c* (cyt *c*) and bovine serum albumin (BSA) [5]. Protein partitioning can be enhanced by affinity labeling [6] as illustrated by attaching *n*-decyl-β-D-glucopyranoside to the protein and using the surfactant to form the two liquid phases [7]. A recent theory was developed to predict protein partition coefficients in two-phase aqueous mixed (nonionic/cationic) micellar systems, and it was used to describe the partitioning behavior of glucose-6-dehydrogenase [8]. A relationship between the hydrophobic character of the protein surface, determined by the binding of a fluorescent probe, and partitioning could not be established for a group of similar mammalian albumins [9]. However, surface hydrophobicity and charge, determined by ammonium sulfate precipitation, correlated well with partition behavior in some cases [10].

Members of the alkyldimethylphosphine oxide (APO) class of nonionic surfactants are pH and temperature stable, water

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soluble, colorless in the near UV and visible region of the spectrum, have critical micelle concentrations (cmc) in the millimolar range, easily prepared in high purity, have a signature ^{31}P NMR signal that is sensitive to the polarity of its environment and are commercially available [11]. Dodecyldimethylphosphine oxide (APO12) is marketed (Calbiochem) for the two-phase separation of proteins, but this potential has not been fully developed. Decyldimethylphosphine oxide (APO10) has been used for the solubilization of cell membranes leading to the isolation of plasmid DNA from *Escherichia coli* [12].

The aqueous phase diagrams for several members of the APO family of compounds have been determined over a broad concentration range [13]. The lower consulate temperature (LCT) for 10% solutions of APO10 and APO12 was approximately 125 and 40 °C, respectively. However, these temperatures are lowered precipitously by the addition of various phospholipids (PL) to as low as 10 °C depending on the mole fraction of APO and PL [14]. The phase separation temperature was modeled using regular solution theory, and it is related to the molecular weight of the micelles and mixed micelles [15]. The composition of the two liquid phases that result from heating aqueous solutions of several aqueous APO and APO/PL mixtures above the lower critical solution temperature (LCST), or cloud point, has been determined [13,16], and it is possible that the liquid–liquid phases that are formed by these mixtures have potential use for protein separations, particularly membrane proteins which require non-conventional methods [17].

The purpose of this investigation was to explore the use of the two-phase system generated in aqueous solutions with a nonionic surfactant (APO12) for the partitioning of several model hydrophilic proteins; cytochrome *c*, lysozyme, bovine β-lactoglobulin (β-L), ovalbumin, bovine serum albumin, and catalase. Partitioning was found to be very sensitive to pH when ionic surfactants are included in the partitioning system according to the net charge of the protein. The thermal stability of β-L is known to be a sensitive indicator of the native structure of the protein as shown by previous DSC investigations involving denaturing agents [18–20], and studies with β-L were useful in determining the mechanism of partitioning. Finally, DSC was used to demonstrate the reversible nature of APO10 and APO12-induced denaturation of β-L below 61 °C.

2. Experimental

2.1. Materials

The sample of β-lactoglobulin (p*I* 5.2, MW = 18.4 kDa) used in this study was the same sample that was isolated from cow's milk and used in a recent calorimetric study [20]. The samples of bovine serum albumin (p*I* 4.9, MW = 65 kDa), lysozyme (p*I* 10.7, MW = 14.1 kDa), ovalbumin (p*I* 4.6, MW = 52 kDa), catalase (p*I* 5.4, MW = 250 kDa), cytochrome *c* (p*I* 10.0, 12.6 kDa), tris(hydroxymethyl)aminomethane (Tris), L-α-dimyristoylphosphatidyl choline (DMPC) and dodecyltrimethylammonium bromide (DTAB) were purchased from Sigma Chemical Co. A sample of sodium dodecyl sulfate was acquired from BDH Chemical Ltd. The samples of *n*-decyldimethylphosphine oxide and *n*-dodecyldimethylphosphine oxide (BioAffinity Systems, Rockford, IL) were the same samples used previously and were free of impurities as determined by gas chromatography [11]. Buffers containing 0.02 M Tris/HCl were used in the pH range of 7–9, and 0.02 M phosphate buffer was used with a pH less than 7. The phosphate buffer was prepared by mixing reagent grade phosphoric acid and either sodium phosphate or sodium hydrogen phosphate. All solutions were prepared with deionized distilled water (17 MΩ cm) and contained 5 ppm of NaN_3 to inhibit bacterial growth. The solid protein and lipid samples were dissolved with gentle shaking or stirring with a magnetic stirrer in order to minimize foaming. Solutions were stored at 5 °C after they were prepared and normally used within 2–3 days.

2.2. Protein concentration

Protein concentration was determined from the absorbance at 280 nm after correction for light scattering due to the presence of micelles with a Hitachi Model 100-40 spectrophotometer. The intensity of scattered radiation for dilute solutions of micelles has an inverse proportional relationship with the fourth power of wavelength and the ratio of the absorbance at 280 and 310 nm for a 7 mg/mL APO12 solution without protein using a water blank was 1.5 ± 0.02 versus the expected value of 1.50. This was the normal APO12 concentration of the protein–surfactant mixtures from the upper phase that were analyzed following phase separation, and further dilution of the samples to below the cmc would have greatly reduced the precision of the measurement. The corrected absorbance of the protein at 280 nm, $OD_{280}^{protein}$, was determined from the absorbance of the APO12–protein mixture since the protein absorbance is much less at 310 than 280 nm by the equation:

$$OD_{280}^{protein} = OD_{280}^{mixture} - 1.5\,OD_{310}^{mixture} \quad (1)$$

The standard curve that was prepared by this method in the presence of 7 mg/mL APO12 over the range of β-L concentrations investigated in this study was linear with a correlation coefficient of 0.986 compared with a value of 0.999 for the standard curve without APO12 over the same concentration range.

2.3. Cloud point

The cloud point temperature, T_{cp}, of β-L solutions containing various lipids was measured with a thermistor/thermometer (Cole Parmer). The average temperature at which a 3 mL sample of solution visibly changed from clear to turbid upon heating or turbid to clear upon cooling was taken as the cloud point. The precision of this measurement was ±0.1 °C.

2.4. Partition coefficients

Stock solutions of APO12, buffer and protein were added consecutively to 3 mL vials at room temperature in order to produce 1.5 mL solutions, which models the way APO12 might be used in other applications (instead of pre-forming the two-phase

system without protein). The tightly sealed vials were placed in a water bath for various periods of time (3–24 h) and inversion intervals (0–3), and equilibrium was reached after 15 h for samples that were inverted once after 3 h. Shaking was not used to shorten the time to reach equilibrium due to unknown possible surface effects. A 100 μL sample from each phase was removed with a digital pipette (Eppendorf) and diluted with buffer to 2 or 3 mL. The concentration of protein in each phase was obtained from the corresponding experimental value of $OD_{280}^{protein}$ along with the volumes of the top (V_T) and bottom (V_B) phases and mass balance for the opposite phase. The recovery of protein from the measured values of $OD_{280}^{protein}$ in each phase was $80 \pm 5\%$ and was independent of the total protein concentration between 1 and 10 mg/mL. The partition coefficient, K_p, is defined as the ratio of the protein concentration in the top phase to the protein concentration in the bottom phase. The average value of K_p from 37 experiments using samples from the top phase, K_p^T, was 13% less than for samples from the bottom phase, K_p^B. Therefore, K_p^B was set equal to K_p since these values were considered to be more accurate. The standard deviation for the average of 18 duplicate determinations of K_p for β-L samples ranging from 1 to 10 mg/ml with 20 mg/mL APO12 solutions was ±6%. Values of K_p determined independently from the ratio of $OD_{280}^{protein}$ in each phase were comparable to the values of K_p obtained using the mass balance procedure.

2.5. Differential scanning calorimetry

DSC studies were performed with a MC-2 differential scanning calorimeter (MicroCal Inc.) that was interfaced to a desktop computer using an analog/digital board (Data Translation, Marlboro, MA). A nominal scanning rate of 60 K/h was used for all of the experiments. The Origin software package version 2.9 supplied by MicroCal was used for data collection, analysis, and plotting. Details of the experimental protocol for the DSC experiments were previously described [21].

3. Results and discussion

3.1. Partitioning

3.1.1. Dependence on protein molecular weight, pH, temperature and concentration

Previous studies have shown that partition coefficients of proteins depend on the molecular mass and concentration of the PEG/Dx used to form the two-phase system and the molecular mass of the proteins, which may vary with pH and temperature [22]. Therefore, some of the same proteins previously investigated were studied under various conditions of pH, temperature and protein/APO12 concentrations. Protein precipitation was encountered with catalase when incubated with an APO12 concentration equal to 20 mg/mL and partition data could not be obtained. The heme was extracted from a sample of cytochrome *c* when it was incubated at 45 °C (pH 6 or 8) and the protein was apparently denatured. However, K_p values were obtained for ovalbumin, lysozyme and bovine serum albumin and the results

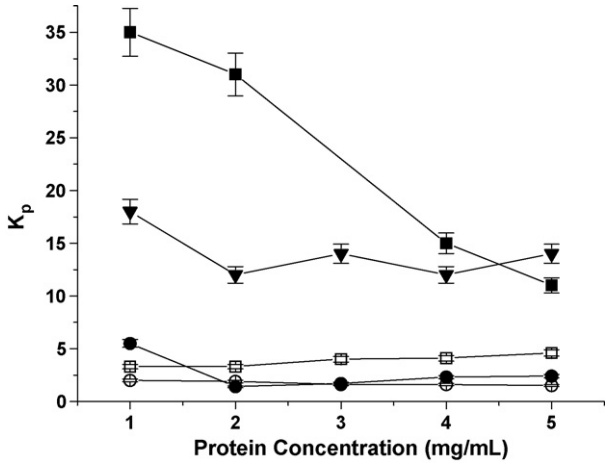

Fig. 1. Variation of K_p with concentration for ovalbumin (squares), serum albumin (down triangles) and lysozyme (circles) at either 55 °C (filled symbols) or 45 °C (open symbols). The concentration of APO12 was 20 mg/mL and the pH was 3.0 for lysozyme or 8.0 for the ovalbumin and albumin samples.

from these experiments are given in Fig. 1. The values of K_p were much higher at 55 than 45 °C for bovine serum albumin than for lysozyme. A similar relationship was previously noted using a PEG/Dx partitioning system [22], possibly reflecting the more hydrophobic surface of serum albumin.

The values of K_p with 1 mg/mL solutions of β-L held at 55 °C were approximately 2–20 times larger than for the other three proteins and β-L was investigated in more detail. Partitioning experiments were conducted at 45 and 55 °C with various concentrations of β-L and the APO12 concentration equal to 20 mg/mL at pH 3.0 and 8.0 where the protein exists as a monomer or dimer, respectively [23]. The results obtained are shown in Fig. 2. The values of K_p determined at 55 °C overlap, which suggests that the normally dimeric state of β-L at pH 8 may now be monomeric in the presence of APO12—if excluded volume plays a significant role in partitioning [22]. However, no systematic correlation of K_p with molecular weight

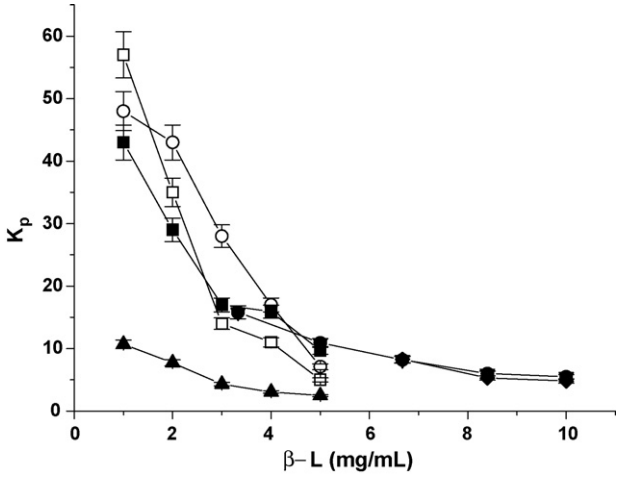

Fig. 2. Variation of K_p with β-L concentration for six separate experiments at either pH 3 (open symbols) or pH 8 (filled symbols). The concentration of APO12 was 20 mg/mL and the incubation temperature was at either 55 or 45 °C (up triangles).

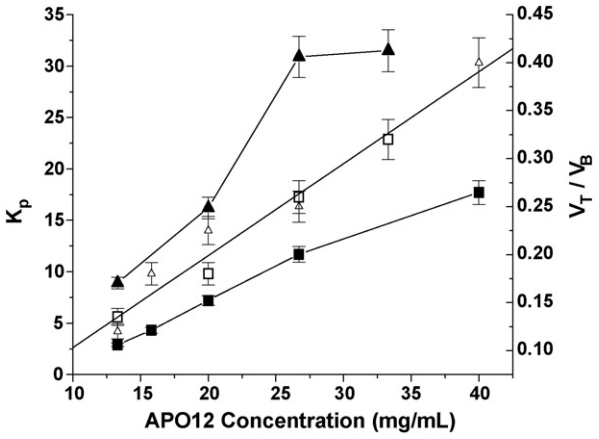

Fig. 3. Variation of K_p with APO12 concentration for 2.61 mg/mL (filled triangles) and 5.04 mg/mL (filled squares) β-L solutions at pH 8 following incubation at 55 °C. The corresponding ratios between the volumes of the top layers, V_T, to the volumes of the bottom layers, V_B, are also shown as open symbols. A least squares fit of the combined volume data gave the solid straight line also included in the figure.

was observed for the four proteins investigated, which indicates that other factors are more important.

The values of K_p determined following incubation at 45 °C are much less than the ones following incubation at the higher temperature. The ratio, V_T/V_B, at 45 °C ($n=5$) and at 55 °C ($n=23$) was 0.29 ± 0.02 and 0.18 ± 0.02, respectively, for all of the data contained in Fig. 2.

3.1.2. Dependence on APO12 concentration

The partitioning of β-L was investigated with various concentrations of APO12 with the concentration of β-L equal to 2.51 or 5.04 mg/mL, and experiments were conducted at 45 and 55 °C in pH 8 Tris buffer. The results of these experiments are given in Fig. 3. The partition coefficient is highest at the lowest β-L concentrations, but the trends at the two temperatures are the same. The relative volume of the top phase (APO12 rich) increased in a linear manner as the APO12 concentration, [APO12], increased ($V_T/V_B = 7.39 \times 10^{-3} \pm 1.62 \times 10^{-2} + 9.58 \times 10^{-3} \pm 6.5 \times 10^{-4}$ [APO12] (mg/mL)).

3.1.3. Model

Excluded volume considerations [22] would predict that hydrophilic proteins would favor the micelle-poor bottom phase, which was not observed. Therefore, an interaction between the proteins and surfactant must be responsible for the partitioning of the proteins into the micelle-rich top phase. The reverse relationship between increasing concentrations of protein or surfactant on K_p (Figs. 1–3) may be explained by the following partial (top phase equilibria not shown) mechanism:

$$\begin{array}{ccccccc} & K_m & & & K_M & & \\ m_{30}P_B & \Leftrightarrow & P_B + m_B & \Leftrightarrow & M_B + P_B & \Leftrightarrow & MP_B \\ \Updownarrow & & & & \Updownarrow & & \\ m_{30}P_T & & & & MP_T & & \end{array} \quad (2)$$

The surfactant monomer, micelle and protein are identified as m, M and P, respectively, for either the top (subscript T) or bottom (subscript B) phases. It is proposed that the equilibria between the monomeric and micellar states of the surfactant lead to the formation to two different protein–surfactant complexes, $m_{30}P_i$ and MP_i. It is assumed that the association constant for forming $m_{30}P_B$, K_m, is greater than the association constant, K_M, for forming MP_B, and the magnitude of the partition coefficients for the two complexes are reversed. ITC studies have shown that approximately 30 APO12 monomers saturate β-lactoglobulin at 37 ± 1 °C with an affinity of $2 \pm 1 \times 10^3\ M^{-1}$, which is enough to cover about 50% of the protein surface, and DSC studies showed that the thermal transition is not completely eliminated until the APO12 concentration is about 15-fold greater than the cmc [20]. Therefore, the ITC and DSC results indicate a favorable interaction between β-L and both monomeric and micellar forms of the surfactant. According to this mechanism, higher protein concentrations with fixed surfactant concentrations would shift the equilibria according to Eq. (2) to the left and favor the formation of increased $m_{30}P_B$ and decreased MP_B concentrations, resulting in lower values of K_p. Increased surfactant concentrations with fixed protein concentrations would shift the equilibria (Eq. (2)) to the right and favor the formation of increased MP_B concentrations, which would result in higher values of K_p.

The value of K_p was calculated with an APO12 concentration of 20 mg/mL following the approach of Lam et al. [7,8] for comparison with the results given in Fig. 2. They give an expression for the affinity contribution to partitioning, K_p^{aff} in terms of the monomer, m_i, and micelle, M_i, concentration in the top (m_T and M_T) and bottom (m_B and M_B) phases and the affinity for the protein (K_{mT}, K_{MT}, K_{mB} and K_{MB}), respectively, as

$$K_p^{aff} = \frac{1 + K_{mT}m_T + K_{MT}M_T}{1 + K_{mB}m_B + K_{MB}M_B} \quad (3)$$

It is assumed that the monomer concentration is the same in each phase and equal to the cmc, 0.3 mM [11]. The value of K_m, was set equal to $2 \times 10^3\ M^{-1}$ [20], and K_M was assumed to be about 95% lower [7]. The concentration of surfactant in each phase was determined experimentally by incubating a solution of APO12 without protein at 55 °C for 16–18 h, and drying an aliquot from each phase to constant weight at 60 °C. Substitution of these values into Eq. (3) gives $K_p^{aff} = 14$, which is close to the value observed with the lowest protein concentrations at 45 °C and the values with the highest protein concentrations at 55 °C. Protein concentration does not appear as a parameter in Eq. (3), but any proposed mechanism must take protein concentration (Figs. 1 and 2) into account, as with Eq. (2). More complex mechanisms, including protein denaturation are possible, but require a structural knowledge of the micelle/protein complex.

According to this model, Eq. (2), calculations using Eq. (3) show that a fivefold β-L concentration increase will increase the concentration of $m_{30}P_B$ in the bottom phase by fivefold, giving a 12-fold reduction for the value of K_p. The reverse would hold true if the APO12 concentration is increased with a fixed concentration of β-L, and an increased value of K_p would result. The data in Figs. 1–3 are in qualitative agreement with this result.

Table 1
Partition coefficients at 45 °C for β-L in various APO12 mixtures at pH 3 and 8[a]

Mixture	pH 3			pH 8		
	K_p	T_{cp} (°C)	V_T/V_B	K_p	T_{cp} (°C)	V_T/V_B
APO12	9.4	39	0.30	10	40	0.31
APO12–DMPC (10:1)	37	12	0.12	13	20	0.13
APO12–SDS–DMPC (10:0.5:1)	Very high	15	0.28	0.35	24	0.28
APO12–DTAB–DMPC (10:0.5:1)	4	4	0.69	170	30	0.25

[a] The incubation mixture contained 0.75 mg/mL of β-L and 0.08 M APO12.

The concentration of APO12 in the top and bottom phases in the absence of protein was calculated at four temperatures with data from the literature [16] between the cloud point and 55 °C. The resulting ratios of APO12 concentrations in the top/bottom phases were found to increase with increasing temperature corresponding to a van't Hoff enthalpy change of 25 ± 4 kJ/mol, but the values for protein partitioning (Figs. 1 and 2) are two to six times larger. Isothermal titration calorimetric studies indicated that the enthalpy change for the interaction of β-L with monomeric APO12 at 38 °C is small, but the interaction is accompanied by a large temperature dependent heat of denaturation, $\Delta C_p = 5.90 \pm 0.23$ kJ/(mol K), corresponding to a enthalpy difference of 59 kJ/mol between 45 and 55 °C [20]. Therefore, the increased size of the APO12 micelles [13] due to their higher concentration in the upper phase [16] as well as protein denaturation might be responsible for the differences between the values of K_p observed at 45 and 55 °C. The mechanism suggested by Eq. (2) would also account for the dependence of K_p on protein concentration observed for other proteins (Fig. 1), but it is not known if these proteins are also reversibly denatured in the presence of APO12.

3.1.4. Addition of ionic surfactants

The effects of the addition of the anionic surfactant, SDS, and the cationic surfactant, DTAB, to APO12 solutions on the phase separation process were investigated. Since the cloud point of both APO12 mixtures at a molar ratio of 1/20 was found to be above 55 °C, DMPC was introduced into the system in order to lower the cloud point below 40 °C based upon previous research [14,16]. The partition coefficient for separations at 55 °C for an APO12–SDS–DMPC mixture (molar ratio of 10:0.5:0.5) with an APO12 concentration of 20 mg/mL decreased as the pH was increased from 3.0, 6.0, 7.0 and 8.0 to values of 109 ± 12, 4.4 ± 0.7, 2.6 ± 0.2, and 0.7 ± 0.1, respectively, as the protein became negatively charged. The results obtained for additional separations at 45 °C are given in Table 1. It can be seen that an opposite trend was observed when DTAB was present as the pH is changed from 3 to 8. The increased values of K_p in the presence of ionic surfactants due to simple electrostatic considerations are much greater than expected—20–50% increases have been observed [24]. The inclusion of phospholipid in the surfactant mixture would increase the size/structure of the mixed micelles [15] and/or structure of the protein [25], and these factors may be responsible.

3.2. DSC

The thermal stability of β-L was determined by DSC experiments in the presence and absence of APO10, a slightly smaller homologue of APO12 with a cloud point of about 125 °C that can be lowered to 10 °C by the addition of PL [16]. The two surfactants are very similar with respect to their affinity for β-L [20], and different chain length APO surfactants [11] may have different partitioning characteristics when used for ATPS separations. Typical results obtained from these studies are presented in Fig. 4. The magnitude of the enthalpy change for the thermal transition of the buffered solution was reduced by 1.25 mM APO10 and completely eliminated in 5 mM APO10. However, a 1:4 dilution of a β-L solution originally containing 5.0 mM APO10 produced the same thermogram as the freshly prepared sample containing 1.25 mM APO10. A similar result was obtained with a series of β-L samples that were held at either room temperature for various lengths of time or heated in the presence of 5 mM APO10, and once again diluted 1:4 at room temperature with buffer. The results from these experiments are given in Table 2. Except for the results from the second scan of the samples with or without APO10 exposure, the values obtained for the thermal midpoint, T_m, calorimetric enthalpy change, ΔH_c, and van't Hoff enthalpy change, ΔH_{vH}, are the

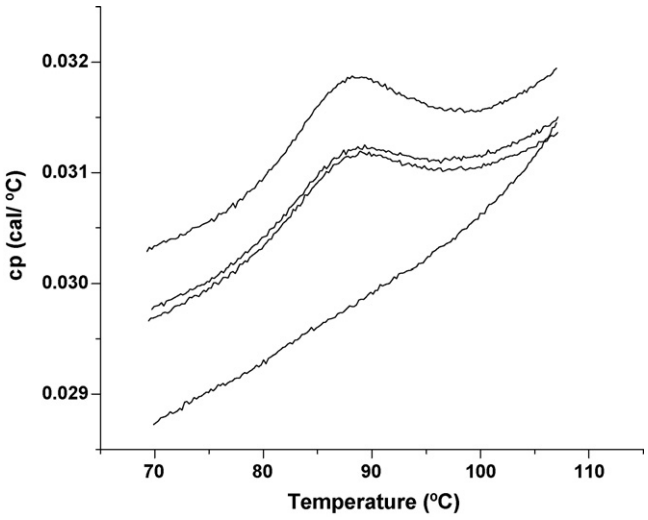

Fig. 4. Raw DSC data obtained at a rate of 60 K/h for 0.129 mM β-L solutions in the presence of 5 mM APO10 (lower curve), 1.25 mM APO10 (solid middle curve), a 1:4 dilution of a 5 mM APO10 solution (broken middle curve) and buffer (top curve).

Table 2
Stability of β-L at pH 2.9 in the presence of various concentrations of APO10 for various periods of time[a]

Sample	T_m (°C)	ΔH_c (kcal/mol)	ΔH_{vH} (kcal/mol)	$\Delta H_c/\Delta H_{vH}$
Buffer	87.4	73	80	0.91
2nd scan	90.2	53	85	0.62
1.25 mM—fresh	87.5	41	83	0.49
1.25 mM—2.5 h/22 °C	86.5	45	86	0.52
5 mM—2 h/22 °C[b]	88.3	51	81	0.63
5 mM—5 min/40 °C[b]	86.9	38	86	0.44
5 mM—30 min/50 °C[b]	87.3	43	88	0.49
5 mM—30 min/60 °C[b]	87.1	42	87	0.48
2nd scan	87.8	28	97	0.29

[a] All samples that were placed into the calorimeter cell contained 0.13 ± 0.01 mM β-L.
[b] Diluted to 1.25 mM after incubation.

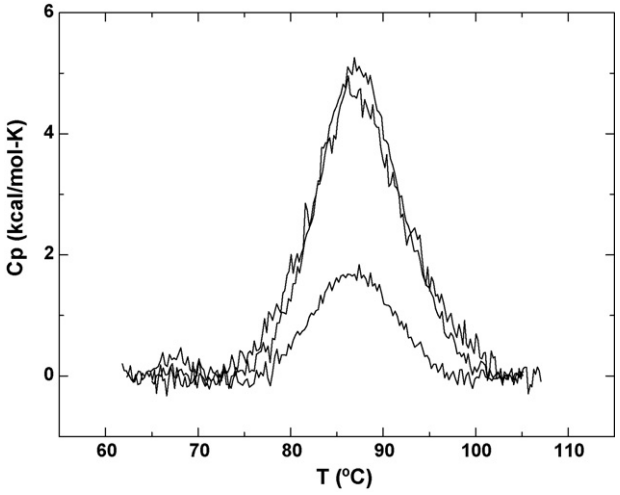

Fig. 5. Molar heat capacity of β-L in the presence of 0.5 mM APO12 (lower curve), buffer (solid upper curve) and a dialyzed sample that originally contained 0.5 mM APO12 (broken upper curve).

same for the heated and unheated samples within the limits of experimental error. These results indicate that the native structure of the protein is disrupted by the addition of 5 mM APO10 (cmc = approximately 3.5 mM), but the process is reversed by simple dilution. The lower values of ΔH_c and $\Delta H_c/\Delta H_{vH}$ obtained for the second scans demonstrate the partial irreversible nature of the thermal denaturation process [18–20].

Over night dialysis at room temperature of β-L that initially contained 5.7 mM APO10 against pH 2.9, 0.2 M glycine buffer completely restored the thermal transition profile that is characteristic of the native form of the protein [18–20]. Dialysis at room temperature of a β-L solution with a concentration of APO12 that reduced the magnitude of the thermal transition enthalpy change by about 50% (0.5 mM), also restored the enthalpy change to that of the native protein (Fig. 5). These experiments provide evidence that the reactions represented by Eq. (2) are reversible.

4. Conclusions

The two-phase partitioning of the proteins investigated in this study was affected by several factors including the partitioning temperature and the concentrations of protein and APO12. The partition coefficients were greater when the proteins were incubated at 55 °C with APO12 than at 45 °C. The value of K_p increased in the order β-L > ovalbumin > serum albumin > lysozyme. Changes in the pH-induced surface properties of β-lactoglobulin were previously detected by comparing the partitioning of the protein in the presence of a two-phase PEG/Dx system with one containing a PEG/palmitic acid ester derivative [26]. These same surface properties are likely responsible for the large values of K_p noted for β-L compared with the other proteins investigated. The heme was extracted from a sample of cytochrome *c*, and this property could be useful for some applications involving heme proteins. Protein precipitation was encountered with catalase and additional studies were not conducted.

K_p increased with increasing APO12 concentrations (13–40 mg/mL) with fixed concentrations of β-L (3.3 or 6.67 mg/mL), decreased with increasing β-L concentrations (1–10 mg/mL) with a fixed concentration of APO12 (20 mg/mL), and was the same within experimental error at pH 3 and pH 8 (monomer or dimer present). A simple model was proposed to account for these observations.

Large partition coefficients resulted with APO12 alone or mixed micelle mixtures containing anionic or cationic surfactants and PL in small molar ratios (5%) when the pH was above or below the isoelectric point of β-L. The elevation of the cloud point by the addition of ionic surfactants to APO12 without DMPC is expected for smaller surfactant mixed micelles, and the effect on the cloud point was reversed by the addition of PL due to increased micellar size [15]. This selectivity may be useful in developing new methods for the purification of proteins, and it is expected that the APO surfactants may be particularly useful for the isolation of membrane-bound proteins. The surfactants would be added at low temperatures to solubilize cell fractions followed by gentle heating to form a two-phase system leading to final separations. Any PL present in the initial mixture, or added later, would produce mixed micelles and lower the minimum temperature needed for phase separation. Ionic surfactants can be included in the initial or subsequent separations along with pH adjustments to adjust the net charge on the proteins to be either the same or opposite the sign of the charge of the mixed micelles.

The denaturation of β-L by APO10 and APO12 was reversed by either dilution or dialysis.

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