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THERMODYNAMIC STUDY OF THE THERMAL DENATURATION OF A GLOBULAR PROTEIN IN THE PRESENCE OF DIFFERENT LIGANDS

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By means of difference UV-Vis spectra, the thermal denaturation of catalase has been studied in the presence of different surfactants: sodium perfluorooctanoate, sodium octanoate and sodium dodecanoate. These results indicate that hydrogenated surfactants play two opposite roles in the folding and stability of catalase, they act as a structure stabiliser at a low molar concentrations (enhancing T_m) and as a destabilizer at a higher concentrations (diminishing T_m). Meanwhile sodium perfluorooctanoate enhances T_m in the whole concentration range. An approach for the determination of the heat capacity, enthalpy and entropy has been made, finding that for the three studied surfactants, at all concentrations, the enthalpy term dominates the entropy term.

Keywords: *catalase, ligands, thermal unfolding, thermodynamics*

Introduction

Only folded proteins are able to execute biological functions. This folded state may usually be unfolded by adding denaturants, increasing the temperature or varying the pH. The role of surfactant as protein denaturants has led to investigations into this area due to their important applications in food and pharmaceutical industry [1].

The unfolding transition can be monitored by different techniques. Differential scanning microcalorimetry experiments can yield a complete thermodynamics characterization of the unfolding transition by determining the heat capacities of native and denaturated protein, the enthalpy and entropy of denaturation, as well as the melting temperatures. Thermal denaturation shows irreversibility much more frequently than solvent denaturation. However, nowadays optical techniques such as absorbance are sensitive and can be performed easily obtaining similar results [2–5].

Perfluorocarbons, surfactants where all the hydrogens in the hydrophobic moiety have been replaced by fluorine, have been much less studied than the corresponding hydrogenated surfactants, despite their technical interest due to their potential usefulness. Comparative studies of perfluorinated and nonfluorinated surfactant systems are interesting in many ways, for example, from a theoretical point of view. These two materials may be used in order to study the hydrophobic effect or trying to predict a priori the physicochemical properties of the perfluorinated on the basis of the properties of their corresponding hydrogenated.

Interactions between proteins and hydrocarbon and fluorocarbon surfactants are interesting from a view to understanding the mechanism responsible for the adsorption of these molecules to such biopolymers, and on the study and comparison of the physicochemical properties of hydrogenated surfactants and their corresponding perfluorinated ones as a function of temperature and alkyl chain [6–9]. Hence, our aim in this article is to build upon the knowledge in this area. We are trying to clarify the thermodynamics (heat capacity, enthalpy, entropy and free energy) of the thermal unfolding of proteins in the presence of different surfactants by using UV spectroscopic methods.

For this purpose, we have chosen catalase, sodium perfluorooctanoate, sodium octanoate and sodium dodecanoate. Catalase (hydrogen peroxide:hydrogen peroxide-oxidoreductase, EC 1.11:1.6) is one of the most potent catalysts known. It is present in the peroxisomes of nearly all aerobic cells, serves to protect the cell from the toxic effects of hydrogen peroxide, a powerful and potential harmful oxidizing agent, by catalyzing its decomposition into molecular oxygen and water. The protein exists as a dumb-bell-shaped tetramer of four identical subunits, each subunit formed by a single polypeptide chain with haemin as a prosthetic group [10].

Sodium perfluorooctanoate, sodium octanoate and sodium dodecanoate will be useful to a complete characterization of the thermodynamic of their interactions with catalase: on the one hand as a function of

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alkyl chain and on the other hand to measure the effect of the substitution of hydrogen atoms for fluorine ones.

Experimental

Materials

Sodium octanoate (C8HONa) and sodium perfluorooctanoate (C8FONa) of at least 97% purity were obtained from Lancaster Synthesis Ltd. Sodium dodecanoate (C12HONa) with purity greater than 99%, was obtained from Sigma Chemical Co. Bovine liver catalase (product no. C-9322) was purchased from Sigma Chemical Co. All of these products were used as received. All measurements were performed using distilled water with conductivity below 3 μS cm^{-1} at 298.15 K. Protein concentration (0.125 mg mL^{-1}) was kept constant in all experiments. Stock solutions of defined protein concentration were prepared by dissolving a certain amount of freeze-dried protein in an appropriate buffer volume.

Methods

Difference spectra were measured using a Beckman spectrophotometer (model DU 640), with six micro-cuvettes, operating in the UV-Visible region, from 190 to 1100 nm of the electromagnetic spectrum wavelength, with a full scale expansion of 0.2 absorbance units. All measurements were made using catalase solutions with a concentration of 0.125 mg mL^{-1} in carefully matched quartz cuvettes (50 mL capacity) in the wavelength range 200–500 nm. For absorbance difference spectra, five of the six microcuvettes were filled with protein plus surfactant solutions. The first microcuvette contained only protein in the corresponding medium and was used as a blank reference. The microcuvettes were filled and placed in the same position for all the tests. Measurements were made after catalase and surfactant had been incubated for over 30 min, time during which the difference spectra did not change. For absorbance measurements with changing temperature, a Beckman (DU Series) temperature controller was used, within a ±0.01 K, following the Peltier methods of controlling temperature, in the range 288 to 373 K.

Results and discussion

Thermodynamically, as a first approximation, the denaturation process can be considered as a transition between the two macroscopic states: The native state (N) and a denaturated state with $\bar{\nu}$ bound surfactant ligands ($DS_{\bar{\nu}}$):

$$N + \bar{\nu}S \Leftrightarrow DS_{\bar{\nu}} \quad (1)$$

where $\bar{\nu}$ is the average number of surfactants molecules bound to denaturated complex $DS_{\bar{\nu}}$. The two-state character of the transition is a consequence of the cooperative nature of the protein unfolding. The analysis of equilibrium unfolding transition requires extrapolation of the baseline for the native and unfolded protein into the transition region to determine the fraction of denaturated molecules, F_D, as a function of the unfolding parameters [11]:

$$F_D = \frac{A_N - A_{OBS}}{A_N - A_D} \quad (2)$$

where A_{OBS} is the absorbance observed and A_N and A_D are the absorbance for the native and denaturated conformations.

The difference in the standard Gibbs energy between the folded and unfolded conformations, can then be calculated as:

$$\Delta G^0 = -RT\ln\left[\frac{F_D}{1-F_D}\right] = -RT\ln\left[\frac{A_N - A_{OBS}}{A_{OBS} - A_D}\right] \quad (3)$$

where R is the gas constant and T is the absolute temperature.

If we now define the equilibrium constant, K, for Eq. (1) as:

$$K = \frac{\lfloor DS_{\bar{\nu}} \rfloor}{[N][S]^{\bar{\nu}}} = \frac{K_s}{[S]^{\bar{\nu}}} = \frac{F_D/(1-F_D)}{[S]^{\bar{\nu}}} \quad (4)$$

The equilibrium constant was deduced from the equation

$$K = \frac{[\text{unfolded}]}{[\text{native}]} \Rightarrow K = \frac{(A_N - A_O)}{(A_O - A_N)} \quad (5)$$

where A_N is the absorbance of the pure native state in the transition zone after extrapolation from the pretransition region, A_D is the corresponding absorbance of the pure denaturated state and A_O is the observed absorbance at any temperature in the transition zone.

Following the procedure created by Kaushik *et al.* [12], Eq. (1) can be rewritten as:

$$A_O = (A_N + KA_D)/(1 + K) \quad (6)$$

and

$$K = \exp(-\Delta G^0/RT) \quad (7)$$

where ΔG^0 is the standard free energy change, R is the gas constant and T is the absolute temperature. Substituting the value of K in Eq. (2) we get

$$A_0 = \left[\frac{A_N + A_D \exp(-\Delta G^0/RT)}{1+\exp(-\Delta G^0/RT)} \right] \quad (8)$$

A form of the Gibbs-Helmholtz equation can be written as

$$\Delta G^0(T) = \Delta H_m(1 - T/T_m) - \\ - \Delta C_p(T_m - T + T\ln(T/T_m)) \quad (9)$$

where ΔH_m is the enthalpy of denaturation calculated at the T_m, ΔC_P is the heat capacity change for denaturation, T_m refers to the temperature at the midpoint if the transiton, and T refers to any temperature where $\Delta G^0(T)$ is calculated. Substituting the value of $\Delta G^0(T)$ obtained from Eq. (5) in Eq. (4) we get

$$A_0 = \frac{A_N + A_D \exp\{-1/R[\Delta H(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m))]\}}{1+\exp\{-1/R[\Delta H(1/T - 1/T_m) - \Delta C_p(T_m/T - 1 + \ln(T/T_m))]\}} \quad (10)$$

Since A_N and A_D have been found to be linear functions of temperature, they can be written as

$$A_n = a_N + m_N T \text{ and } A_D = a_D + m_D T \quad (11)$$

where a_N and a_D are the intercepts of A_N and A_D *vs.* temperature plots and m_N and m_D are the corresponding slopes. Substituting A_N and A_D as obtained from Eq. (11) in Eq. (10) we get

$$A_0 = \frac{a_N + m_N T}{1+\exp\{-1/R[\Delta H(1/T - 1/T_m) - \Delta C_P(T_m/T - 1 + \ln(T/T_m))]\}} + \\ + \frac{(a_D + m_D T)\exp\{-1/R[\Delta H(1/T - 1/T_m) - \Delta C_P(T_m/T - 1 + \ln(T/T_m))]\}}{1+\exp\{-1/R[\Delta H(1/T - 1/T_m) - \Delta C_P(T_m/T - 1 + \ln T/T_m))]\}} \quad (12)$$

Figures 1 and 2 show the unfolding temperature, T_m, of catalase as a function of the concentration of the surfactants under study. T_m were calculated by fitting the experimental difference absorbance (the inset in Fig. 1 shows, as an example, three typical thermal unfolding curves) to Eq. (12). The numerical analysis of the data was made by means of the Levenberg–Marquardt least-squares fitting algorithm which consists of the combination of two classical methods to minimize χ^2, the approximation to the minimum by a Taylor expansion and by a gradient approximation.

These results indicate that C8HNa and C12HNa play two opposite roles in the folding and stability of catalase, they act as a structure stabiliser at a low molar concentrations (enhancing T_m) and as a destabilizer at a higher concentrations (diminishing T_m). A similar behaviour has been corroborated with BSA and SDS [13]. However, for C8FNa its stabiliser functions increases with concentration which may be attributed to the effect of the CF_3 group altering the hydration shell of the peptides [14]. This fact has been interpreted on the basis that small amount of surfactants induced a protective effect due to some cross-linking function of the surfactant ions. In other words, the native conformation is stabilized by a cross linking function of the surfactant ion between a group of nonpolar residues and a positively charged residue located on different loops of the protein [15, 16]. Nuclear magnetic resonance studies of systems pro-

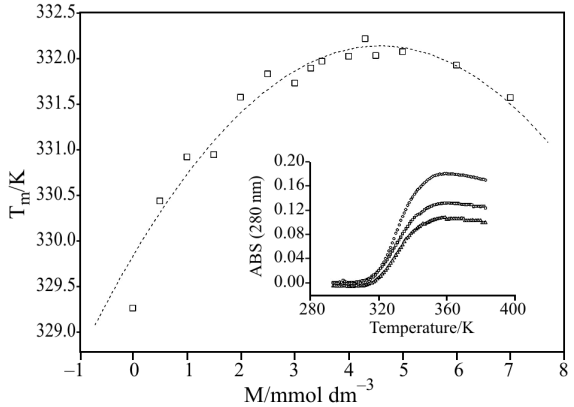

Fig. 1 Catalase unfolding temperature (T_m) as a function of sodium octanoate concentration. The inset shows two typical unfolding curves for catalase (in the presence of three different octanoate concentrations) by using the difference absorbance

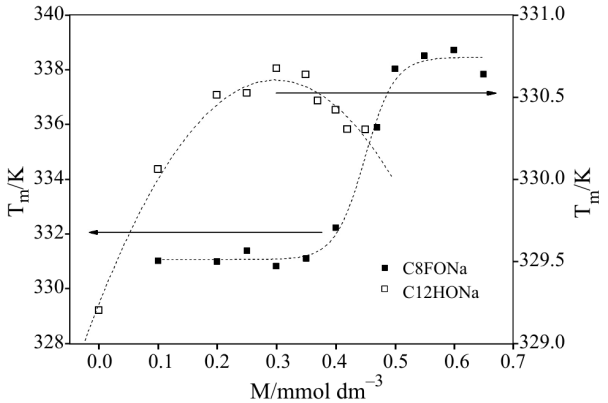

Fig. 2 Catalase unfolding temperature (T_m) as a function of sodium perfluorooctanoate (C8FONa) and sodium dodecanoate (C12HONa) concentrations

tein-fluorinated surfactant have pointed out the interactions between the fluorocarbon and the apolar moiety of the protein. Meanwhile, volumetric measurements support the existence of electrostatic ones [17].

Tables 1 to 3 represents the results obtained for the thermodynamic parameters for fitting of experimental point to Eq. (12). For the three studied surfactants, at all concentrations, the enthalpy term dominates the entropy term. Enthalpy tends to decrease with C8FONa. However, this function increases with C8HONa and C12HONa concentration. Such behaviour could be explained on the basis of confor-

Table 1 Thermodynamic parameters for catalase in sodium perflurooctanoate aqueous solutions obtained for Eq. (12)

[C8FONa]/mM	T_m/K	ΔH_m/kJ mol^{-1}	ΔS_m/kJ mol^{-1} K^{-1}	ΔC_p/kJ mol^{-1} K^{-1}
0.00	329.20	149.34	0.45	11.05
0.10	331.11	146.83	0.44	6.85
0.20	331.08	153.05	0.46	5.98
0.25	331.65	142.90	0.43	6.35
0.30	330.81	140.25	0.42	6.57
0.35	331.10	149.47	0.45	7.86
0.40	332.22	143.66	0.43	9.55
0.45	331.24	138.14	0.42	10.76
0.50	331.11	124.78	0.38	12.52
0.55	338.50	126.51	0.37	15.16
0.60	338.80	121.45	0.36	10.75
0.65	337.00	102.20	0.30	8.56

Table 2 Thermodynamic parameters for catalase in sodium octanoate aqueous solutions obtained for Eq. (12)

[C8HONa]/mM	T_m/K	ΔH_m/kJ mol^{-1}	ΔS_m/kJ mol^{-1} K^{-1}	ΔC_p/kJ mol^{-1} K^{-1}
0.0	329.20	149.34	0.45	11.05
0.5	330.56	160.61	0.49	7.31
1.0	331.23	170.64	0.51	5.34
1.5	330.90	174.88	0.53	3.97
2.0	331.66	172.56	0.52	4.68
2.5	332.14	183.76	0.55	6.32
3.0	331.53	181.29	0.55	6.84
3.3	331.83	185.21	0.56	7.66
3.5	331.91	190.62	0.57	8.51
4.0	331.91	187.12	0.56	12.08
4.3	332.23	195.24	0.59	15.68
4.5	331.86	189.33	0.57	12.47

Table 3 Thermodynamic parameters for catalase in sodium dodecanoate aqueous solutions obtained for Eq. (12)

[C12HONa]/mM	T_m/K	ΔH_m/kJ mol^{-1}	ΔS_m/kJ mol^{-1} K^{-1}	ΔC_p/kJ mol^{-1} K^{-1}
0.00	329.21	149.34	0.45	11.05
0.10	330.06	157.74	0.48	6.98
0.20	330.54	169.15	0.51	4.56
0.25	330.42	158.77	0.48	12.49
0.30	330.71	155.61	0.47	18.82
0.35	330.65	170.96	0.52	24.94
0.37	330.44	221.79	0.67	17.93
0.40	330.42	200.05	0.61	11.66
0.42	330.25	256.09	0.77	10.06
0.45	330.40	231.61	0.71	12.41

mational changes induced by the fluorinated moiety. Thus, the loss of ordered water coating around the peptide, indirectly induced by structuring of water around the $–CF_3$ groups and by the presence of CF_3 near the peptide, causes the –NH and –C=O groups of the backbone to interact on short distances with each other, favouring the α-helix. Short-range interactions between nearby amino acid residues stabilize the α-helical structure in contrast to long-range interactions stabilizing the β-sheet structure [14]. It is commonly assumed that the heat capacity is strictly dependent on the ordering of water molecules around exposed hydrophobic groups, it is evident that the change of the heat capacity at denaturation should be connected with the disruption of apolar contacts in proteins [18, 19].

Conclusions

It has been shown that both hydrogenated and fluorinated surfactant can acts as a stabilized (at low surfactant concentrations) or as a denaturant (at high surfactant concentrations) of catalase. Greater changes in the unfolding temperature were found for C8FONa, meanwhile C8HONa and C12HONa exhibit similar changes in T_m. Obviously, this event is related with the effect of the perfluorinated alkyl chain. Although, the positive sign of heat capacity found for all the surfactants and concentration studied indicates apolar solvation, the presence of fluorine atoms in the alkyl chain induces small conformational changes in the protein structure which is reflected in the different enthalpy behaviour.

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