Short communication

The Unfolding Process of Apo-Human Serum Transferrin in the Presence of Cetylpyridinium Chloride: An Isothermal Titration Calorimetry Study

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Abstract

Isothermal titration calorimetry (ITC) was utilized at conditions close to physiological (50 mM HEPES buffer, pH 7.4 and 160 mM NaCl) and at various temperatures of 25, 30, 35 and 40 °C to evaluate the thermodynamic parameters, enthalpy and heat capacity changes, and subsequently the unfolding process of apo-human serum tarnsferrin (apo-hTf) in the presence of cetylpyridinium chloride (CPC) as a cationic surfactant. The precise thermograms and heat capacity curves obtained and interpreted in terms of molecular events such as specific and non-specific binding and the unfolding process. The analysis of obtained enthalpograms and heat capacity changes profile showed a distinct extreme region close to [CPC]/[apo-hTf] mole ratio of 20 indicated that predominant cooperative unfolding occurs at this mole ratio via a two states mechanism.

Keywords: Apo-human serum transferrin, Cetylpyridinium chloride, Isothermal titration calorimetry, Heat capacity, Interaction enthalpy

1. Introduction

Transferrins (Tfs) are a homologous group of iron-binding glycoproteins present in all vertebrates whose primary function is iron sequestration and transport and include serum transferrin, lactoferrin and ovotransferrin.[1–3] Mammalian and avian serum transferrins are known to deliver iron ions into target cells via transferrin receptor.[4] Tfs are monomeric proteins of 76–81 kDa, depending on the extent of glycosylation, and consist of two structurally similar lobes (termed the N- and C-lobes) connected by a short peptide linker. Each lobe contains a single iron-binding site.[1,5]

Apo-human transferrin (apo-hTf), a bilobal single chain protein, is the main Fe(III) transport protein in human serum. It is capable of tight reversible binding of two equivalents of Fe(III) ions and may play a defensive role against systemic infections by withholding iron from potential pathogens.[6] This protein can also bind well a wide variety of other metal ions and some of their complexes.[7–10] This capability reveals the essential role of this protein in transporting of these chemicals in blood and is becoming more important when these chemicals used as drug. For instance, vanadyl ion and some of its organometallic compounds that are known as anti-diabetic drugs, efficiently transported in the blood by apo-hTf.[11]

Ionic surfactants as amphiphilic molecules can interact with globular proteins via both electrostatic and hydrophobic interactions. Such interactions have been extensively studied since many industrial, biological, pharmaceutical and cosmetic products contain both proteins and surfactants. This is revealed that such interactions depend strongly on protein and surfactant type, as well as on

medium and its physico-chemical properties such as pH, ionic strength and temperature.[12–24] It is generally accepted that binding of ionic surfactant molecules to proteins can disrupt the native structure of most globular proteins.[23] Therefore, an understanding of the mechanism involved in protein–surfactant interactions provides a basis for the evaluation of protein conformational stability and for rational strategies to optimize the applications of surfactants.[25–29]

Protein–surfactant interactions can be evaluated by many different techniques to approach the full view of the structural, stoichiometric and calorimetric changes accompanying different binding stages. Among these techniques, isothermal titration calorimetry (ITC) is one of the valuable tools for this purpose. In this technique, surfactant is titrated into a solution of protein and the heat flow associated with binding is monitored.[25,27,28,30–32] All binding processes are accompanied by an enthalpic change that can very often be measured as a heat flow. ITC is faster, more convenient and more informative than e.g. equilibrium dialysis in this regard, since it provides the full picture of the different steps of surfactant binding, which may typically differ in terms of the magnitude and sign of the enthalpic change. This change is typically exothermic for electrostatically driven binding reactions and endothermic when binding is coupled to protein unfolding.[33–35]

In the present study, the binding of CPC to apo-hTf has been investigated at conditions close to physiological using ITC at various temperatures to determine enthalpy and heat capacity changes. The obtained results can be very informative due to importance of heat capacity change as a major thermodynamic quantity that is one of the richest potential sources of information in physical terms. The obtained precise thermograms and heat capacity curves have been interpreted in terms of molecular events such as specific and non-specific binding and the unfolding process.

2. Materials and Methods

2. 1. Materials

Apo-human serum transferrin and cetylpyridinium chloride used in this study were obtained from Sigma Chemical Co. Sodium chloride and *N*-(2-hydroxyethyl) piperazine-*N*´-(2-ethanesulfonic acid) (HEPES) were purchased from Merck Chemical Co. All the purchased reagents were highly pure. The double distilled water was used to prepare all of the solutions. All the experiments were carried out at conditions close to physiological (50 mM HEPES buffer pH 7.4 containing 160 mM NaCl). All the protein and surfactant solutions were used freshly after preparation. Apo-hTf was dialyzed using Pierce regenerated cellulose dialysis cassettes with a molecular weight cutoff of 10 kDa against HEPES buffer treated with Chelex-100 resin. The surfactant concentrations in the reaction cell were below their critical micelle concentration (cmc) in all experiments.

2. 2. Isothermal Titration Calorimetric Experiments

An isothermal titration calorimeter (VP-ITC, Microcal, Inc., Northampton, MA) was used to measure enthalpies of mixing at 25.0, 30.0, 35.0, and 40.0 °C. The enthalpy changes per mole of CPC at each temperature was determined from sequential injections of 5 μL aliquots of 1.0 mM CPC into 1460 μL reaction cell containing initially stock buffer solution (50 mM HEPES buffer pH 7.4, 160 mM NaCl) or apo-hTf solution. The concentration of apo-hTf in the reaction cell was 2.0 μM in 50 mM HEPES buffer pH 7.4, 160 mM NaCl. Each injection was lasted 5 s and there was an interval of 180 s between every successive injection. The solution in the reaction cell was stirred at a speed of 315 rpm throughout the experiments. All the solutions were degassed before the measurements. All the experiments were carried out at least twice using freshly prepared samples and the results were reported as the average. Typically, the reproducibility of the enthalpy changes that measured on a particular sample by ITC, was acceptable with less than 10% of error.

3. Results and Discussion

3. 1. Influence of Temperature on Micellization of CPC

When micellar surfactant solution is titrated into buffer solution, ITC records the differential enthalpy changes associated with demicellization phenomenon and surfactant dilution. Both cmc and ΔH_{mic} can be directly achieved from one ITC experiment. Using ITC technique, the cmc of CPC in the HEPES buffer was obtained about 0.1 mM at 25 °C. It has been found that the cmc tends to increase with increasing temperature.[27]

3. 2. Enthalpy of CPC Binding to Apo-hTf at Different Temperatures

ITC technique was utilized to find out the interaction enthalpy of CPC binding to apo-hTf at a range of temperature, using surfactant solution in the injector and apo-hTf solution in the reaction cell. The ITC experiments were done at temperatures below the T_m of protein that is about 60 °C.[31] In this case, the CPC molecules were interacted with native form of protein at all studied temperatures. The plot of heat flow *vs* time profile resulting from interactions of CPC to apo-hTf at 40 °C is shown in Figure 1. The same profiles were obtained at other temperatures.

The enthalpy change per mole of CPC in the absence and in the presence of apo-hTf was calculated from the integral heat. The difference between these two calculated

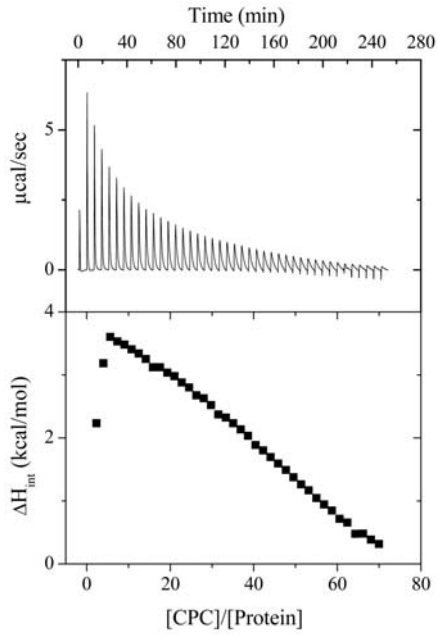

Figure 1. Heat flow versus time profiles resulting from sequential injections of 5 µL aliquots of CPC solution (1.0 mM) into a 1460 μL reaction cell containing apo-hTF solution (2.0 µM) at 40 °C and its interaction enthalpy change (ΔH_{int}) versus mole ratios of [CPC]/[apo-hTf] at pH 7.4, 50 mM HEPES buffer and 160 mM NaCl. The similar profiles were obtained at other temperatures.

enthalpies should be equal to enthalpy of interaction between CPC ions and apo-hTf. The variation of ΔH_{int} *vs* [CPC]/[apo-hTf] mole ratio, an integral titration isotherm, at 40 °C is shown in the lower part of Figure 1. The similar enthalpograms were obtained at other temperatures that are shown in Figure 2. As it shown in this figure, it can be declared that the interaction of CPC to apo-hTf was highly endothermic at low mole ratios and then gradually became less endothermic at higher mole ratios. The same profile was observed at all studied temperatures. It is

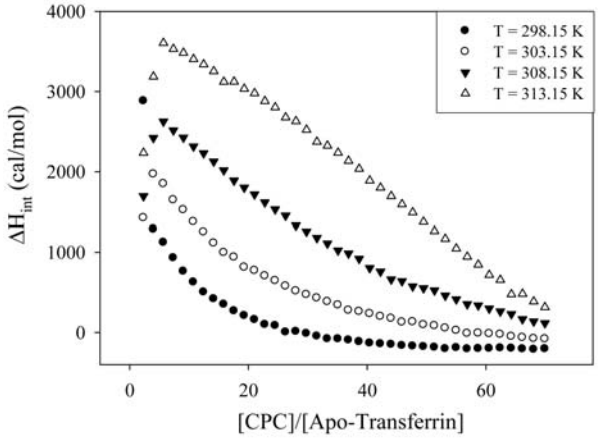

Figure 2. The variation of interaction enthalpy change (ΔH_{int}) *vs* mole ratios of [CPC]/[apo-hTf] in the reaction cell for 1.0 mM CPC injected into apo-hTf solution (2.0 µM) at pH 7.4, 50 mM HEPES buffer, 160 mM NaCl, and at 25, 30, 35 and 40 °C.

evident from this figure that ΔH_{int} is increased with increasing temperature (Figure 2).

It is possible to interpret the observed profiles of ΔH_{int} *vs* [CPC]/[apo-hTf] mole ratio in terms of enthalpy changes associated with the various physicochemical phenomena occurring, e.g. binding and folding changes of apo-hTf. It has been generally accepted that the driving force in the initial binding of ionic surfactants to globular proteins, is a combination of electrostatic and hydrophobic interactions. In the other word, the binding begins initially with coulombic interactions of ionized fragments of surfactant with opposite charges on the protein surface that is accompanying with hydrophobic interactions of non-polar group of surfactant tail with hydrophobic patches at protein surfaces or hydrophobic clefts.[13,23,35–37] The ratio of these two opposite interactions (electrostatic and hydrophobic) determines the sign of interaction enthalpy. It has been known that electrostatic and hydrophobic interactions are recognized with exothermic and endothermic enthalpies, respectively, and hydrophobic interactions are mainly entropy driven. Hence, the endothermic nature of the initial interaction represents the higher contribution of hydrophobic interaction. However, the increasing of endothermisity with increasing of mole ratio (Figure 2) in the initial binding stage at 303, 308 and 313 K, can be related to the contribution of unfolding process as an endothermic phenomenon, in the net enthalpy. It looks that the contribution of unfolding is increased with increasing surfactant concentration and reaches to an extreme value due to the nature of unfolding process. However, as it shown in Figure 2, these extreme regions are not so clear in obtained enthalpograms (there are just one or two points before the extreme point that made difficult the recognition of this extreme point). Hence, the interaction heat capacity change ($\Delta C_{P,int}$) has been calculated for clear determination of these extreme regions in this study.

3. 3. Heat Capacity Changes of Binding of CPC to Apo-hTf

The values of interaction heat capacity changes ($\Delta C_{P,int}$) were determined in order to get better insight of interactions of CPC with apo-hTf, using the standard approach for determining $\Delta C_{P,int}$ by ITC. Figure 3 shows the variation of ΔH_{int} *vs* temperature at any specified [CPC]/[apo-hTf] mole ratios. Various plots in this figure are related to various mole ratios. The slope of each linear plot should be equal to $\Delta C_{P,int}$ at the specified mole ratio that is implied by the definition of heat capacity, $\Delta C_{P,int} = \frac{d\Delta H_{int}}{dT}$, i. e., the increase in energy (heat) with temperature. The linearity of plots in Figure 3 indicates the invariability of $\Delta C_{P,int}$ with temperature. The $\Delta C_{P,int}$ value at any specified [CPC]/[apo-hTf] mole ratio should be equal to the slope of the corresponding line in Figure 3. However, it should

be mentioned that all of the curves corresponding to the all examined mole ratios have not been shown in Figure 3 for better appearance.

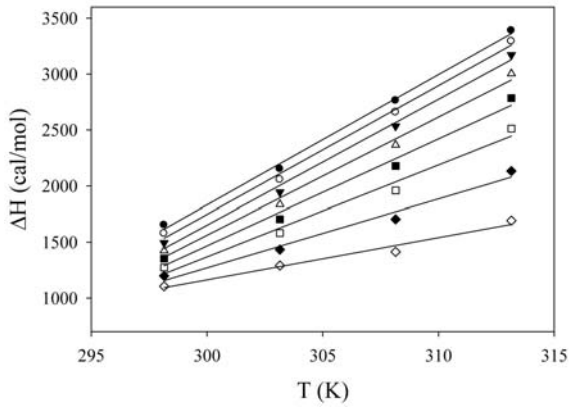

Figure 3. Temperature dependence of interaction enthalpy at some specified [CPC]/[apo-hTf] mole ratio. The slope of each linear plot equals to $\Delta C_{P,\text{int}}$ as is implied by the definition of heat capacity, $\Delta C_{P,\text{int}} = \frac{d\Delta H_{\text{int}}}{dT}$, due to interaction with apo-hTf. The molar ratios of SDS/apo-hTf equal to 8.2 (●), 17.4 (○), 26.1 (▼), 32.9 (▽), 40.3(■), 48.4 (□), 55.8(◆) and 61.7 (◇). The similar curves were obtained at other molar ratios.

The changes of calculated $\Delta C_{P,\text{int}}$ with mole ratio of [CPC]/[apo-hTf] is shown in Figure 4. As it shown in this figure, a distinct extreme region can be observed for the interaction of CPC with apo-hTf. This region is identified nearly at [CPC]/[apo-hTf] mole ratio of 20.

As it obvious in this figure, a sharp increase of $\Delta C_{P,\text{int}}$ at initial stages of binding can be observed and a decrease in $\Delta C_{P,\text{int}}$ values is evident from [CPC]/[apo-hTf] mole ratio of 20 until higher [CPC]/[apo-hTf] mole ratios. The values of $\Delta C_{P,\text{int}}$ remain positive at all [CPC]/[apo-hTf] mole ratios. The positive values of $\Delta C_{P,\text{int}}$ is a sign for the hydrophobic interactions throughout protein-surfactant interactions (the association of non-polar parts in aqueous medium). The shift of $\Delta C_{P,\text{int}}$ to less positive values at higher mole ratios of [CPC]/[apo-hTf] is a sign for the decreasing role of hydrophobic interactions.[27] It means that the nature of binding at higher mole ratios of [CPC]/[apo-hTf] was less hydrophobic than at [CPC]/[apo-hTf] mole ratio of about 20. The conformational changes in apo-hTf due to its interactions with CPC could have also influence on $\Delta C_{P,\text{int}}$ values. The protein unfolding was proceeded due to the addition of CPC ions to the protein solution until reaching [CPC]/[apo-hTf] mole ratio of about 20. However, the values of $\Delta C_{P,\text{int}}$ was decreased by increasing more surfactant ions after this mole ratio.

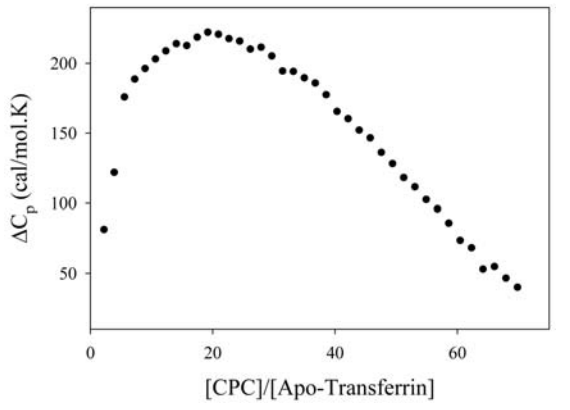

Figure 4. The profile of heat capacity changes versus mole ratios of [CPC]/[apo-hTf] at pH 7.4, 50 mM HEPES buffer and 160 mM NaCl.

The heat capacity changes for both hydrophobic binding of CPC tail and unfolding of apo-hTf domains were positive but due to cooperative nature of unfolding, its contribution to overall binding capacity should pass from a maximum. The number of distinct maxima should be related to unfolding mechanism and number of intermediates.[27] The $\Delta C_{P,\text{int}}$ profile (Figure 4) had a distinct maximum at [CPC]/[apo-hTf] mole ratio of about 20, indicating a two states mechanism for the unfolding process of apo-hTf in the presence of CPC. Moreover, by considering the high cooperative nature of unfolding process; that is so called all-or-none process, it can be concluded that apo-hTf completely lost its tertiary structure due to its interaction with CPC.

4. Conclusions

Heat capacity is one of the major thermodynamic quantities measured in proteins. This quantity provides physical, mechanistic and atomic-level insight into how proteins fold and how they are interact with small molecules (ligand). It is the richest potential source of thermodynamic and structural information and the hardest of the thermodynamic quantities to understand, in physical terms. The endothermic nature of the interaction and positive values of $\Delta C_{P,\text{int}}$ for interaction of CPC with apo-hTf represent the predominate role of hydrophobic interaction in the complex formation process. The variations of $\Delta C_{P,\text{int}}$ with [CPC]/[apo-hTf] mole ratios showed a distinct extreme region close to [CPC]/[apo-hTf] mole ratio of 20. This represents that the major unfolding occurs at this region and the unfolding of this protein in the presence of CPC is most probably a two states process.

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6. References

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Povzetek

Z uporabo izotermne titracijske kalorimetrije (ITC) smo pri 25, 30, 35 in 40 °C določili termodinamske parametre za procese razvijanja apo-človeškega seruma ternsferin (apo-hTf) v prisotnosti kationskega surfaktanta cetilpiridinijevega klorida (CPC) pri fizioloških pogojih (50 mM HEPES pufer, pH 7.4 in 160 mM NaCl). Dobljene termograme in vrednosti toplotnih kapacitet smo analizirali z uporabo modelov za specifično in nespecifično vezavo ter proces razvijanja. Pri razmerju molskih deležev [CPC]/[apo-hTf] ≈20 je opazno ekstremno območje, ki kaže na prevladujoče kooperativno razvijanje.