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# Thermodynamic domain analysis of fresh and incubated human apotransferrin

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**Abstract**

The thermal denaturation of human apotransferrin was studied by a differential scanning calorimeter (DSC) in 100 mM Hepes buffer at pH 7.0. Deconvolution analysis of excess molar heat capacity was adjusted by four transitions of which two transitions were linked to N-domain and the remaining two were connected to C-domain. The same experiment was carried out for the incubated sample for a period of 24 h at 27 °C. The deconvolution subpeaks exhibited five transitions. The free energy, $\Delta G^{\circ}_{H_2O}$, in the absence of sodium *n*-dodecyl sulphate (SDS), as a criterion of stability measurement, was obtained based on Pace theory on UV spectrophotometry. The result indicates that incubated sample was stabilized by 14 kJ mol$^{-1}$ relative to the fresh sample. The circular dichroism (CD) study confirms the presence of additional secondary structures for the incubated sample compared with the fresh one because of Hepes molecule interaction with protein domains. 

*Keywords:* Incubated apotransferrin; Differential scanning calorimetry (DSC); Deconvolution analysis; Additional subpeak; Hepes buffer

## 1. Introduction

Transferrins, including serum transferrin (TF), lactoferrin and ovotransferrin, are a class of proteins [1] that bind extracellular iron, which they ultimately supply to metabolizing cells through interaction with TF receptors on the cell surface [2,3]. The half-life of TF in human circulation is 7.6 days; whereas the lifetime of TF-bound Fe is 1.7 h. A molecule of human serum transferrin (hTF) undergoes ca. 100 cycles of iron bindings during its lifetime [4]. It should be noted that hTF is a single strand glycoprotein of Mr = 80,000 [5] containing 678 amino acid residues consists of two domains (N and C lobes). These lobes are connected by a single short bridging peptide [6]. Nevertheless, the glycan chain in hTF is positioned in its external position where it is believed to provide recognition signals [7]. Each domain (lobe) consists of two dissimilar subdomains that forms a cleft where it encloses the binding site for Fe ion [8]. The gradual addition of ferric ion (chelated with nitrilotriacetate,

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NTA) to hTF saturates C-site before the N-site begins to saturate [9]. In spite of the fact, that N and C lobes of TF exhibit 40% homology, a suggestive of gene duplication, the release of iron from both binding sites exhibits kinetic and thermodynamic heterogeneity [4,10]. In fact, the extended X-ray absorption fine structure spectroscopy provides an accurate and acceptable means of information on the metal environment; and often this information is used to study and differentiate between two or more metal binding sites. Interestingly, iron ($Fe^{3+}$) binding sites consisting of two Tyr, one His, one Asp, and one bidentated carbonate ion are similar to one another [11–14] but not identical [15,16]. Upon iron-binding and its release, each lobe of the TFs undergoes a large-scale conformational changes; where subsequently, both interdomain clefts begin to open up before the uptake of iron, and afterward they begin to close down upon the incorporation of iron into its binding site [17,18].

Calorimetric studies of TF show separate transitions for the two domains of apotransferrin and a single domain for TF [19,20]. This investigation was carried out to compare the domain status of fresh human apotransferrin in Hepes buffer with the sample that was incubated for a period of 24 h in the same buffer condition. The incubated sample under the study adopts a new secondary structure in Hepes buffer.

## 2. Materials and methods

### 2.1. Materials

Apotransferrin and sodium *n*-dodecyl sulphate (SDS) were obtained from Sigma. The Hepes buffer was purchased from Merck, and it was utilized at 100 mM, pH 7.0. Apotransferrin solution was used as fresh sample and the incubated sample was held at 27 °C for a period of 24 h.

### 2.2. Methods

Differential scanning calorimeter (DSC) experiments were carried out on a Scal-1 microcalorimeter (Russian), the heating rate was fixed at 1 K $min^{-1}$. An additional pressure of 1.5 atm was applied during all DSC runs; in order to prevent any possible degassing of the solutions during heating. A dos-based software package (Scal-2) was also supplied and used for data analysis.

Circular dichorism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter (Japan). The results are expressed as ellipticity [$\theta$] (deg $cm^2$ $dmol^{-1}$), based on a mean amino acid residues weight (MRW) having the average weight of 118 for TF. The data was smoothed by applying the Jasco J-715 software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without distorting their peak shapes.

Spectrophotometry experiments were performed by using a recording spectrophotometer (UV-3100), Shimadzu model, Japan. The sample cell contained 0.8 ml of apotransferrin solution at a concentration of 0.3 mg $ml^{-1}$ with different fixed concentrations of SDS ranging from 0.00 to 16.00 mM. The buffer was used in the place of surfactant in the reference cell; otherwise, both reference and sample cells contained the same materials. Both cells were set at 27 °C, and the absorption of the sample cell was recorded at 280 nm wavelength versus reference cell.

All experiments were repeated for three times, and the concentrations of protein solutions were 1 mg $ml^{-1}$ for both DSC and CD experiments.

## 3. Results and discussion

The profiles of absorption changes versus SDS concentrations for both fresh and incubated samples are shown in Fig. 1. The single sigmoidal curve designates the fresh apotransferrin and the dual sequential sigmoidal curves (I and II) represent the incubated sample.

The determination of free energy ($\Delta G^{\circ}$), as a criterion of conformational stability of a globular protein, is based on two-state theory as follows:

$$\text{Native (N)} \leftrightarrows \text{Denatured (D)}. \quad (1)$$

This theory was developed by Pace [21–23]. He describes the process as a single denaturant-dependent step according to the two-state theory [24]. By assuming two-state mechanisms for apotransferrin denaturation by SDS, one can determine the process by monitoring the changes in the absorbance, and hence

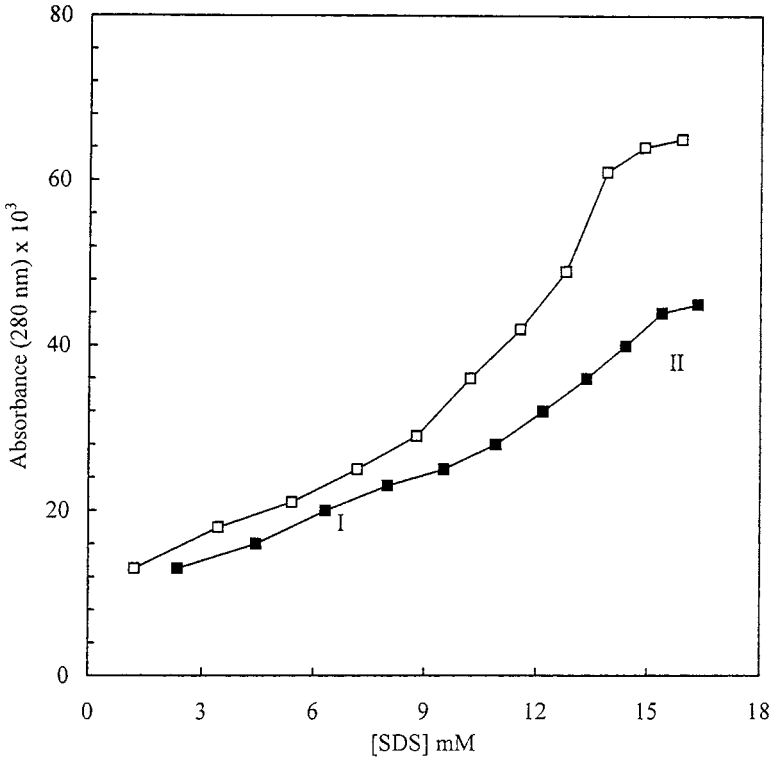


Fig. 1. Absorbance changes at 280 nm for the fresh and the incubated apotransferrin versus different concentrations of SDS. Fresh sample (□); incubated sample (■); first transition I; second transition II.

calculates the denature fractions of protein ($F_d$) as well as the determination of the equilibrium constant ($K$).

$$F_d = \frac{Y_N - Y_{obs}}{Y_N - Y_D} \quad (2)$$

$$K = \frac{F_d}{(1 - F_d)} = \frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)} \quad (3)$$

where $Y_{obs}$ is the observed variable parameter (e.g. absorbance) and $Y_N$ and $Y_D$ the values of $Y$ characteristic of a fully native and denatured conformations respectively.

The free energy change ($\Delta G^{\circ}$) for the Eq. (1) is given by the following equation:

$$\Delta G^{\circ} = -RT \operatorname{Ln} K \quad (4)$$

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

Fig. 2 shows free energy changes, $\Delta G^{\circ}_{H_2O}$, for fresh and incubated samples in the absence of ligand [25,26]. The free energies, $\Delta G^{\circ}_{H_2O}$, for fresh TF, transitions I and II in the incubated sample were determined to be 19, 11 and 22 kJ mol$^{-1}$, respectively. The reason that incubated TF is stabilized by 14 kJ mol$^{-1}$ relative to the fresh sample is because of the occurrence of an additional transition (the transition I).

Fig. 3 depicts CD spectra for the fresh and the incubated TFs. The spectra shows an additional secondary structures for the incubated sample compared with the fresh one. The amount of α-helix, β-sheets, and the turn for both samples are tabulated in Table 1. The percentage of the secondary structures for fresh sample is consistent with the previously reported data [27]. The CD data in Table 1 indicates an increase in β turn (10%) for the incubated TF relative to the fresh one. It seems that the overall percentage of the α-helix and the β-sheets structures have not changed dramatically.

Fig. 4(a and b) (insets) illustrates the DSC profiles of the fresh and the incubated samples. However, each sample shows a similar patterns with two apparent thermal transitions such as the first apparent transition ($T_m = 60$ °C) and the second transition ($T_m = 70$ °C),

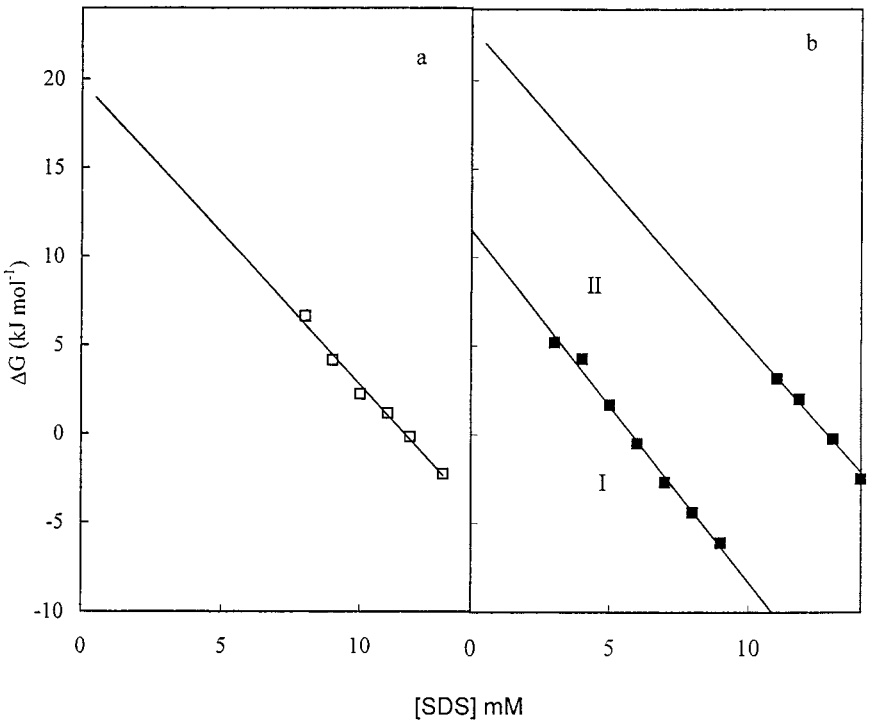


Fig. 2. Free energy changes for the chemical denaturation of the fresh (a) and the incubated (b) apotransferrin (transitions I and II depicted in Fig. 1).

according to the literature, the thermal profiles are linked to C and to N lobes, respectively [20].

Fig. 4(a) shows four transitions corresponding to their four subdomains of fresh sample; whereas, the Fig. 4(b) indicates five transitions for the incubated sample of which four subpeaks show resemblance to the fresh protein sample with an additional transition (transition V). The transition ($T_m$) and the enthalpy of

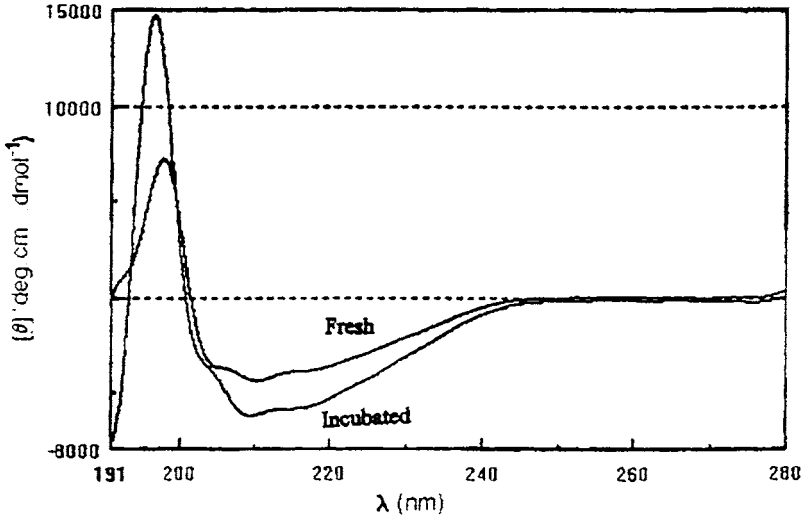


Fig. 3. Far-UV, CD spectra (elipticity ($\theta$)) of fresh and incubated apotransferrin.

Table 1
The percentages of the secondary structures of fresh and incubated apotransferrin

| Apotransferrin sample | α-Helix | β-Sheet | Turn | Other structure |
|---|---|---|---|---|
| Fresh | 27 | 32 | 17 | 24 |
| Incubated | 29 | 34 | 27 | 10 |

unfolding of each subpeaks are tabulated in Table 2. The amounts of enthalpy of denaturation for subpeaks I and II are consistent with the melting of the two subdomains of C lobe, while the subpeaks of III and IV are linked to two subdomains of N lobe. This finding indicates that two subdomains of each lobe are not identical with one another. In fact, this result is consistent with prior report [15,16] showing the presence of two unidentical subdomains of each lobe of TF.

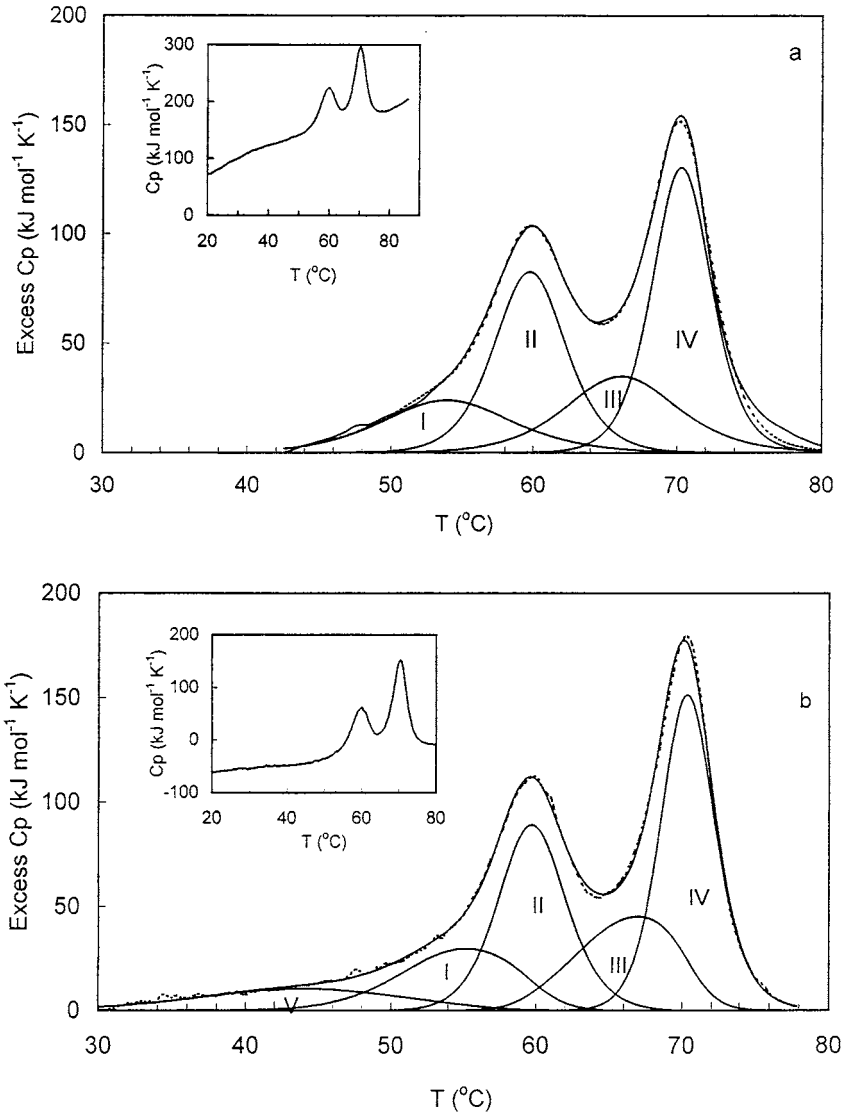


Fig. 4. DSC profiles of fresh and incubated apotransferrin (inset). (a) Deconvuloted excess molar heat capacity of fresh sample(subpeaks, I, II, III, IV). (b) Incubated sample (subpeaks I, II, III, IV, V). The transition V is consistent with the transition I in Fig. 1.

Table 2
Enthalpy and $T_m$ for the subpeaks of fresh and incubated apotransferrin

| Apotransferrin sample | Subpeak | $\Delta H$ (kJ mol$^{-1}$) | $T_m$ (°C) |
|---|---|---|---|
| Fresh | I | 292.6 ± 3.8 | 54 |
| | II | 552.6 ± 7.2 | 60 |
| | III | 363.7 ± 4.7 | 66 |
| | IV | 714.8 ± 9.3 | 70 |
| Incubated | I | 301.0 ± 3.0 | 54 |
| | II | 539.2 ± 5.4 | 60 |
| | III | 392.9 ± 3.9 | 66 |
| | IV | 740.0 ± 7.4 | 70 |
| | V | 183.9 ± 1.8 | 44 |

The results from CD and UV absorption indicate that incubated TF has a more stable conformation relative to the fresh one because of its secondary increment structure. The transition V in Fig. 4(b) (is consistent with the transition I in Fig. 1) is also related to the secondary increment structure. This transition which is occurring in the lower thermal, and the chemical stability of remaining transitions are shown in the Figs. 1 and 4(b). The enthalpy of unfolding of this additional transition of incubated sample is 184 kJ mol$^{-1}$.

This finding leads to the conclusion that the Hepes molecule, as a buffer, induces the new secondary structures of incubated apotransferrin that stabilizes the protein sample during a period of 24 h.

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