CHAPTER VII

THERMAL UNFOLDING OF PORCINE LACTOFERRIN: DIFFERENTIAL SCANNING CALORIMETRIC STUDIES

7.1 Introduction

Lactoferrin is an iron binding secretory mammalian glycoprotein (see chapter 2). Studies conducted till date on the stability and nature of unfolding of lactoferrins is less extensive. Hadden et.al.,(1994) has reported differential scanning calorimetric and spectroscopic studies on the pattern of thermal unfolding in RbTf, HLf and HTf. Harrington (1992) analyzed the effect of metal ion substitution on the stability of lactoferrin, using electronic spectroscopy. Paulsson et.al., (1993) reported the thermal stability of apo and diferric CLf. The only available report giving detailed account on the unfolding dynamics of transferrin proteins is by Lin et.al.,(1994). They have studied the thermal unfolding of HTf and HOTf (intact molecule [diferric and apo], and of individual lobes).

This section reports the results of the Differential Scanning Calorimetric tracking of thermal unfolding of porcine lactoferrin (intact DPLf [diferric and apo], individual lobes [ferric and apo lobes] as well as that of reassociated lobes).

7.2 Structure and Unfolding of Protein

7.2.1 Stability of the Folding

The rigidity of the "optimally folded state" of the protein is a direct variable of various environmental factors (pH, temperature, pressure, additives etc.) (Karplus and McCammon 1983; Karplus and McCammon 1981; Puett 1973). This "native state" will collapse to the "denatured state" when the environmental features alter the structural determinants holding the optimally preferred form (Tanford 1970). The native state is maintained by a thermodynamically favoured orientation of the polypeptide backbone (Privalov 1989).

Unfolded state is characterized by disordered structure, with predominating random coil structure (Tanford 1970). However, as the energetics of interaction among different parts of the unfolded protein may not be exactly balanced by interactions with the medium (due to the differences in the physical properties of the amino acid side chains), the unfolded state need not be true random coil (Shortle and Meeker 1989). Another form of unfolded state is "molten globule", where neither fully unfolded nor true folded form exists (Kuwajima 1989).

7.2.2 Techniques Used to Monitor Protein Unfolding

Generally used techniques for tracking protein unfolding are electronic spectroscopy (Pace et.al., 1989; Mach et.al., 1995), fluorescence spectroscopy (Eftnik 1995; Royer 1995), nuclear magnetic resonance (Wuthrich 1986; Eftnik 1995), circular dichroism (Pace et.al.,1989; Kuwajima 1995) and differential scanning calorimetry (Privalov 1979; Sanchez-Ruiz 1995).

7.3 Differential Scanning Calorimetry and Protein Unfolding

DSC is a powerful tool to monitor temperature induced conformational changes of proteins. This technique is precisely used for analyzing the general conformational stability (Burova et.al.,2001; Shnyrov et.al.,2000; Kamen et.al.,2000; Kretschmar and Jaenicke 1999), energetics (Gonzalez et.al.,2001; Honda et.al.,1999; Rosengarth et.al.,1999), intermolecular interactions (Kaspieva et.al.,2001; Levitsky et.al.,2000; Oberer et.al.,1999) as well as structural determinants of folding stability (Kato et.al.,2000; Ginsburg et.al.,2000; Chakraborthy et.al.,1999; Lassale and Hinz 1999).

Principle and Data Analysis in Scanning Calorimetry

Excellent reviews are available on the concept and interpretation of DSC data (Sanchez-Ruiz 1995; Privalov 1980). The output from DSC experiment is obtained as a plot of excess heat capacity (C_p^{ex}) versus temperature, where C_p^{ex} is the partial heat capacity of the protein. From the heat capacity difference and the partial volume of the protein, absolute values representing the partial heat capacity of the protein can be derived. The resulting negative value after buffer-baseline subtraction, indicates that heat capacity of the reference is higher than that of the sample, which is a consequence of the lower water content of the latter. Some water is displaced by the protein, and the heat capacity of liquid water is higher that of the protein.

Excess enthalpy is given by the temperature of integration of C_p^{ex}-

$$\langle \Delta H \rangle = \int_{T^0}^{T} \Delta_N^D C_p^{ex} \, dT \qquad \text{----------(1)}$$

where $\mathbf{T^o}$ is the temperature where all the protein is in its native conformation. At the temperature **T,** all the protein is in the denatured state, and $< \Delta H >$ represents the calorimetric enthalpy.

Reversibility and Two State Equilibrium Model

When reversible transition from native (N) to denatured state (D) occurs, this model is applied,

$$N \overset{K}{\rightleftharpoons} D$$

where $K = \dfrac{[D]}{[N]}$

where k is the equilibrium constant.

Excess enthalpy is derived as follows:-

$$\langle \Delta H \rangle = X_D \times \Delta_N^D H = \frac{K \times \Delta_N^D H}{(1+K)} \quad \text{---------- (2)}$$

Excess heat capacity is given by the following equation-

$$Cp^{ex} = \frac{d\langle \Delta H \rangle}{dT} = \frac{(\Delta_N^D H)^2}{(RT^2)} \times \frac{K}{(1+K)} + X_D \cdot \Delta_N^D C_p \text{ ----- (3)}$$

where X_D = [K/(1+K)] and amounts the quantity of the denatured protein.

Relationship between K and $\Delta_N^D H$ can be expressed by van't Hoff and Kirchoff equation as,

$$\frac{d \ln K}{dT} = \frac{\Delta_N^D H}{(RT^2)} \text{ and } \frac{\Delta_N^D H}{dT} = \Delta_N^D C_p \text{ ----------(4)}$$

At the denaturation temperature (T_m), X_D = ½ (and hence K = 1 and $\Delta_N{}^D G = 0$).

The area encircled in between the transition and the chemical baseline represents the calorimetric enthalpy $\Delta_N{}^D H$. It is related to temperature (Kirchoff equation), as well as to the denaturational heat capacity increment $\left[\Delta_N^D C_p\right]$

For any temperature T

$$\Delta_N^D H(T) = \Delta_N^D H(T_m) + \int_{T_m}^{T} \Delta_N^D C_p \, dT \text{ ----------(5)}$$

$$\Delta_N^D S(T) = \frac{\Delta_N^D H(T_m)}{T_m} + \int_{T_m}^{T}\left[\frac{\Delta_N^D C_p}{T}\right]dT \text{ ----------(6)}$$

$$\Delta_N^D G(T) = \Delta_N^D H(T) - T\Delta_N^D S(T) \text{ ----------(7)}$$

If the values for T_m, $\Delta_N{}^D H(T_m)$, and $\Delta_N{}^D C_p$ can be determined from experimental DSC transition, the thermodynamic parameters for the protein denaturation can be derived from these.

The denaturation enthalpy, called apparent van't Hoff enthalpy (ΔH_{vH}), is calculated from the width of the transition.

So if we take $T = T_m$ (hence K = 1)

$$\Delta H_{vH} = 4RT_m^2\left[\frac{\Delta C_p^m}{\Delta_N^D H(T_m)}\right] \text{ ----------(8)}$$

where $\Delta C_p{}^m$ is the heat capacity at the temperature T_m and measured from the chemical baseline. $[\Delta C_p{}^m / \Delta_N{}^D H(T_m)]$ is sensitive to the width of the transition.

When DSC transition is broader, van't Hoff enthalpy is smaller (compared to the calorimetric enthalpy). Depending on the ratio of $\Delta_N{}^D H(T_m)/\Delta H_{vH}$, much about the intramolecular cooperativity of the protein can be deduced. The ratio of $\Delta H_{cal}/\Delta H_{vH}$ is denoted by 'R',

$$R = \frac{\Delta_N^D H(T_m)}{\Delta H_{vH}}$$

which tells about the structural state of a protein. $R < 1$ denotes that co-operativity is involved in the denaturation process. $R > 1$ hints at the presence of intermediate states.

Multi-state Equilibrium

R > 1 (or when additional transitions are observed in the DSC scan) indicates the presence of intermediate states. Such complex DSC transitions can be conceived as the sum of independent, two state transitions:

$$C_P^{cv} = \sum_j \frac{(\Delta_N^D H_j)^2}{RT^2} \times \frac{K_j}{(1+K_j)^2}$$

where $\Delta_N{}^D H_j$ and K_j represent the enthalpy change and the equilibrium constant respectively of the denaturation of the thermodynamic domain j.

Models that take into account the effect of inter-domain interactions have been applied to the interpretation of the unfolding of multi-domain proteins (Freire et.al., 1992).

Kinetic Analysis of DSC Thermograms

In cases where the non-attainment of equilibrium arises due either to irreversibility or slow unfolding kinetics, the quantity of the significantly populated states at any given temperature will be a direct variable of the time required to attain that temperature, i.e., there will be scanning rate dependency of transitions.

Although protein stability is generally conceived as the Gibbs energy difference between the denatured and the native states, for practical purposes, the parameter "denaturation temperature (T_m)" is a more useful scale of protein stability (Sanchez-Ruiz 1995). Thermal stability of proteins can be subjected to kinetic constraints, especially in irreversible transitions (Hernandez-Arana et.al.,1993).

7.4 Materials and Methods

7.4.1. Materials

Preparation of diferric lactoferrin as well as that of lobes is described in chapter 4. Protocols for making apo-protein is elaborated in chapter 6. Preparation of reassociated N- and C- lobes was as per the procedure given in chapter 4.

Buffers used for scanning are sodium acetate (for pH 5 and below) and HEPES (for pH above 5), at 20mM concentration.

7.4.2. DSC Measurements

The experiments were carried out on a Microcal Scanning Microcalorimeter (Microcal Inc., Northampton, Madison, USA). Data analysis and plotting were done using the package Origin, supplied by Microcal.

Scan rate was 60°C/hour, and the temperature range was 25°C to 95°C. Protein concentration used was 1.5mg/ml. DPLf was scanned at pH values 8,7,6,5,4,3.5,3 and 2.5; apo-intact protein was scanned at pH values 5,4,3.5 and 3. Ferric N- lobe at pH 3.5 and 3, Ferric C- lobe at pH 7, 5 and 3, and apo N- lobe at pH 5 and 3.5.Reassociated N- and C- lobe complex was scanned at pH 3.5.

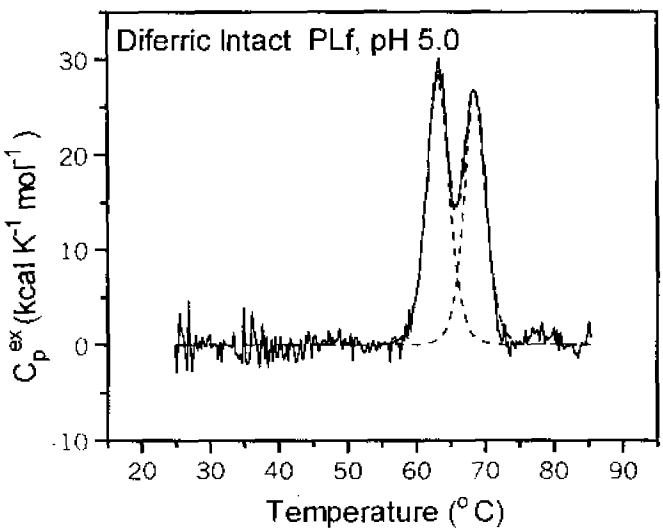
Thermodynamic calculations were done using the non two-state model. Reversibility of the protein unfolding was checked by re-heating the samples from a previous upscan, after cooling.

7.5 Results

7.5.1. Scanning of DPLf

DPLf aggregated at all the pH values above 5 during heating (plot not shown) (Fig: 7.1 A & B). This behaviour of DPLf is against that of CLf and

HLf (Paulsson et.al.,1993; Hadden et.al., 1994) At pH 5, it showed two transitions with the Tm of 63.19°C (ΔH 122 kcal/mol) and 68.5°C (ΔH 108 kcal/mol). These transition peaks were partially merged. There is also a very small peak diffused in the base line. At pH 4, in addition to the shift in the Tm values (to 57.04°C (ΔH 201 kcal/mol) and 63.36°C (ΔH 107 kcal/mol), respectively), there is also the appearance of a new minor peak at 78.33°C (ΔH 93 kcal/mol). Also, a reduction in the area of the second peak is visible. The same trend continues at pH 3.5 showing shifts in Tm values to 56.79 (ΔH 178 kcal/mol), 63 (ΔH 102 kcal/mol), and 77.67 (ΔH 105 kcal/mol). At pH 3 there are only two transitions, at 44.5°C (ΔH 222 kcal/mol) and 64.9°C (ΔH 60.2 kcal/mol). Below pH 3, the protein is completely denatured, failing to show any transitions, but only a flat line (curve not shown).



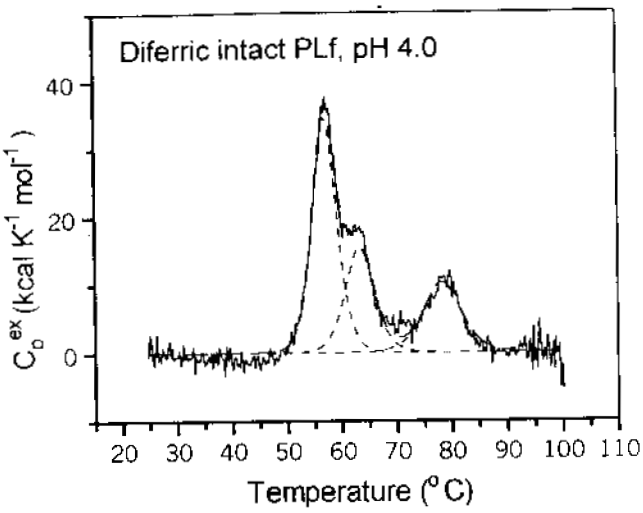


Fig: 7.1 A. DSC curve of DPLf at pH 5 (above) and 4 (below).

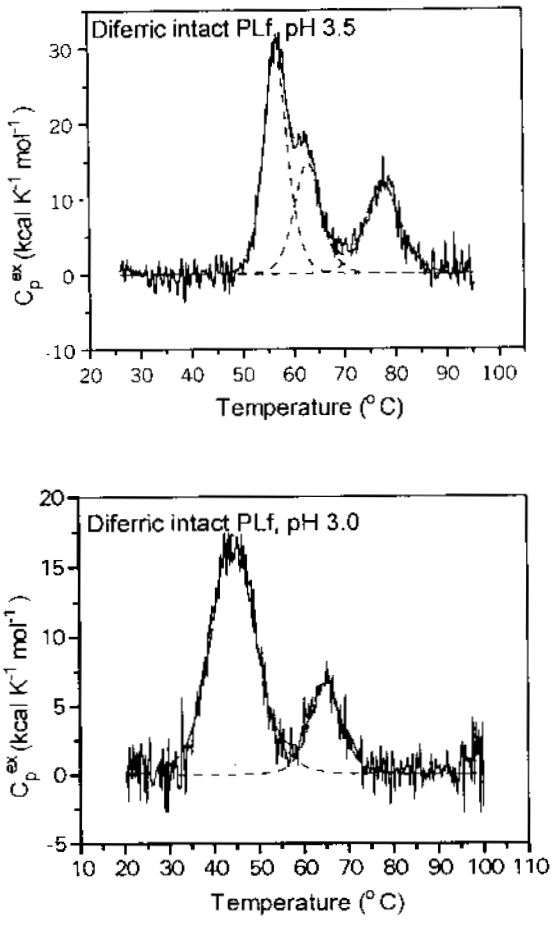


Fig: 7.1 B. DSC curve of DPLf at pH 3.5 (above) and 3 (below).

7.5.2. Scanning of Apo-intact Protein

At all the pH values where experiments were done (i.e., pH 5,4 and 3.5), there was only one transition (Fig: 7.2 A & B) with a Tm of 58.2°C (ΔH 179 kcal/mol), 51.77°C (ΔH 177 kcal/mol), and 46.21°C (ΔH 187 kcal/mol). At pH 3 complete denaturation was observed (not shown).

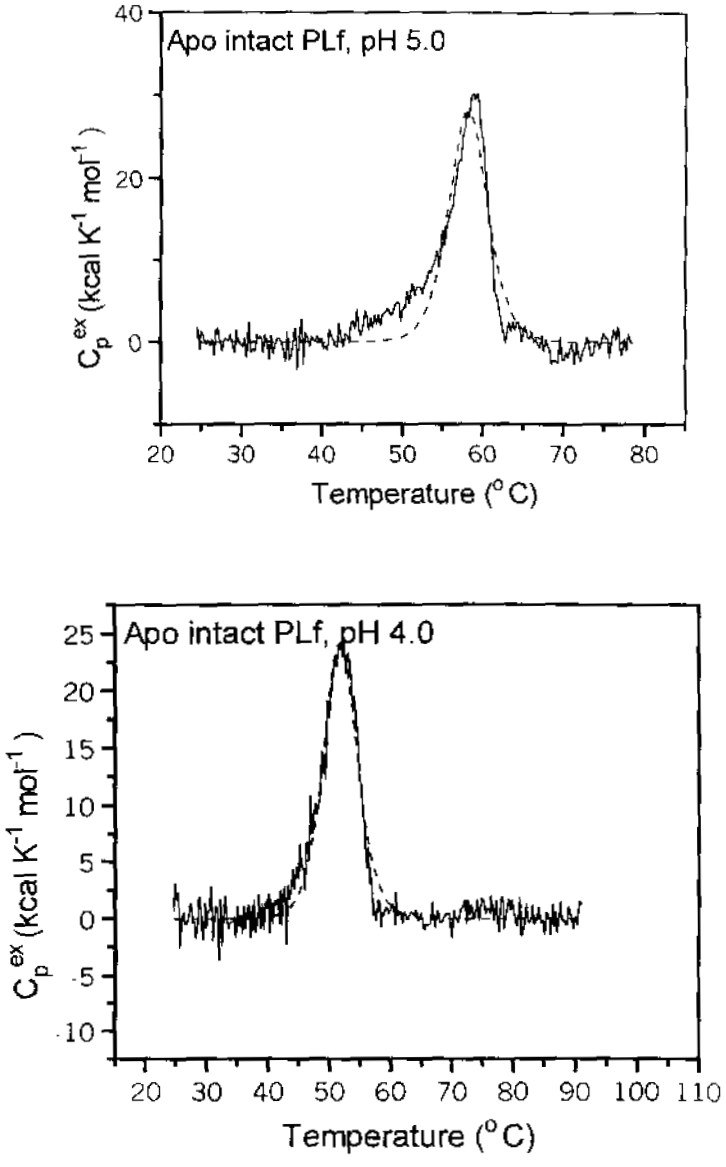


Fig: 7.2 A. DSC curve of apo intact PLf at pH 5 (above) and 4 (below).

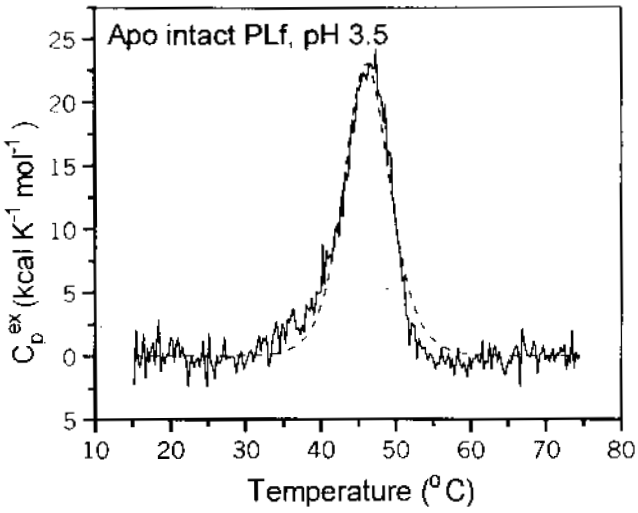
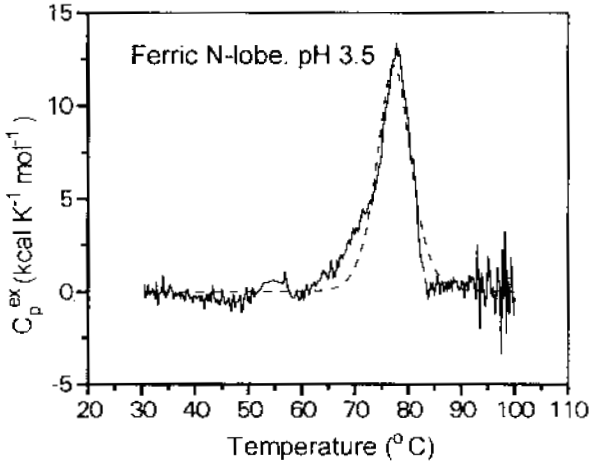


Fig: 7.2 B. DSC curve of apo intact PLf at pH 3.5.

7.5.3. Scanning of Ferric and Apo-N- Lobes

Ferric N lobe gave a single transition at pH 3.5 with a Tm of 77.43°C (ΔH 101 kcal/mol). At pH 3, the Tm shifted to 63.91°C (ΔH 78.5 kcal/mol) (Fig: 7.3).

Apo N- lobe presented a Tm of 62.34°C (ΔH 164 kcal/mol) and 50.48°C (ΔH 103 kcal/mol) at pH values 5 and 3.5 respectively (Fig: 7.4).



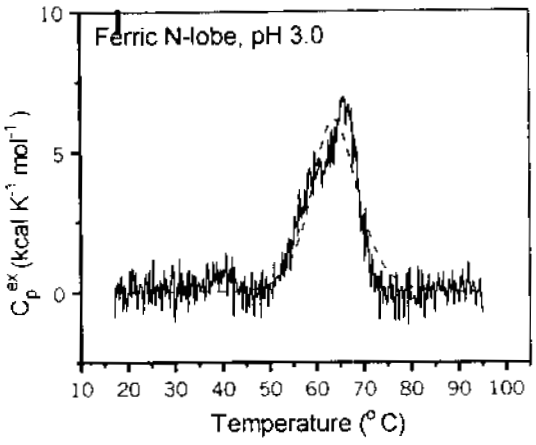


Fig: 7.3. DSC curve of ferric N- lobe at pH 3.5 (above) and 3 (below)

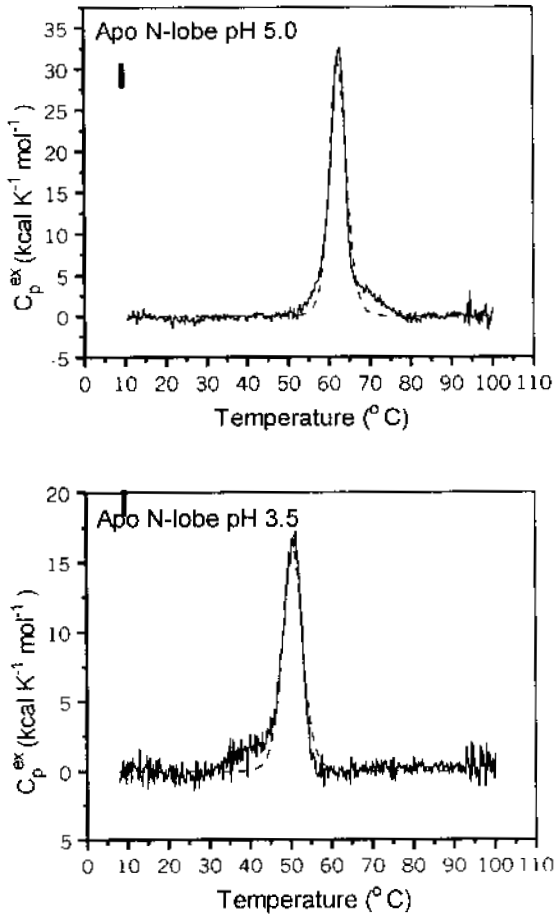


Fig: 7.4. DSC curve of apo N- lobe at pH 5 (above) and 3.5 (below).

7.5.4. Scanning of C- Lobe

C- Lobe failed to give any transition at the pH values of all the experiments done (plot not shown).

7.5.5. Scanning of Reassociated N- and C- Lobe Complex

At pH 3.5 the complex gave a transition at 56.6°C (Fig: 7.5). Beyond that aggregation was observed.

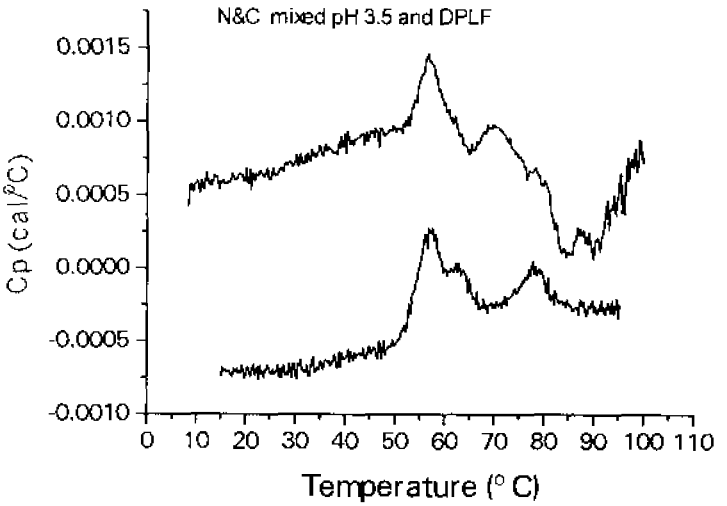


Fig: 7.5. DSC curve of reassociated N- and C- lobe complex at pH 3.5 (upper curve). Lower is the transition for DPLf.

7.6 Discussion

Ascribing the Thermal Transitions to the Molecular Species

Lin et.al., (1994) has reported the DSC analysis of the thermal unfolding of HTf and HOTf. They had conducted the experiments at a pH of around 7.5. Unlike HTF and HOTf, DPLf showed aggregation during heating at all the pH values above 5. In the Tm vs heat capacity plot of DPLf, there is a clear and progressive change in the Tm values and the number of transition peaks at pH values from 5 to 3 (Fig: 7.1A & B).

At pH 5, the major peaks are in the form of a partially merged but still with distinct, transitions, having the Tm values of 63.19°C and 68.55°C. But there is also a very small peak, rather diffused on the base line, as the third transition at 78°C. At pH 4, the high-Tm peak of the merge peaks decreases. But the minor transition peak observed at 78°C increased drastically and dramatically with a Tm of 78.33°C. The same trend continued to pH 3.5 also, except for the very minor shift in Tm values (from 57.04°C, 63.36°C and 78.33°C, to 56.79°C, 63.0°C, and 77.67°C respectively). But at pH 3, the partially merged form of the early transitions disappeared giving only one transition at 44.5°C. The one corresponding to 77.67°C (at pH 3.5) shifted to 64.95°C. The enthalpy of transitions are mentioned in the section 7.5.

How can the individual transition peaks obtained in the thermal unfolding of DPLf be assigned to particular molecular species or intramolecular segments of lactoferrin? Biochemical studies indicate that at pH 5 there is a significant loss in the bound iron from both the lobes. Naturally, at pH $\leq$5, a mixed population of apo and iron saturated molecules exists, with the latter predominating. Apo form itself can be either without iron in both the lobes or only one lobe; in the latter case, either N or C lobe will be without iron while the other lobe will be having iron (Lin et.al., 1994). Considering the spontaneity and reversibility of the iron binding reaction, it is possible that some fraction of the released iron is rebinding to protein at any given time (unless the released iron is not removed instantaneously). Such theoretical possibilities have to be considered while analyzing the DSC scan results of DPLf. Testifying this view, the thermogram of DPLf (Fig: 7.1 A & B) presents complex thermal transitions. Apo-intact protein has a Tm of 58.2°C, at pH 5. But no corresponding transition is seen in DPLf at the same pH, as well as at other pH values also. This indicates that the true apo-form with both the

lobes devoid of iron does not exists in significant quantity at the scanning conditions. Apo N- lobe has a Tm of 62.34°C (at pH 5), which is more or less similar to the Tm of first transition of DPLf at pH 5, i.e., 63.19°C. But the situation is further complicated at pH 3.5, where N-apo has a Tm of 50.4°C, the first transition of DPLf corresponds to 56.79°C. Interestingly, the Tm of ferric-N lobe (77.43°C and 63.91°C at pH 3.5 and 3, respectively) corresponds exactly to that of the third obtained in the scan of DPLf. But lack of the Tm values of C lobe (ferric and apo-forms) critically limits the efforts to identify the molecular determinants of the transition peaks.

Previously, Lin et al (1994) has shown through titration of human serotransferrin with varying degrees of iron saturation that (in intact molecule) when one lobe is apo and the other is saturated, free energy of pair-wise interaction induces significant shifts in the Tm values of apo-lobe. Viewing in the light of this report, the complex thermal transition peaks obtained in the case of DPLf is not surprising; it is highly probable that the overlapping peaks observed in the DSC scan of DPLf is the transition(s) of a mixture of apo-lobes influenced by iron loaded pair-lobe. Still the question of "why true apo-form (both the lobes iron-less) does not exist" remains. The absence of any transition peak corresponding to full apo-intact protein appears to be inconsistent with biochemical data, which demonstrates that at pH $\leq$5, lactoferrin releases a significant quantity of bound iron. The above inconsistency between the DSC and biochemical observation could be due to the proteins' "structural readiness", that the iron binding and release at pH $\leq$5 is only an environment driven process. A deeper look at the problem throws some hints.

In biochemical studies, the released iron is removed so that the "system" is deprived of ligand. But in the DSC scan, the sample is in a closed chamber, where the released iron is distributed uniformly with the protein. Moreover, the elevation of the kinetic energy (and mobility) of

the dissolved materials due to the increase in temperature during heating enhances inter-molecular (- atomic) collision rates. Considering the reversibility of the iron binding process and the ability of the apo-lactoferrin to rebind the iron (Legrand et al 1990), the absence of Tm values corresponding to true apo-forms can be explained as possibly due to the spontaneous rebinding of the released iron to the protein. It should also be noted that there is no significant collapse of secondary structures at the pH values where transitions obtained (as evidenced in the CD data), indicating that the structural scaffold required for iron binding is still intact (Fig: 7.6).

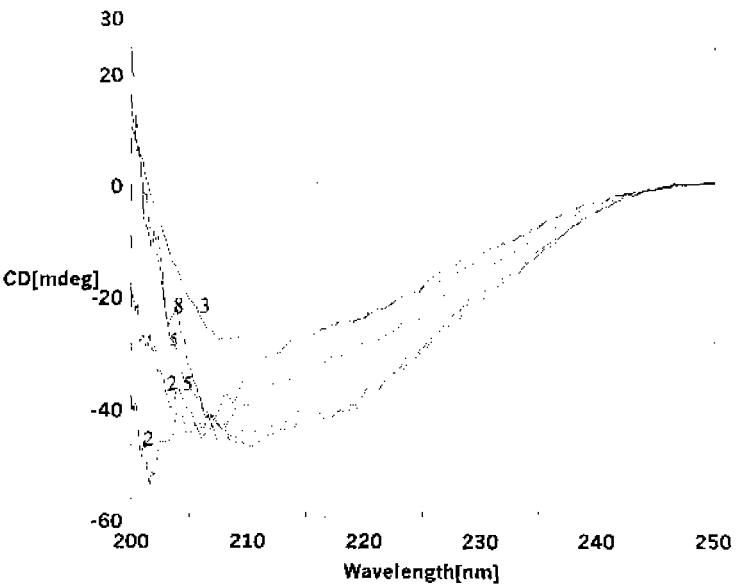


Fig: 7.6. CD spectra of DPLf at the pH values 8, 5, 3, 2.5 and 2 (marked on the curves). 4 hours of equilibration was given to the protein at each pH. The spectra were recorded on a Jasco make Spectropolarimeter

The high Tm-peak observed in the DSC scan of DPLf at pH 3.5 matches with that of ferric N-lobe. Since the Tm of apo-intact protein is 46.12°C (at pH 3.5), and that of apo-N lobe is 50.4°C (at pH 3.5), it can be deduced that the Tm of apo-C lobe will be below 46.21°C (because

the single transition of apo-intact protein is a representation of the transitions of both the lobes).

Separated lobes of lactoferrins have the unique property of reassociating in a functional manner, if mixed together (Legrand et.al., 1990) (see chapter 4 for an elaborate discussion). Underlining this concept of reassociation, the DSC profile of the mixed ferric N- and C- lobes of porcine lactoferrin tended to give a transition similar to that of the intact DPLf. As evidenced from the Fig: 7.5, the first peak in the scan of N- and C- lobe complex matches with that of DPLf, but a sudden onset of protein aggregation prevents further transitions.

Overall, the thermal unfolding behaviour of diferric porcine lactoferrin is highly complex. Identifying the transition peaks with the corresponding molecular species (or segments of the protein), as well as the biochemical/structural reasons for the aggregation of the DPLf and the C- lobe during heating requires further experiments.

Influence of pH on Thermal Transition(s)

Figs: 7.2 A & B reveal that when pH is altered the Tm also shifts. More exactly, lowered pH causes early transitions. For example, the Tm of apo-intact protein is 58.2°C at pH 5, while the same has shifted to 46.21°C at pH 3.5. Similar changes can be seen in the thermal unfolding of DPLf also (Fig: 7.1A & B).

It is well documented that ionizable groups and ionic interactions are among the key contributors to the stability and functioning of proteins (Perutz 1978). As the level of ionization is a direct variable of pH, changes in the hydrogen and hydroxyl ion concentrations can significantly influence the charge distribution on protein surface. Those residues having ionizable groups are the structural factors of origin of pH dependent protein stability. Changes in pH can alter the electrostatic

bonding (as well as net charge on the molecule), affecting the rigidity of folding. The change in stability has been found to vary inversely with that of net charge of the protein- the greater the net charge, the lower the stability (Hollecker and Creighton 1982). This view conforms to the model proposed by Linderstrom-Lang (1924), which conceives protein as a charged sphere whose electrostatic free energy is proportional to the square of the net surface charge.

Reversibility of the Thermal Unfolding of Porcine Lactoferrin

The reversibility has been checked by reheating the same protein which is used for the first upscan. In the case of DPLf, the third transition (Tm 77.67°C at pH 3.5) is reversible; the first peak is partially reversible, while second peak (Tm 63°C at pH 3.5) is irreversible (Fig: 7.6). Thermal unfolding of apo-intact protein is completely irreversible, as in apoHLf (Hadden et.al.,1994) (Fig:7.6). While ferric-N lobe unfolded irreversibly, the refolding of apo-N lobe is rather insignificant (Fig: 7.7). In Fig:7.6, it can be seen that the Tm values of reheat are the same as that of the firstscan of the same sample.

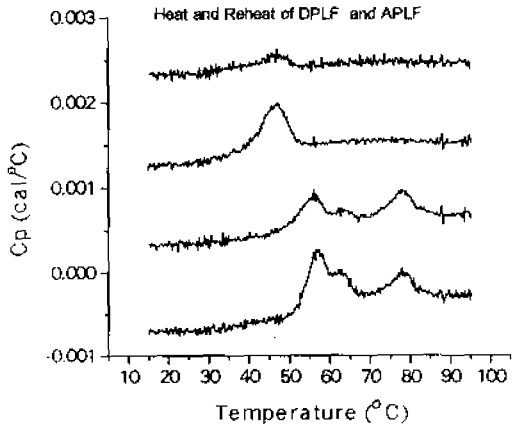


Fig: 7.7. DSC curve for the heat and reheat of DPLf and apo intact PLf. Curves from below are (1) first upscan of DPLf, (2) second upscan of DPLf, (3) first upscan of apo protein, and (4) second upscan of apo protein.

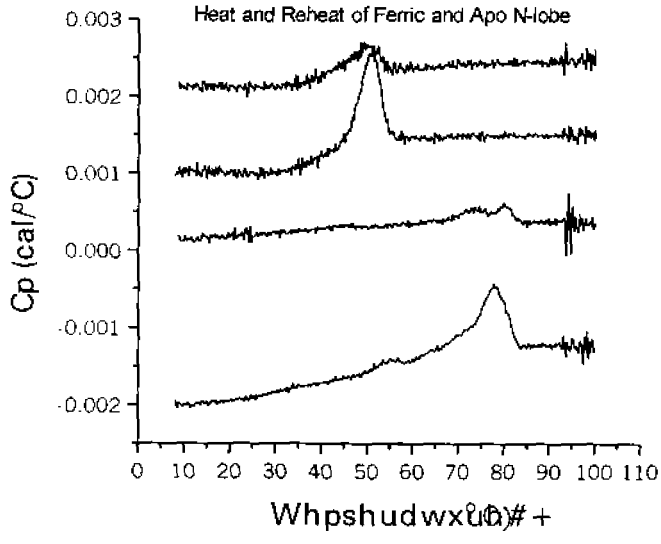


Fig: 7.8. DSC curve for the heat and reheat of ferric and apo N- lobe. Curves from below are (1) first upscan of ferric N- lobe, (2) second upscan of ferric N- lobe, (3) first upscan of apo N- lobe, and (4) second upscan of apo N- lobe.

Conclusions

1. **Thermal unfolding of porcine lactoferrin is highly complex.**
2. **Diferric protein aggregated at all the experimental pH values above 5.**
3. **C- lobe aggregated at all the experimental pH units.**
4. **In the DSC scan profiles of DPLf, at pH $\leq$5, no transition corresponding to true apo form is seen. This may be due to spontaneous rebinding of iron, and the free energy of pair wise interaction between a ferric lobe and its apo pair-lobe.**
5. **Reassociated N- and C- lobe complex tended to yield a transition similar to that of DPLf, except for an early aggregation.**
6. **Apo intact protein, apo N- lobe and ferric N- lobe gave a single transition during unfolding.**