CHAPTER V

IRON-RELEASE BEHAVIOUR AND THE NATURE OF DOMAIN-DOMAIN INTERACTIONS OF PORCINE LACTOFERRIN

5.1 Introduction

Various lactoferrins differ significantly in their pH induced iron release profile (Legrand et.al., 1990; Khan et.al., 2001). Both the lobes of the same lactoferrin are also dissimilar in their iron-releasing property. Though the existence of domain-domain interactions between both the lobes of the lactoferrin molecule has been reported (Lin et.al.,1994; Legrand et.al., 1990), extensive studies involving all lactoferrins are required to establish the universality of such interactions. These differences in the functional properties may have an underlying structural basis dictated by the diversity in the sequences between various lactoferrins.

Considering the variability in the properties of Lactoferrins, it would be worthwhile to study the iron binding stability and nature of domain-domain interactions of PLf. This section reports:-

1. **pH induced iron-release from intact PLf.**
2. **pH induced iron-release from C– lobe of PLf.**
3. **pH induced iron-release from N– lobe of PLf.**
4. **pH induced iron-release from reassociated N_{Fe}-C_{Fe} lobe complex.**
5. **pH induced iron-release from reassociated N_{Fe}-C_{Apo} lobe complex.**

5.2 Materials and Methods

All the experiments for the study of pH induced iron release were done in two steps. The first set was to identify the iron release pH by downgrading the dialysis by pH units of 0.5. After arriving at the coarse pH of iron release by the above step, it was refined further by the second sets of equilibration around the same pH of iron release by pH units of

0.2 units. At each pH, 48 hours of dialysis was done, with buffer change at every 6hours. The weight of the protein was determined by weighing lyophilized sample.

5.2.1 pH Induced Iron Release From Intact PLf

1mg/ml of iron-saturated intact PLf was dialyzed successively against a series of decreasing pH values from pH 8 to 2.5 (by 0.5 units) of Citrate-Sodium hydroxide buffer, 10mM, at 4°C. Absorption at 465nm was used to monitor the changes in iron-saturation.

5.2.2 pH Induced Iron Release From C– Lobe

1mg/ml of iron-saturated PLf C- lobe was dialyzed successively against a series of decreasing pH values from pH 8 to 2.5 (by 0.5 units) of Citrate-Sodium hydroxide buffer, 10mM, at 4°C. Absorption at 465nm was used to monitor the changes in iron-saturation.

5.2.3 pH Induced Iron Release From N– Lobe

1mg of iron-saturated PLf N- lobe was dialyzed successively against a series of decreasing pH values from pH 8 to 2.5 (by 0.5 units) of Citrate-Sodium hydroxide buffer, 10mM, at 4°C. Absorption at 453nm was used to monitor the changes in iron-saturation.

5.2.4 pH Induced Iron Release From Reassociated N_{Fe}- C_{Fe} Lobe Complex

1mg/ml of reassociated N_{Fe}-C_{Fe} complex was dialyzed successively against a series of decreasing pH values from pH 8 to 2.5 (by 0.5 units) of Citrate-Sodium hydroxide buffer, 10mM, at 4°C. Absorption at 465nm was used to monitor the changes in iron-saturation.

5.2.5 pH Induced Iron Release From Reassociated N_{Fe}-C_{Apo} Lobe Complex

1mg/ml of reassociated N_{Fe}-C_{Apo} complex was dialyzed successively against a series of decreasing pH values from pH 7.5 to .5 (by 0.5 units) of Citrate-Sodium hydroxide buffer, 10mM, at 4°C. Absorption at 465nm was used to monitor the changes in iron-saturation.

5.3 Results

5.3.1 pH Induced Iron Release From Intact PLf

Fig: 5.1 shows the iron release from intact PLf. Although 24 hours were enough for the change, 48 hours were given to make sure that the release is complete. There are two dips in the graph, first one at pH 6.1, and the second at pH 5.1.

5.3.2 pH Induced Iron Release From C– Lobe

Fig: 5.1 shows the iron–release from C– lobe. A monophasic curve with a slope having pH_{50} of 5.1 was obtained.

5.3.3 pH Induced Iron Release From N– Lobe

As shown in Fig: 5.1, release from N lobe has a pH_{50} of 5.5.

5.3.4 pH Induced Iron Release From Reassociated N_{Fe}-C_{Fe} Lobe Complex

Fig: 5.1 shows the iron–release from N_{Fe}-C_{Fe} Reassociated Complex. A pattern similar to that of intact PLf was obtained. First slope is around 6.1 and the second slope lies at pH 5.1.

5.3.5 pH Induced Iron Release From reassociated N_{Fe}- C_{Apo} Lobe Complex

The iron release profile varies from that of intact molecule and that of reassociated complex of N_{Fe}-C_{Fe} (Fig: 5.1), the pH_{50} being at pH 5.7.

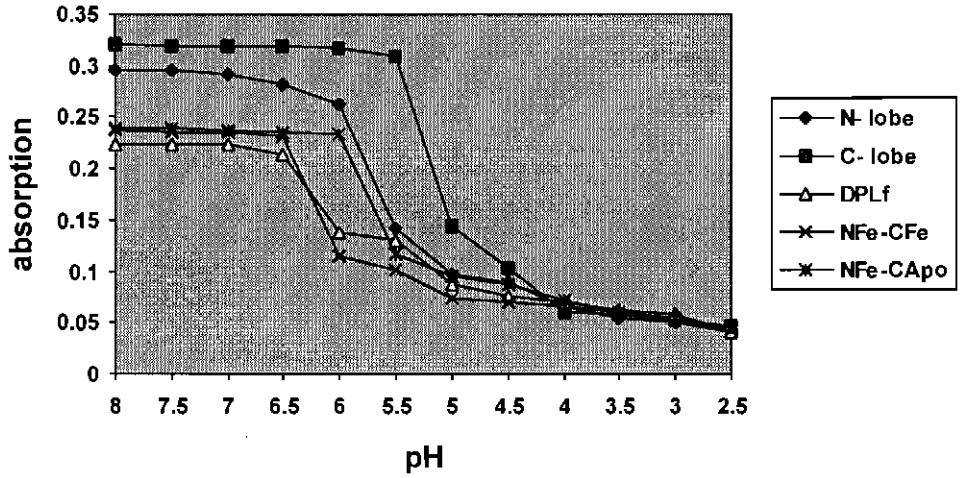

Fig:5.1 pH_{50} of iron-release of DPLf , N- & C- lobes and Nfe-Cfe & Nfe-CApo

5.4 Discussion

It has been reported previously that isolated N- lobe of HLf (generated by proteolysis or recombinant DNA technology) releases iron at pH 6, and C- lobe at pH 4.5 (Day et.al., 1992; Legrand et.al., 1990). Separated N- and C- lobes of CLf also behave in a similar manner, releasing at pH 6.3 and pH 4.6 (Legrand et.al.,1990). **But, as evidenced in the current study, there exists a substantial variation in the iron binding stability of isolated N- and C- lobes of PLF, compared to that of HLf and CLf.** Separated N- lobe of PLf has a higher binding stability, releasing iron only at pH 5.5. On the other hand, the iron binding by C- lobe of PLf is less stable compared to that of HLF and CLf, with a pH_{50} of 5.1. (pH_{50} is the pH at which 50% of iron is released).

Why C- lobe of PLf releases iron at a pH higher than that of the C- lobe of other lactoferrins? An observation of the amino acid sequence of PLf shows that the iron and anion coordinating residues as seen in other lactoferrins are conserved; hence it is not a change in these residues which makes porcine C- lobe iron binding more weak. Another unique feature of PLf is it's basic pI, which is different from the commonly acidic (~pH 5) pI of the C- lobe of other lactoferrins (see chapter 4). This change in the charge profile may have a profound influence on the bonding environment of the C- lobe, causing it to release iron at a lower pH.

Equally intriguing is the structural feature(s) governing the altered iron-binding stability of the N- lobe of PLf. N- lobes from all the lactoferrins studied, except that of ULf, releases iron only at a pH above 6 (Table: 5.1). ULf_N loses iron at pH 4 only (Khan et.al., 2001). Sequence analysis reveals that N- lobe of PLf has a significantly higher identity with ULf_N, compared to that of the other lactoferrins (see chapter 3). This remarkable correspondence in the iron release behaviour and sequence identity between the N- lobes of PLf and ULf may be indicative of the presence of an underlying dictating structural feature. Similarly, the C- lobe of PLf also shows increased residue identity with that of ULf, compared to that of other lactoferrins. Interestingly, it has been reported that ULf_C releases iron at pH 6.5 (Khan et.al., 2001). An extended analysis of the sequence pattern of the N- and C- lobes of all lactoferrins together perplexes the problem further (chapter 3). The residue identity of the N- and C- lobes of ULf with corresponding lobes of GLf, CLf and ELf are higher than that with PLf lobes (Tables 3.2 and 3.3). Yet the N- lobes of GLf, CLf and ELf releases iron at pH ~6.5, and the C- lobe at a much lower pH (Table 5.1). At present, the data required to propose a structural explanation for such mutually contradictory diferences in the iron releasing pattern of different lactoferrins, is not sufficient. Baker et.al., (2000)

has proposed the involvement of second shell residues in determining the iron binding affinity.

	Intact Molecule	Isolated N- Lobe	Isolated C- Lobe
BLf	4.5, 3.8	6.1	4
CLf	4.5, 3.5	6.3	4.6
GLf	6.8, 4	6.8	4
PLf	6.1, 5.1	5.5	5.1
HLf	4, 3	6	4.5
ULf	6.5, 4	4	6.5

Table: 5.1 pH_{50} of Iron Release From Lactoferrins

The iron-release curve (Fig: 5.1) of intact PLf is biphasic. First dip on the curve appears at pH 6.1, followed by the second at pH 5.1. As the isolated N- lobe releases at a higher pH than that of C- lobe, the first fall must be that of iron release from N- lobe of intact molecule. But there is a remarkable shift in the iron releasing pH. The point corresponding to the iron-release from the N- lobe-release stands at pH 6.1, which is 0.6 units towards the basic pH from that of isolated N- lobe. This contradicts the common trend, as seen in HLf and CLf, in which N- lobe always releases only at a lower pH when it is part of the intact molecule, compared to that of the isolated N- lobe. This indicates that the stability of the iron of the N- lobe is enhanced in someway in the intact molecule, in HLf and CLf, compared to that in their separated form. **Contrary to this, the iron release pattern of PLf shows that there exists no positive modulatory interactions between its lobes.**

Legrand et.al., (1986,1990) reported that the separated N- and C- lobes have the property of reassociating to an 80Kd molecule, showing features similar to that of intact Lf. Reassociated N- and C- lobe complex of HLf and CLf has shown a lower pH of iron release as that of the intact molecule. Similar to the previous reports, **reasssociated PLf**

N- and C- lobes also shows an iron release behaviour comparable to that of intact PLf. Iron-release curve of PLf N_{Fe}-C_{Fe} complex shows falls at pH 6.1 and 5.1, respectively for N-and C- lobes. (The slight variations in the points of release of iron as observed during some of the experiments in the range of 0.1 units of pH [data not shown] may be due to fine changes in the interaction between the lobes consequent to cleavage; but the values are comparable with that obtained from the intact molecule).

The increase in the iron releasing stability of intact lactoferrins compared to that of separated lobes has been explained as a result of the stabilizing interactions between the lobes. In the intact lactoferrin, the N- lobe has a significantly higher stability than in the separated state, due to the modulatory interaction with the C- lobe. C- lobe also is reported to be reciprocated by N- lobe (Legrand et al. 1990). Some kind of positive cooperativity exists between the lobes in modulating their binding stability (Baker et.al., 2000) , which may be a factor evolved to confer efficiency to lactoferrins. Such modulatory interactions are absent in serotransferrins (Line back_Zins and Brew 1980). Many structural features have been proposed to be responsible for this inter-domain interaction. One of this includes a stretch of hydrophobic residues seen on both the lobes (Anderson et.al., 1990). A more logical proposal can be drawn from the crystal structure of a mutant apoHLf with open N- lobe (resembling an open state) and closed C- lobe (resembling iron loaded state) (Jameson et.al., 1998). In this structure it has been observed that an overhanging C-terminal helix makes increased contacts with N-domain. This helix may be an important structural factor involved in conferring the N- lobe with an increased stability when associated with the C- lobe.

The failure of the intact PLf to show increased pH stability compared to that of separated N- lobe **is a deviation from the concept of the existence of stabilizing domain-domain interactions**. Iron release behaviour of intact PLf attracts more attention as it looses iron at

a pH even higher than that at which free N- lobe does, indicating the lack of stabilizing interactions. **This also hints at the exertion of a kind of destabilizing influence on the N- lobe when it is part of the intact molecule.** How can this be interpreted? Has the unique basic pI (see chapter 4) of the PLf C- lobe got any role in this? It is logical to surmise that the unusually basic C- lobe may be interfering with the N- lobe iron-coordination, making its iron and/or anion binding less rigid. By virtue of it's position and basic charge, C- lobe when complexed with N- lobe, may destabilize the liganding environment of N- lobe.

Results from the iron release studies on PLf $\mathbf{N_{Fe}}$-$\mathbf{C_{Apo}}$ complex reveal more about this problem. While an iron- loaded C- lobe destabilized the N- lobe, apo C- lobe exerted only less effect. **The PLf $\mathbf{N_{Fe}}$-$\mathbf{C_{Apo}}$ complex released iron at pH 5.7, which lies in between that of free N- lobe and intact PLf.** Previously Ward et.al., (1996) have shown through studies with mutant HLf (having functional N- lobe but mutant C- lobe lacking iron binding residues), that an iron- loaded C- lobe is essential for modulating N- lobe. This feature, along with the structure of a mutant apoHLf with closed C- lobe and open N- lobe, tends to offer some insight into the underlying factors of the domain-domain interactions (Jameson et.al., 1998). The conformational changes accompanying iron binding increase the proximity of the C-terminal helix (or an adjoining region) to the N-domain so that it can make more contacts, thereby elevating the stability of the domains. Viewing from this angle, in PLf $\mathbf{N_{Fe}}$-$\mathbf{C_{Apo}}$ complex, the apo-C may not be having increased proximity to the N-domain, resulting in a lowering of the destabilizing influence on the N- lobe liganding environment, compared to that of PLf $\mathbf{N_{Fe}}$-$\mathbf{C_{Fe}}$ complex, leading to an iron release pattern more near to that of the free N- lobe. This proposal offers an explanation for why N- lobe of PLf $\mathbf{N_{Fe}}$-$\mathbf{C_{Apo}}$ complex releases iron more similar to that of isolated N- lobe.

The current study provides definitive evidence for the absence of stabilizing domain-domain interactions in PLf. More eliciting is the inference

of the existence of a rather destabilizing effect of C- lobe on N- lobe. Recently, Khan et al. (2001) have reported the absence of the cooperative iron binding modulation between the lobes of ULf. Similarly, unpublished results of the iron release pattern from GLf (intact molecule and separated lobes) also reveal the absence of any kind of modulation between the lobes (Mohanty et.al., personal communication). **Hence it is explicitly clear from these reports that the positive modulatory interaction between the lobes is not a general characteristic of lactoferrins.** These observations testify that the lactoferrins differ remarkably from each other with respect to their properties. The obvious question that remains is whether these variations in the properties of different lactoferrins bear any relation with species specific physiological requirements. All these observations are conducive to the notion that the structural underpinnings governing the property variations of lactoferrins are not discernible through any single feature. The bilobed nature of Transferrin-family of proteins is proposed to be evolved by duplication from an original monolobal archetypal iron binding protein (Williams 1982). The bias for the evolution of such a bilobal structure is not known. It has been proposed earlier that the evolution of a C- lobe capable of enhancing the stability of the N- lobe is one of the factors that confer lactoferrins a higher iron binding stability than that of serotransferrins (Ward 1996). But the iron release behaviour of lactoferrins from pig, camel and goat limits the scope of such a notion.

Conclusions

1. **Isolated N- lobe of porcine lactoferrin releases iron at pH 5.5, and C- lobe at pH 5.1. Intact molecule at pH 6.1 and 5.1 respectively.**
2. **Reassociated complex (N_{Fe}-C_{Fe}) releases iron at 6.1 and 5.1, respectively.**
3. **Reassociated complex (N_{Fe}-C_{Apo}) releases iron at 5.7.**

4. **N- lobe iron binding is destabilized in intact porcine lactoferrin.**

5. **It is proposed that, as both the lobes are of basic nature, the increased proximity of the lobes together may destabilize the iron liganding environment of N- lobe.**

6. **Current study proves that the positive cooperativity between the lobes of lactoferrins is not a universal feature of lactoferrins.**