CHAPTER VI

CRYSTALLIZATION OF PORCINE LACTOFERRIN
6.1 Introduction

The crystal structures of lactoferrins from five sources have been reported so far (Protein Data Bank). Each lactoferrin structure has come up with new and exciting features, similar to the diversity in their functional properties (Khan et.al., 2001; Lonnerdal and lyer 1995). Additionally, the nature of crystallization of these lactoferrins showed many interesting patterns. For example, lactoferrins from bovine, buffalo and goat tended to be very difficult to crystallize. In sequence identity also they are very close to each other (see chapter 3).

In this context porcine lactoferrin is interesting as it is the least glycosylated of all lactoferrins, and it stands alone in the sequence identity, without any significant identity with any other lactoferrin.

6.2 Materials and Methods

6.2.1 Preparation of Iron Saturated Lactoferrin

The purified lactoferrin (10mg/ml) was equilibrated by dialysis against a solution of 0.02mM ferric chloride in 100mM sodium bicarbonate and trisodium citrate, pH 8 for 48 hours, at 4°C. The concentrated and desalted (by ultrafiltration) protein was used for the crystallization of diferric form of PLf.

6.2.2 Preparation of Apo Lactoferrin

Dialysis against 0.1M citric acid with regular changes at every 12 hours until the absorption at 465nm becomes negligible was done to demetalize diferric-PLf. The apo-protein was desalted and concentrated by ultrafiltration before crystallization. All steps were carried out at 4°C.
6.2.3 Deglycosylation

PNGase (peptide-N-glycosidase) purchased from SIGMA Chemical Company was used for deglycosylation. A previously reported method was followed (Baker HM et. al., 1994). 25 mg of protein (@10mg/ml) in 0.1 M sodium phosphate, pH 6.5 was incubated with 2mg of the enzyme PNGase for 24 hours. Following the PNGase-incubation, the protein was re-purified by ion-exchange chromatography (see section 4.1.2).

6.2.4 Primary Screening

A sparse matrix screening was done using all the proven and popular precipitants like various salts, polymers as well as organic solvents against different pH values (Jancarik and Kim 1991). The concentration of the protein used for initial screening was 70mg/ml. The purified and desalted protein was concentrated by ultrafiltration (on Amicon) or lyophilization. The samples were filtered through 0.22μm (Millipore filters) before using for crystallization. Both apo and diferric proteins were tried for crystallization. The methods employed for primary screening include both hanging drop and microdialysis. Experiments were set up at both room temperature and 4°C.

6.2.5 Optimization Screening

From the results of primary screening, promising conditions were selected, and used for further narrowing down of the crystallization conditions through grid screening by varying the pH, concentration of precipitant and protein.

6.3 Results

6.3.1 Preparation of Iron Saturated Lactoferrin

Percent saturation was monitored by the ratio of absorbance at 465nm/280nm. An absorbance value of around 0.462 was estimated to
be indicative of complete saturation (Peterson et.al., 2000). Sodium citrate has been found to be effective in preventing protein aggregation when otherwise mixed with Fe₂Cl₃. Iron saturated sample was having deep red colour.

6.3.2 Preparation of Apo Lactoferrin

With the increase in dialysis time, the diferric protein lost its reddish colour, to completion. At the completion, absorption at 465nm remained negligible and stable.

6.3.3 Deglycosylation

Fig: 4.3 shows the migration profile of native and deglycosylated forms of diferric porcine lactoferrin. A hairline-thick difference in the migration distance between the two can be seen.

6.3.4 Primary Screening

Organic solvents and polyethylene glycol 4000 were found to be more effective in inducing phase separation. As the concentration of protein used was high, samples with PEG 4000 tended to dry out. This problem was absent in the cases where ethanol, methanol and methyl pentane diene (MPD) were used as precipitants. A pH of 8 to 8.5, with the buffers Tris-HCl and HEPES were selected.

6.3.5 Optimization Screening

Further screening using ethanol, MPD and methanol, alone and in combination, against Tris-HCL and HEPES of varying pH at different protein concentrations led to the identification of a combination of ethanol and MPD in Tris-HCL, 25mM, pH 8.5 as the most responding condition.
Crystals of diferric PLf appeared after around 5 months. They were pink in colour, plate or rod like, and thin (Fig: 6.1). Attempts to diffract them failed as the crystals were highly unstable, and melted during transfer to diffractometer. The reproducibility of PLf crystallization was extremely low.

6.4 Discussion

Overall, it is evident from the time required for the crystals to appear and the uneven nature of response, that PLf is not an easily crystallizing molecule. Additionally, the crystals formed were rather flaky and thin.

The unfriendliness of PLf to crystallize deserves serious consideration as it is against certain general presumptions. Of all the reported lactoferrins, bovine, buffalo and goat lactoferrins were found to be the most difficult to crystallize. One common feature of these three is their high glycosylation content (11 to 14%) (Spik et.al., 1994). From the point of view of glycosylation PLf is the least glycosylated (3.4%) (Spik et.al., 1994). If it is glycosylation which hinders crystallization of lactoferrins, then PLf should crystallize without much difficulty. Surprisingly, PLf has disproved this notion as evident in the current study, turning out to be one of the most difficult lactoferrins, to crystallize. To check whether the glycosylation prevents (by its position if not by % content) crystallization, deglycosylated PLf has been used for crystallization experiments; this also failed to respond, prompting to think of alternate reasons retarding crystallization.

Sequence analyses of the whole molecules reveals that PLf does not form any distinct subgroup with other lactoferrins. Though seems to be minor, there exists a slightly high sequence identity with BLf, CLf and GLf (Table: 3.1); but in dendrogram it stands far away from the three (Fig: 3.3). The highest residue identity of PLf is with camel lactoferrin
Fig: 6.1. Crystals of diferric (upper) and apo (lower) porcine lactoferrin.
(72.1%); still it is far apart from camel lactoferrin in dendrogram. It has been generally found that BLf, CLf and GLf are rather difficult to crystallize. Does the slight increase in the residue identity of PLf with them indicate that it shares some critical structural features with BLf, CLf and GLf? At present, the existence of any common structural feature between BLf, CLf and GLf, retarding their crystallization is not known.

To tackle this problem, further experiments were carried out. Generally, the N-lobes of lactoferrins have a basic pl (pH 8 to 10), and C-lobes are acidic (pl around pH 5). Proteolytically generated C- lobe of PLf (see chapter 4) was found to differ remarkably from this common pattern, showing a unique pl of 7.5. PLf N- lobe has a pl of 9.8; and that of intact molecule is 9.6. Does the basic nature of both the lobes (as against the common acidic C- lobe and basic N- lobe pattern) acts as a hindrance to crystallization by interfering with the molecular aggregation and packing?

A careful re-observation of those crystallization dishes which gave diferric crystals led to the detection of a number of colourles crystals among the usual pink crystals (Fig: 6.1). They must be crystals of apo-lactoferrin. How the crystals of apo-lactoferrin appeared in a diferric sample?

Is the diferric protein loosing iron someway? This can perhaps be answered through the iron-release profile of PLf. Further experiments on the iron binding stability of PLf (intact molecule and separated lobes) as a function of pH (see chapter 5) gave intriguing but revealing results. Separated N- lobe released iron at pH 5.5, while C- lobe at pH 5.1. Intact molecule released at pH 6.1 and 5.1, successively. This is a marked deviation from the common trend of human and bovine lactoferrins (Legrand et.al., 1990) (Table: 5.1). While separated N- lobes of HLf and CLf lose iron at pH 6 and 6.3 respectively, PLf releases only at pH 5.5, indicating an increased binding stability. But the intact PLf molecule started releasing iron at pH 6.1 (as against pH 4 and pH 4.5 of HLf and
CLf respectively), followed by a second fall in the iron release profile at around pH 5.1. The first point corresponds to the release from N-lobe and the second that of C-lobe. In the case of HLf and CLf, N-lobe of intact molecule releases iron only at a lower pH (compared to the higher iron releasing pH of separated N-lobes), showing increased stability (Table: 5.1). This change in the iron release pattern of N-lobe in the intact molecule is interpreted as due to stabilizing inter-domain modulatory interactions (see chapter 5). The iron binding stability of N-lobe increases due to the positive cooperativity with C-lobe due to inter-lobe interaction (Legrand et al., 1986, Day et al., 1992). Contrary to this, in the intact PLf molecule, N-lobe releases iron at pH 6.1, 0.6 units higher than that of free N-lobe, This indicates that iron binding by N-lobe is destabilized in intact PLf. What destabilizes the N-lobe, and how does it affect crystallization?

Such a reduction in inter-lobe modulation of iron binding is possibly related to the unique basic pl of the C-lobe. An unusually basic C-lobe may negatively influence the liganding environment of N-lobe, making the iron-N-lobe complex rather less stable, leading to the release of iron even at a higher pH. Due to this destabilizing interaction, N-lobe iron binding can be less firm, causing some fraction of it to loose iron spontaneously. Such a loss will create heterogeneity in the protein sample, leading to the formation of monoferric/apo molecules among diferric molecules. This increase of conformational heterogeneity will decrease the physical purity of the sample, consequently making the system less favourable for crystallization. Moreover, the obtained crystals of ferric form may also have minor representation of monoferric forms, resulting in improper crystal growth, leading to poor stability. This proposal can account for the crystallization behaviour of porcine lactoferrin.
Conclusions

1. Diferric pig lactoferrin requires much time to crystallize, and is not reproducible. The crystals are thin and flaky.

2. Small crystals of apo-lactoferrin are also formed in the dishes of diferric protein.

3. It is not the glycosylation which prevents PLf crystallization.

4. It is proposed from this study that:-

   The difficulty of porcine lactoferrin to crystallize arises possibly due to the spontaneous iron loss from the N-lobe, whose iron binding stability is diminished by the destabilizing interaction with basic C-lobe. The partial iron loss from a single lobe will lead to conformational heterogeneity of the sample, thereby decreasing the physical purity of the protein.