Reductive release of Fe(III) from Bovine Lactoferrin by Ascorbic Acid

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Summary: The kinetics of the reduction of Fe^{3+} from bovine diferric Lactoferrin (Fe_2Lf) and monoferric *C*-terminal (Fe_C-Lf) and *N*-terminal (Lf-Fe_N) sites is investigated at pH 6.1 and 3.5 under pseudo first-order conditions using ascorbic acid (AA) as a biological reducing agent and 1, 10-Orthophenanthroline (*O*-phen) as Fe^{2+} chelator. Pseudo first-order rate constants as a function of ascorbic acid concentration are measured. Second order rate constants (k_2) for Fe_2Lf, Lf-Fe_N, Fe_C-Lf at pH 6.1 and 27 °C are 0.1527 $M^{-1}.min^{-1}$, 0.0381 $M^{-1}.min^{-1}$, 0.1381 $M^{-1}.min^{-1}$, respectively. While, at pH 3.5 and 27 °C these values are 0.1915 $M^{-1}.min^{-1}$, 0.1116 $M^{-1}.min^{-1}$, 0.4434 $M^{-1}.min^{-1}$, respectively. A linear dependence of k_{obs} on ascorbic acid concentration is suggestive of simple pseudo first-order pathway for reduction of iron under the conditions applied for all the three forms of protein. Moreover, the results show that the *C*-terminal site is more labile toward reduction by ascorbic acid than the *N*-terminal site.

Introduction

Lactoferrin is an iron binding protein that belongs to the same family as serum transferrin and binds two high spin Fe^{3+} ions with very high affinity [1]. It provides numerous benefits to the immune system, to good intestinal health, as antioxidant, protector against harmful microbes, and regulator of iron levels [2]. The protein is bilobal and each lobe contains an iron-binding site. The two sites are too distant (35 nm) interact directly. However, both the sites are similar but not chemically identical [3]. Unlike transferrin, only traces of lactoferrin are found in serum. The highest levels in humans are found in colostrums (~7 g/L) and milk (~1 g/L). It is also found in nearly all exocrine secretions that bathe the mucosal surfaces of bronchial, nasal, lachrymal, and genitourinary passages of the body. Lactoferrin is a single-chain glycoprotein with a molecular weight of about 80 kDa. It is folded into two lobes. Each lobe consists of two domains, containing single, high-affinity metal binding site which binds very tightly but reversibly, one ferric ion together with one carbonate as the synergistic anion. Each iron-binding site consists of the same set of six ligating groups: the phenolic oxygens of two tyrosines, the imidazole group of a histidine, the carboxylate group of an aspartic acid residue and two oxygens of the bidentate carbonate synergistic anion. It closely resembles transferrin although its affinity for iron is somewhat higher, allowing iron to be retained at lower pH values. The crystal structures of lactoferrin [4-8], rabbit serum transferrin [9, 10] and ovotransferrin [11-13] have been reported. However, the two sites are not chemically equivalent. They differ in many aspects, such as ESR spectra, thermodynamic stability and kinetic lability [14].

Lactoferrin is also highly basic with pI of 8–9, probably due to a unique basic region in the *N*-terminal region of the molecule that is not found in transferrin. One important consequence of this property is that lactoferrin can bind in a "pseudospecific" way to many acidic molecules, including heparin and various cell surface molecules [15].

Because of its close resemblance to transferrin, initial research on lactoferrin function was directed toward establishing functions related to its iron-binding properties viz-iron absorption, antimicrobial activity and modulation of iron metabolism during inflammation. Subsequent researches however, have revealed a large number of other possible functions, many of which do not appear to involve iron binding. The iron coordinating property of lactoferrin is central to its role as a bacteriostatic agent. Lactoferrin retains iron down to pH 2, whereas, iron is lost quantitatively from transferrin by pH 4. At physiological pH, the formation constant for the ferric complex of lactoferrin is about 260-fold greater than that of transferrin. This greater stability is attributed to differences in the relative stability of the favored conformations for the metal complex and free protein [16].

Several chemical factors may be involved in promoting iron release from transferrin. These include: chelation, reduction, protonation, disruption of the carbonate-iron stabilization interactions and possible conformational factors [17].

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Numerous studies on the kinetics of iron removal from transferrin by a variety of chelating agents have been reported [1, 18-43] but the mechanism for this reaction is still poorly understood. Iron removal process seems to be dependent on several factors, namely, the type of chelator, concentration of chelator, pH, temperature, ionic strength and concentration and type of added salts [44]. A comparison of different ligands' ability to remove iron from transferrin is given in Table-1.

Table-1: Comparison of ligand ability to remove iron from transferrin [45, 46]
$k_{obs} = k_2[L]/1+(k_2/k_{max})[L]$ for pM values only.

Competing Ligand	k_{max} ($10^{-2}min^{-1}$)	k_2 ($M^{-1}min^{-1}$)	pM
Enterobactin	**2.1**	**8.1**	**35.5**
TRENCAM	**1.5**	**10**	**27.8**
Desferrioxamine B	**~0**	**~0**	**26.6**
TRENCAM-3,2-HOPO	**2.7**	**23**	**-**
TRPN-3,2-HOPO	**3.1**	**5.9**	**24.2**
TREN-1,2,3-HOPO	**3.7**	**6.3**	**-**
TREN(Me-3,2-HOPO)	**3.2**	**16**	**26.7**
BU-O-3,4-HOPO	**7.0**	**4.5**	**20.8**
5LIO-3,2-HOPO	**8.3**	**7.2**	**-**
Deferiprone	**8.1**	**7.8**	**21.1**
TREN(Me-3,2-HOPO)$_2$(TAMmeg)$_2$	**3.0±0.6**	**15.3±1.1**	**30.9**
TREN(Me-3,2-HOPO)(TAMmeg)$_2$	**2.3±0.3**	**18.4±0.5**	**33.6**
TREN(TAMmeg)	**1.9±0.1**	**20.7±0.1**	**34.2**
5-LIO(Me-3,2-HOPO)$_2$(TAM)	**10.1±2.1**	**9.3±0.5**	**30.4**
5-LIO (TAMmeg)$_2$(TAM)	**1.4±0.1**	**12.6±0.1**	**34.4**
3,4-LI(Me-3,2-HOPO)	**8.0±3.8**	**8.5±2.4**	**25.5**
3,4-LI(Me-3,2-HOPO)$_2$(TAMmeg)	**6.0±1.6**	**9.1±0.8**	**27.3**
3,4-LI(Me-3,2-HOPO)(TAMmeg)$_2$	**3.7±0.3**	**9.7±0.1**	**28.7**
3,4-LI(TAMmeg)	**1.1±0.1**	**9.2±0.1**	**32.5**

Due to the physiological importance of these proteins, their possible roles in hydroxyl radical catalysis have been investigated. The observation that the iron-binding protein lactoferrin occurs in neutrophils has led to many studies examining the catalytic potential of this protein in Haber-Weiss-Fenton chemistry but with contradictory results [47]. Ambruso and Johnston [48] and Bannister *et al.* [49] have observed $OH^{\bullet}$ formation in the presence of lactoferrin. In contrast, Winterbourn [50] and Baldwin *et al.* [51] reported no significant $OH^{\bullet}$ production. Similar conflicting results were obtained for transferrin [52].

In this present investigation, we report the spectrophotometric studies of the kinetics of reductive release of iron from diferric (Fe_2Lf) and monoferric lactoferrin (Lf-Fe_N, Fe_C-Lf) by ascorbic acid (AA). The reduction from Fe^{3+} to Fe^{2+} presents a potential pathway of iron release. It has been reported earlier that the binding of Fe^{2+} to apotransferrin is weak and in rapid equilibrium [53, 54] therefore, the reduction process might lead to a rapid release of iron from transferrin.

Results and Discussion

Addition of AA solution to a solution of ferrilactoferrin containing *o*-phen yielded an increase in the absorbance at 510 nm, indicating the reduction of iron, its removal from protein and subsequent formation of Fe^{2+}-(*o*-phen)$_3$. The plots of $\ln|A_\infty-A_t/A_\infty-A_0|$ are linear over many half lives ~ 90% of the reaction. However, the high concentrations of AA necessary to remove iron produce an artificially high ionic strength, and ionic strength is known to play a key role in iron removal [16].

It was difficult to obtain accurate, stable infinite time values for absorbance due to the long half-lives required for 99% completion of reaction, at low concentrations of the reducing agent. Values of absorbance after ~24 hours have been considered A_∞. Rate constants for the reaction were obtained from the linear plots of the $\ln|A_\infty-A_t/A_\infty-A_0|$ vs time (R^2=0.98-0.998) [Fig. 1].

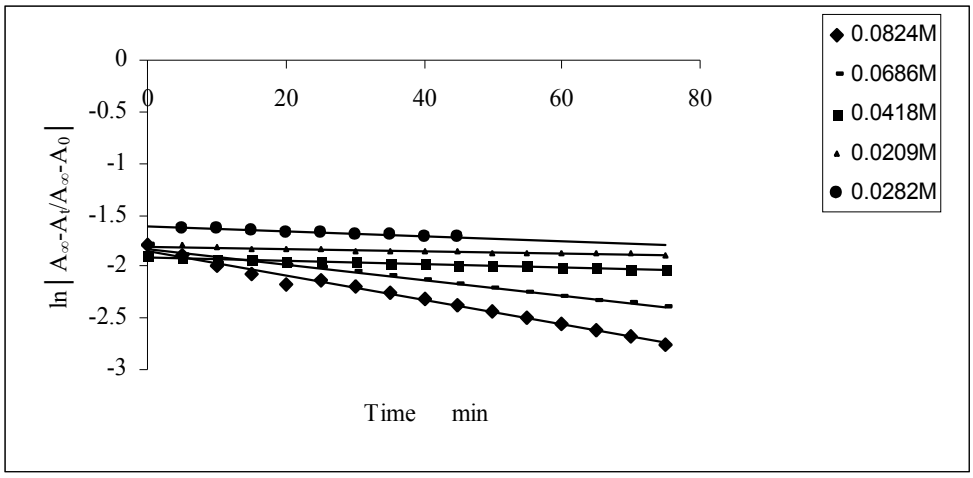

Fig. 1: Plots of $\ln|A_t-A_\infty/A_0-A_\infty|$ versus time for the reductive release of Fe(III) from Fe_2Lf by different concentrations of Ascorbic at pH 6.1 and 27 °C.
$[Fe_2Lf]$ = 3.133E-5M

The reduction of Fe^{3+} of Fe^{3+}-Tf-CO_3^{2-} to Fe^{2+}, its removal from the protein and subsequent formation of Fe^{2+}-(*o*-phen)$_3$ is depicted by the following reaction sequence:

$$Fe^{3+}\text{-Tf-}CO_3^{2-} + AA \rightleftharpoons Fe^{2+}\text{-Tf-}CO_3^{2-} + AA^-$$
$$Fe^{2+}\text{-Tf-}CO_3^{2-} \rightleftharpoons Fe^{2+} + Tf + CO_3^{2-}$$
$$Fe^{2+} + 3(o\text{-phen}) \rightleftharpoons Fe^{2+}\text{-}(o\text{-phen})_3$$

Scheme I: Reductive Release of Iron

Both lactoferrin and transferrin possess two iron atoms that are not readily reduced by physiological oxidants, probably because of stabilization of the Fe^{3+} by coordination of each iron atom to two tyrosine residues [47].

The reductive release of iron from the two terminals of lactoferrin at a preliminary stage as presented here provides some insight of the heterogeneity of the two binding sites in terms of their redox properties under the conditions applied.

Kinetic parameters for the reduction process are given in Table-2. Values of rate constants indicate that the reduction process is highly pH dependent.

Table-2: Reductive Release of Fe(III) from different forms of Lactoferrin by Ascorbic Acid.

Form of Protein	k $M^{-1}.min^{-1}$	pH	T °C
Fe_2Lf	0.1527	6.1	27
	0.2183		33
Fe_2Lf	0.1915	3.5	27
	0.2492		33
Lf-Fe_N	0.0381	6.1	27
	0.1116	3.5	
Fe_C-Lf	0.1381	6.1	27
	0.4434	3.5	

The redox potential of the transferrin-Fe^{3+}/transferrin-Fe^{2+} couple, which is proposed to be from -280 mV [55] to -400 mV(For an ionic strength near 0.1 M at 25 degrees C and pH 7.3 under 0.048 atm. CO_2), half of the iron is reduced at a potential near -0.40 V (vs SHE) [56].

Harris [55] estimated the formal reduction potential of *C*-terminal as -0.340V and that for *N*-terminal iron – transferrin -0.280V vs NHE. Assuming an outer-sphere electron transfer mechanism, Marcus theory and on the basis of this difference (60mV) one can anticipate a difference of 0.507 in the values of $\Delta\log k_{12}$, and more than a factor of 3 in the values of rate constant, for that of *N*-terminal to *C*-terminal within single specie of transferrin. Our data follows the same trend in values of the rate constant for both the monoferric forms of lactoferrin at each pH (Table-2).

The results of the reductive iron release from monoferric (Lf-Fe_N, Fe_C-Lf) and diferric (Fe_2Lf) complexes of Lactoferrin at different pH are indicative of the fact that the reduction potential of ascorbic acid at a particular pH is not the only factor, which is responsible for variation of rate constant as a function of pH. Rate constant for the reduction of Fe (III)-Lf-CO_3^{2-}changes due to the different degree of protonation of the iron binding groups that is increased at lower pH values, and consequently the iron-lactoferrin bond is weakened. It makes iron more susceptible toward reduction. Both these factors are opposite to each other and the changes in rate constants are modest. This fact is obvious from the modest increase in the rate constants for the reaction when pH was 3.5 (Table-2)

It appears from the results that *C*-terminal iron is kinetically more reducible than *N*-terminal. Even though, from consideration of the reduction potential alone as estimated by Harris, reduction of Fe bound to *N*-terminal should be faster. For this discrepancy, it can be said that apart from the larger ε° values, intrinsic reactivity of *N*-terminal is less than that of *C*-terminal. Since the rates of reaction at the pH higher than 7.0 are unmanageably very slow, we did not study the reductive release of iron from lactoferrin in alkaline condition.

In an earlier report [57] from this laboratory the same trend in rate constants was observed except that the studies were conducted on serum transferrin and conalbumin. As, the *C*-terminal iron has been observed as more reduction labile than *N*-terminal and it is already known that *N*-terminal is more acid labile than the *C*-terminal. Therefore, it is possible, in the light of proposal of Fletcher and Hehns [58] that *C*-terminal site transfers its iron to growing red cells by a possible reductive mechanism and the *N*-terminal transfer it in the liver and intestinal mucosal tissues by a mechanism favored by presence of acid.

It is attempted to make a comparison of results based on present study and the one reported by Ahmed [55], for serum transferrin. But a clear assessment could not be established due to large differences in the ionic strength. The large concentrations of ascorbic acid required to study a measurable reduction reaction produce an artificial ionic strength. It has been stated earlier in this article that the ionic strength is one of the key factors in iron removal process from the transferrin group of proteins. This effect is very obvious in the values of k_{obs}, found from lower to higher concentrations of ascorbic acid.

Kinetic study of the two terminal sites of bovine lactoferrin shows definite differences in their reactivity toward the reduction with ascorbic acid. It seems that the two sites may have functional heterogeneity in release of iron, if the release mechanism involves reduction, in biological systems.

The studies of redox reaction between Fe_2Lf and AA were conducted at two different temperatures for each pH value. The results indicate a very small change in the values of rate constants, which is indicative of small values of enthalpy of activation, characteristics of outer-sphere electron transfer mechanism [59].

Our data (Table-2) shows that the vacancy at any of the sites resists the reduction of Fe^{3+} at the other site at pH 6.1 and hence show a negative cooperativity. But, this trend is not followed at pH 3.5 where the values of rate constant for Fe_C-Lf are larger than the expected. This is attributed to low pH of the medium which effects the removal of iron from lactoferrin and reduction is not the only pathway of iron removal process.

Experimental

Reagents

Bioferrin or Apolactoferrin from bovine whey was purchased from "LIFE EXTENSION" in form of capsules and was used after further purification. For purification, contents of the capsules were mixed with *tris*-HCl (*tri*(hydroxyl-methyl)aminomethane) buffer solution (0.15 M, pH = 7.5). The suspension was then centrifuged at 4000 r.p.m for 30 minutes. The supernatant solution was separated from the residue and dialyzed extensively against two changes of 0.1 M citrate-acetate buffer (pH 4.5), followed by the dialysis against two changes of distilled water. This preparation was further dialyzed against 20 mM $NaHCO_3$ in 40 mM *tris* buffer pH 7.4 to provide HCO_3^- for binding of iron. The concentration of the apoprotein was determined through scanning on a Hewlett Packard 8452A Diode Array Spectro-photometer, distributed by OLIS-Global works, using a molar extinction coefficient of $8.85x10^4$ M^{-1} cm^{-1} at 279 nm. All other reagents were of AR Grade and were used without further purification. Distilled water was deionized by passing through cation exchange column and further through a column of Chelex-100 to make it completely iron free. Iron free water was used for the preparation of all solutions of reagents and buffers. All pH measurements were done on HANNA, HI 83141 pH meter.

Preparation of Diferric Lactoferrin

For the preparation of diferric lactoferrin the apolactoferrin at pH 7.5 was saturated to 95% of total available sites by the addition of 400 μM of $Fe(NTA)_2$ (freshly prepared)and incubated for 2-3 hours at 37 °C [60]. The resulting Solomon pink solution was loaded on a sephadex G-25 column, pre-equilibrated with *tris*-HCl buffer (pH = 7.5) and eluted with the same buffer. This process removes excess of free iron, NTA and all other small molecules. The ferritransferrin solution was stored in freezer until further use.

Loading of Iron at N-terminal Site

N-Terminal monoferric lactoferrin was prepared by 45% saturation of the total available sites, with 400 μM freshly prepared $Fe(NH_4)_2.(SO_4)_2$ $.7H_2O$ at pH 7.5 or higher and incubating for about 2 hours at 37 °C. It was then loaded on sephadex G-25 column, pre-equilibrated with the working buffer. The band of iron-lactoferrin complex, distinguished by salmon-pink color, was eluted with the working buffer of pH 7.5.

Loading of Iron at C-terminal Site

For the preparation of the iron complex of lactoferrin at the *C*-terminal site, iron is loaded by $Fe(NTA)_2$. The proteins were saturated to 45% by addition of 400 μM freshly prepared Fe $(NTA)_2$, to avoid excess iron. The solution turned red immediately and was stirred for 5 min at room temperature [61]. It was then loaded on sephadex G-25 column, pre-equilibrated with the working buffer and eluting salmon-pink band with the same working buffer.

All the protein solutions were diluted with buffer as per requirement and stored in freezer until further use.

Purity Check and Concentration Determination

The concentrations of the protein solutions were determined using the molar extinction coefficients of different forms of the proteins on the wavelength of their maximum absorbance (λ_{max}).The extinction coefficient used at λ_{max} 279 was $8.85x10^4$ $M^{-1}.cm^{-1}$ for apolactoferrin [62, 63].

Metal site concentrations were determined at λ_{max} 466 nm from ε, 2300 $M^{-1}.cm^{-1}$ for diferric lactoferrin, based on the formation of the carbonate complex [64].

The distribution of different protein species was then checked by DISC – PAGE following an already well-established method [65] except that electrophoresis apparatus used was provided by SCIE-PLAS, 2D-v100. A sample gel scan is given in Fig. 2

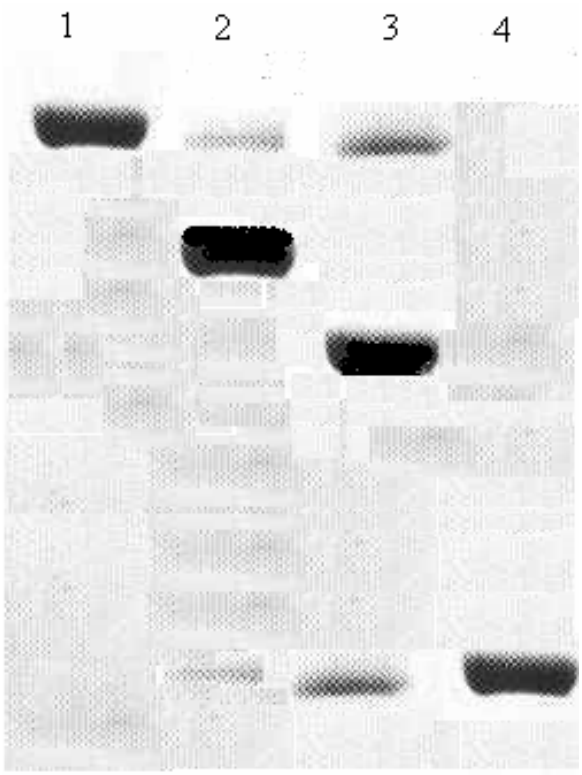

Fig. 2: A sample DISC-PAGE gel for the electrophoresis of different preparations of lactoferrin through different iron donor compounds.
Lane 1= ApoLf
Lane 2= Fe_C-Lf (loaded by $Fe(NTA)_2$)
Lane 3= Lf-Fe_N (loaded by $Fe(NH_4)_2.(SO_4)_2 .7H_2O$)
Lane 4= Fe_2Lf (loaded by $Fe(NTA)_2$)

Gels containing 3% (stacking gel), 8.0 % acrylamide were prepared from a stock solution of 30 % by weight of acrylamide and 0.8% by weight of *N*,*N*-bis-methylene acrylamide. The final concentrations in the separation gel (resolving gel) were as follows: 0.37M Tris-HCl (pH 8.8), 0.1% SDS. The gels were polymerized chemically by the addition of 0.025% by volume of tetramethyle-ethylenediamine (TEMED) and ammonium per sulphate. The stacking gels of 3% acrylamide contained 0.125M Tris-HCl (pH6.8) 0.1% SDS and were polymerized chemically in the same way as for the separating gel. The electrode buffer (pH 8.3) contained 0.025M Tris and 0.192M glycine and 0.1% SDS. The sample (0.2-0.3) contained the final concentration (final sample buffer). 0.0625 M *Tris* HCl (pH 6.8) 2% SDS, 10% glycerol, 5% 2-mercaptoethanal and 0.001% bromophenol blue as the tracking dye.

Electrophoresis was carried out with a current of 3mA per gel until the bromophenol blue marker reached the bottom of the gel (about 7 h). The proteins were fixed in the gel with 50% trichloroacetic acid (TCA) overnight, stained for 1 h at 37 °C with a 0.1% coomassie brilliant blue solution made up freshly in 50% TCA. The gels were diffusion destained by repeated washing in 7% acetic acid [65].

Preparation of Buffers

Preparation of Tris-HCl Buffer

6.057 g of *Tris*(hydroxymethyl) methyl-amine was accurately weighed out and dissolved in 700 mL of Fe-free DI water. The pH of the solution was adjusted to 7.4 by drop wise addition of 6M HCl. To this solution, about 8.766 g NaCl and 1.6802 g $NaHCO_3$ were added and volume was topped up to 1000 mL with Fe free DI water. The pH of the resulting solution was 7.4 and the ionic strength was 0.22.

Preparation of Succinic Acid-Sodium Hydroxide Buffer (0.125M, pH 6.0)

Accurately 14.7625 g of succinic acid was dissolved in 1000 mL of Fe free DI water already containing 8.7 g of NaOH. 0.84 g $NaHCO_3$ and 8.766 g NaCl were added to this solution to give the final ionic strength of 0.3.

Preparation of Formic Acid-Sodium Hydroxide Buffer (0.125M, pH 3.5)

4.9 mL of 96%, 1.22 g/mol formic acid was added to 600 mL of Fe free DI water and pH was adjusted to 3.5 by 1 M NaOH and volume was topped up to 1000 mL by Fe-free DI water. 0.84 g $NaHCO_3$ and 8.766 g NaCl were added to this solution to give the final ionic strength of 0.3.

Kinetics of Iron Removal

The iron removal reactions were followed using visible spectrophotometry. The rate of iron removal was measured by recording the increase in absorbance of Fe^{2+}-$(Opt)_3$ at 510 nm. Reactions were performed at pH 6.1 (succinate buffer) and 3.5 (formate buffer), using plastic cuvettes (4 mL total volume) with a 1 cm path length. The total volume of the reaction mixture in the cuvettes was maintained at 3.0 mL. All the cuvettes contained 1.0 mL Protein solution and different proportion of solution of Ascorbic acid, 0.1 mL of 1, 10-orthophenanthroline (used as a thermodynamic sink) and a balance of buffer solution to give a final volume of 3.0 mL. Micropipettes 10-100 μL and 100-1000 μL were used to transfer the volume to the cuvette.

All solutions were maintained at the same temperature and reactions began by rapid transfer of reductant solution with eppendorf pipettes to the protein-buffer-opt solutions followed by several inversions of the capped cell to effect mixing. Progress curves (Fig. 3) were monitored on a Hewlett Packard 8452A Diode Array Spectrophotometer, distributed by OLIS-Global works. Repetitive scans were recorded at constant time intervals.

Kinetic studies of iron removal from diferric lactoferrin were performed with a large excess of reducing agent over ferric lactoferrin and were treated as pseudo first-order reactions.

Kinetic Data Analysis

Observed pseudo first-order rate constants (k_{obs}) were obtained from the spectrophotometric progress curves by linear least-square fitting. The raw data were fit to the equation (1) and a sample plot is presented in Fig. 1

$$A_t = (A_\infty - A_0)\exp(-k_{obs}\,t) \qquad (1)$$

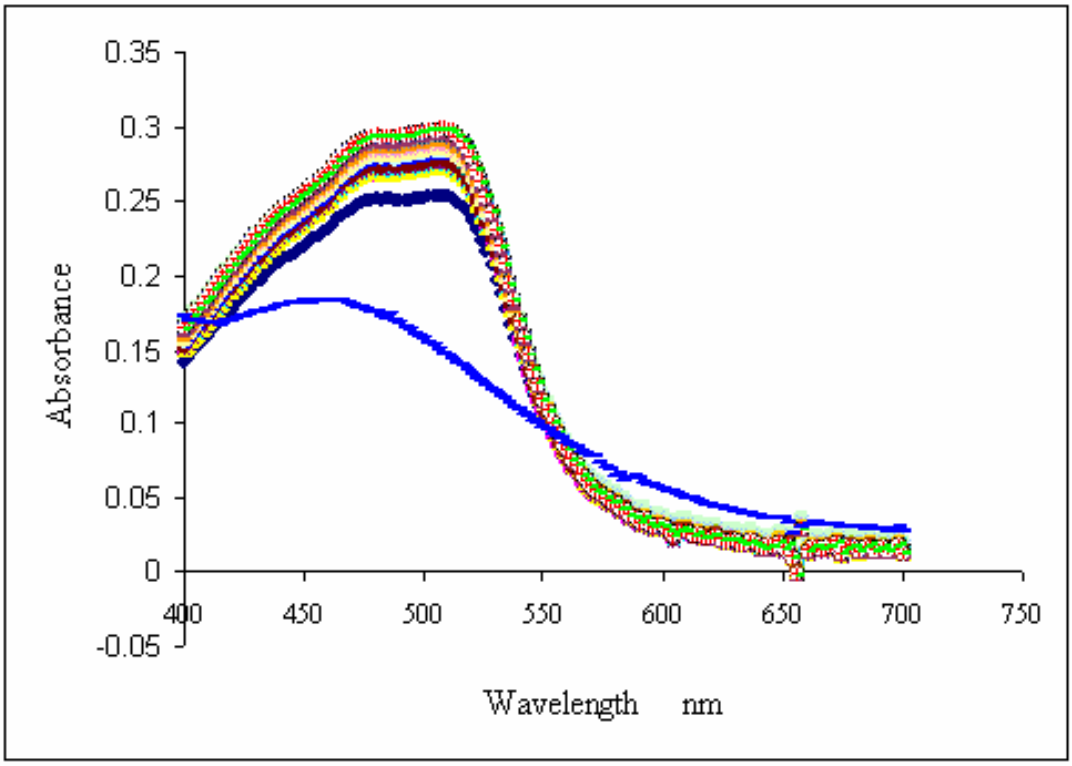

Fig. 3: Progress curve for the reaction of Diferric lactoferrin (Fe_2Lf) with AA at pH = 3.5 and T = 33 $\pm$ 0.5 $^{\circ}$C. Each scan was recorded after 5 minutes interval. The lowest spectrum is purely for Diferric lactoferrin.

In which A_t is the absorbance at time t, A_∞ is the equilibrium absorbance at the end of each reaction, and A_0 is the initial absorbance. The observed rate constants reported here are the averages of two experiments. Plots of k_{obs} versus ascorbic acid (AA) concentration were obtained for each form of protein. A linear relationship between k_{obs} and AA concentration was observed in all the cases indicating pseudo first-order dependence with respect to AA concentration (Fig. 4-7). Rate constants were calculated from the slopes of these plots. Simple pseudo first-order kinetics is governed by equation:

$$k_{obs} = k_2.[AA] \qquad (2)$$

where, [AA] is the concentration of ascorbic acid. In this model, parameter k_2 is the 2nd order rate constant under the pseudo first order conditions of ascorbic acid concentration.

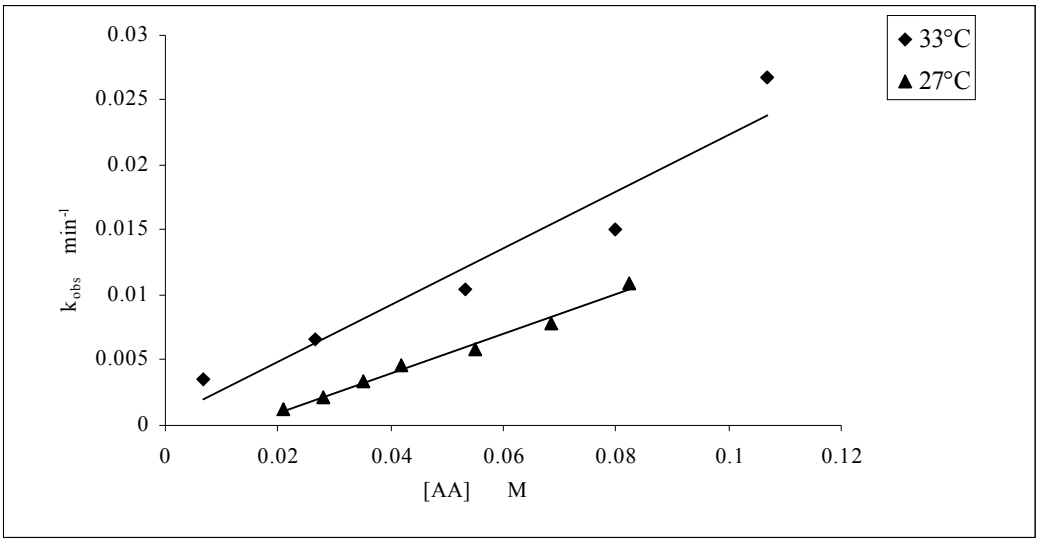

Fig. 4: Plots of the observed pseudo first-order rate constants for the reductive release of Fe(III) from Fe_2Lf versus ascorbic acid concentration.
pH = 6.1
[Fe_2Lf] = 3.133E-5M

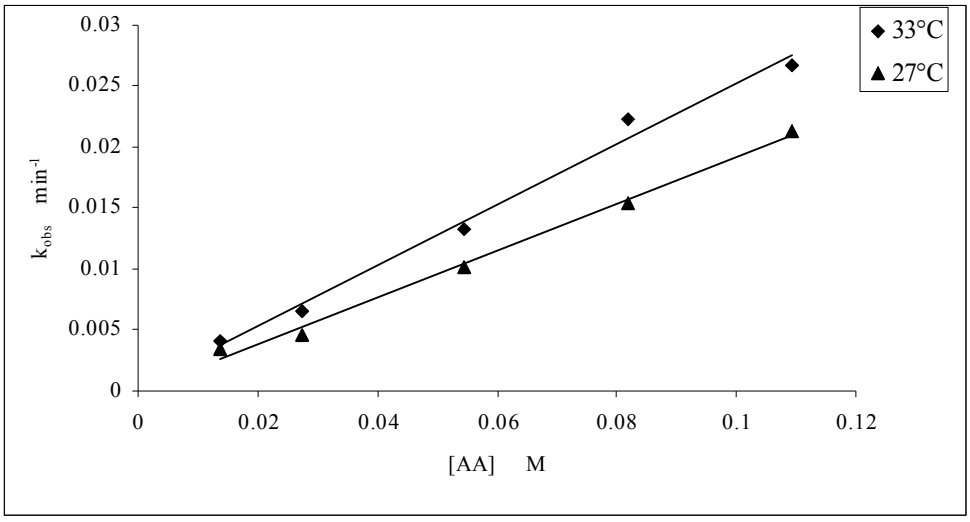

Fig.5: Plots of the observed pseudo first-order rate constants for the reductive release of Fe(III) from Fe_2Lf versus ascorbic acid concentration.
pH = 3.5
[Fe_2Lf] = 3.133E-5M

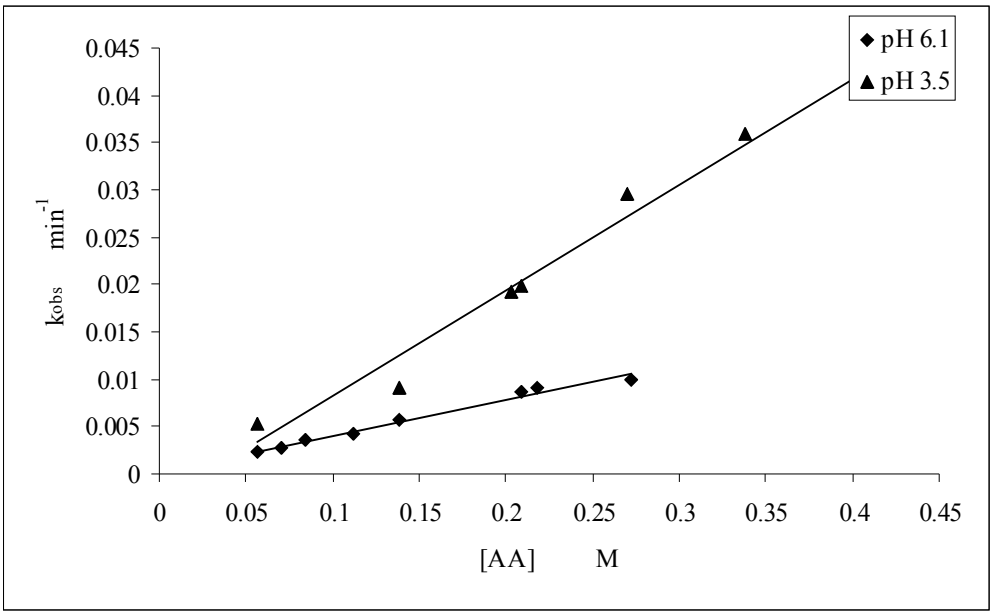

Fig. 6: Plots of the observed pseudo first-order rate constants for the reductive release of Fe(III) from Lf-Fe_N versus ascorbic acid concentration.
T = 27± 1 °C
[Fe_NLf] = 3.36E-5M

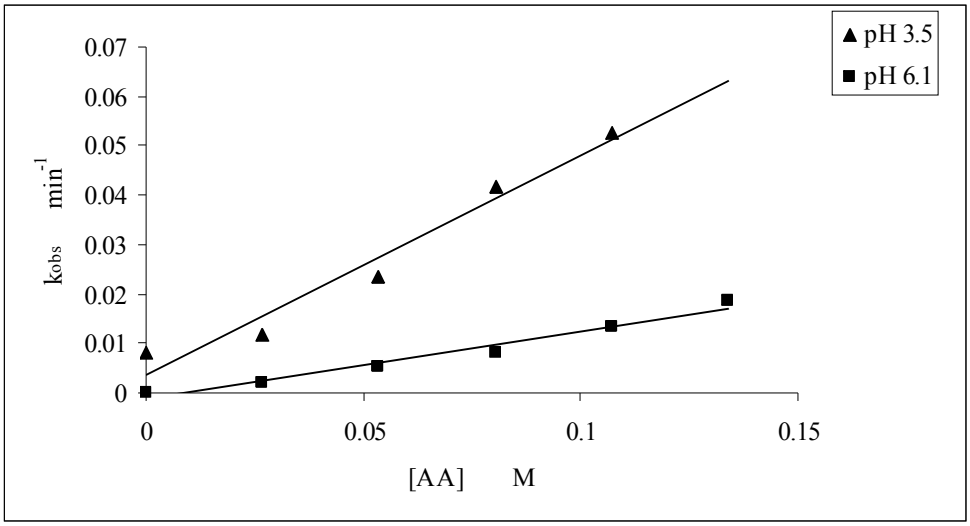

Fig. 7: Plots of the observed pseudo first-order rate constants for the reductive release of Fe(III) from Fe_C-Lf versus ascorbic acid concentration.
T = 27± 1 °C
[Fe_CLf] = 3.342E-5M

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