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Comparative Study of Kinetic Parameters for Abstraction of Fe^{3+} from Monoferric (C- and N-terminal) and Diferric Forms of Bovine Lactoferrin by Acetohydroxamic Acid

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Abstract. This study reports the kinetic parameters for the removal of Fe^{3+} from both the metal binding sites of bovine lactoferrin by acetohydroxamic acid (AHA). The values of observed rate constants (kobs) were determined for iron removal from diferric, N- and C-terminals of monoferric lactoferrin under pseudo first-order conditions of [L] at pH values 7.5, 5.0 and 3.5. A saturation behavior for the dependence of k_{obs} on ligand concentration at pH 7.5 was observed. At pH 5.0, both the monoferric forms show a saturation behavior with diferric lactoferrin following a combination of linear and saturation pathways. For a saturation pathway k_{obs} are:

$$k_{obs} = \frac{k_{max}[L]}{k_d + [L]}$$

where:

[L] is the concentration of acetohydroxamic acid, k_{max} is the rate constant for the removal of iron at plateau, k_d is ligand concentration required to reach half saturation.

While at pH 3.5, k_{obs} follows:

$k_{obs} = k_o + k''' [L]$

where:

k''' is the rate constant for iron abstraction from ferrilactoferrin through linear pathway, while k_o is the rate constant for deferration of iron from ferrilactoferrin at lower pH and is independent of [L]. Moreover, the trends in the values of Kinetic parameters clearly show the inter-conversion of one type of pathway to another under varying conditions of pH.

Keywords: lactoferrin, monoferric lactoferrin, kinetics, acetohydroxamic acid, pseudo first order, rate constant

Introduction

Lactoferrin, a member of transferrin group of proteins is known to have strong iron binding properties (Yajima *et al*., 2000; Aisen and Listowsky, 1980) and hence binds two high spin Fe^{3+} ions (Baker and Baker, 2004) through its two lobes. The metal ion binding sites in the protein are chemically different from each other (Baker and Baker, 2005; Shimazaki *et al.*, 2000). Studies have shown that at physiological pH lactoferrin binds iron with a 260 fold greater formation constant than transferrin (Baker *et al*., 1991; Aisen and Leibman, 1972) hence, keeping iron bound with it even at lower pH. The reports on the crystal structure of lactoferrin are also available (Anghel *et al*., 2018; Vogel, 2012; Baker *et al*., 2000; Haridas *et al*., 1995; Day *et al*., 1993). Kinetic lability, thermodynamic stability and ESR spectra of the N- and C-terminal of this protein are dissimilar (Li and Harris, 1998). N-lobe is primarily involved in the antimicrobial function. Conversely, C-lobe has diverse therapeutic functions, therefore, it has a potential to be used for ailments like diabetes, gastropathy and wound healing (Sharma *et al*., 2013). In addition, the bacteriostatic action of lactoferrin is

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well known and is a consequence of its iron coordinating efficiency (Hong *et al*., 2024; McCarthy and O'Callaghan, 2024; Adlerova *et al*., 2008). Hence, it has capability to retain iron down to pH 2, however, iron is lost quantitatively from transferrin by pH 4 (Chung and Raymond, 1993). This greater stability of iron-lactoferrin complex is attributable to the differences in the relative stability of the favored conformations for the free protein and its metal complex (Chung and Raymond, 1993). Consequently, a few reports on the kinetics of iron release from lactoferrin are available, so far. Moreover, iron retaining ability of lactoferrin especially at low pH has significant role in controlling levels of iron at sites of infections and inflammation, where metabolic activity of bacteria lowers the pH even below 4.5. Consequently, lactoferrin binds the iron released from transferrin. This control on free iron prevents the bacterial proliferation (Bali and Harris, 1990). As far as the kinetic studies of abstraction of iron from lactoferrin are concerned, several ligands have been known to follow a simple saturation pathway (He *et al*., 1997; Kretchmar and Raymond, 1986; Cowart *et al*., 1986; Cowart *et al*., 1982) and proceed through a rate limiting conformational change between apo and ferric lactoferrin. Crystallographic studies have confirmed this phenomenon (Qian *et al*., 2002). There are other ligands such as nitrilotriacetic acid, aminocarboxylates (Bali *et al*., 1991) and diethylenetriaminepentaacetic acid (Harris *et al*., 1992) that have a first order dependence on [L]. Moreover, another group of ligands, including a series of phosphonic acid and citrate is known to show a complex ligand concentration dependence *i.e*. saturation kinetics at low [L] and first- order at high ligand concentration (Bertini *et al*., 1998; Harris and Bao, 1997; Harris *et al*., 1992; Bali *et al.*, 1991; Bali and Harris, 1989; Harris *et al*., 1987). Acetohydroxamic acid is a bidentate model of siderophores of hydroxamate type including ferrioxamines and ferrichromes. It effectively treats urinary tract infections and is also an efficient inhibitor of bacterial urease (Griffith and Musher, 1973; Griffith *et al*., 1973). It has a strong binding capability for trivalent ions including Fe^{+3} with which it can form 1:1, 1:2 and 1:3 complexes (Schwarzenbach and Schwarzenbach, 1963). Together with the lactoferrin's bacteriostatic action and strong affinity towards iron chelation this study was designed where AHA was used to investigate and compare the kinetic parameters for abstraction of iron from both the monoferric and diferric forms of lactoferrin. The present research was conducted through spectrofluorophotometry due to its sensitivity in the detection limits of 10-100 nM concentrations.

Materials and Methods

Bioferrin or apolactoferrin from bovine which was purchased from "LIFE EXTENSION" in form of capsules and was used after further purification. For purification purpose, contents of the capsules were made to mix with Tris-HCl (tris (hydroxymethyl) aminomethane) buffer solution (0.15 M, pH=7.5). The suspension was centrifuged at 4000 r.p.m for 30 min that separated the apolactoferrin solution from the residue. Furthermore, the concentration of the apoprotein was determined through scanning on a Hewlett Packard 8452A diode array spectrophotometer, given by Olis-global works, using a molar extinction coefficient of 8.85^4/M/cm at 279 nm. All the other reagents were AR Grade and used directly without further purification. Iron free water was used throughout the work, which was made by passing distilled water through cation exchange following the chelex-100 column to make it completely iron free. For pH measurements HANNA, HI 83141 pH meter was used.

Preparation, purification and concentration determination of diferric and monoferric (N-terminal and C-terminal) lactoferrin. For the preparation and purification of these proteins already published procedures were followed (Nisar and Kazmi, 2010; Turcot *et al*., 2000). A sample Disk-page gel obtained during the purification of protein preparation is given in Fig. 1.

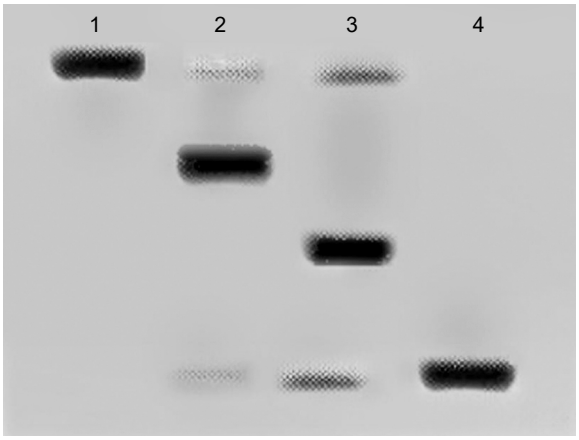

Fig. 1. A sample Disc-page gel for the electrophoresis of different preparations of lactoferrin through different iron donor compounds. Lane 1=ApoLf, Lane 2=Fe_C-Lf (loaded by Fe(NTA)2), Lane 3=Lf-Fe_N (loaded by $Fe(NH_4)_2.(SO_4)_2 .7H_2O$) and Lane 4= Fe_C-Lf- Fe_N (loaded by $Fe(NTA)_2$.

Iron removal kinetics. These studies were carried out at 25±0.5 °C and μ=0.22 M in 0.15 M Tris-HCl buffer (pH=7.5), 0.05 M acetate buffer (pH=5) and 0.05M formate buffer (pH=3.5). The increment in fluorescence intensity of apolactoferrin at 334 nm was used to determine the rate of iron removal. To monitor the progress curves, RF-5301PC (Shimadzu) spectrofluorophotometer was set with the excitation wavelength set at 280 nm and the emission wavelength at 330 nm. Removal of iron from diferric lactoferrin was studied kinetically by using chelator concentration range 67.7-333 mM. However, [L] range for monoferric type proteins was 66.8-334 mM, while the protein concentrations were fixed at [FeC-Lf] = 6.41E-5 M, [Lf-FeN] = 7.07E-5 M, [FeC-Lf- FeN] = 4.25E-5 M. The reaction was carried out in 4 cm^3 quartz cuvettes designed specifically for fluorometric research. All reactions were treated as pseudo first-order as they were routed with substantial excess of ligand over ferric lactoferrin.

Kinetic data analysis. Least-square fitting of the data yielded the observed pseudo first-order rate constants (k_{obs}) from the spectrofluorometric progress curves. The following equation was used to fit the data:

$$F_{(t)} = F_\infty-(F_\infty-F_o)\exp(-k_{obs}\, t) \quad \text{..... (1)}$$

where:

$F_{(t)}$ stands for the fluorescence intensity at time t, F_∞ represents equilibrium fluorescence intensity when each reaction ends and F_o is the initial fluorescence intensity. The fixed value of F_∞ was set based on what was observed at higher ligand concentrations. Here, the reported k_{obs} is the average of three experiments. Kinetic parameters were calculated for the conditions applied. For each type of the protein, plots of k_{obs} versus AHA concentration were generated. At pH 7.5, all three types of ferrilactoferrin showed a hyperbolic relationship between k_{obs} and AHA concentration, indicating saturation behavior with regard to AHA concentration, as shown in Fig. 2a, b and c.

Depending on the nature of various processes (whether linear, saturation or combination pathway) the rate constants were computed using nonlinear least-square fitting of the data set to the following equation:

$$k_{obs} = k_{max}\,[L]/(k_d + [L]) \quad \text{..... (2)}$$

where:

[L] is the concentration of AHA, k_{max} is the rate constant for the removal of iron at the plateau, which is achieved at higher ligand concentrations, while, k_d parameter in the above equation corresponds to that concentration of ligand, a requisite for half saturation (Haris *et al*., 2003).

$$\text{Fe-CO}_3\text{-Lf} \underset{k_1}{\overset{k_1}{\rightleftharpoons}} \text{Fe-CO}_3\text{-Lf}^*$$

$$\text{Fe-CO}_3\text{-Lf}^* + \text{L} \underset{k_2}{\overset{k_2}{\rightleftharpoons}} \text{L-Fe-CO}_3\text{-Lf}^*$$

$$\text{L-Fe-CO}_3\text{-Lf}^* \xrightarrow{k_3} \text{FeL} + \text{HCO}_3^- + \text{apoLf}$$

Scheme 1. Bates mechanism for Iron release from Ferrilactoferrin, (Fe-CO_3-Lf is the closed conformation while, Fe-CO_3-Lf* is the open conformation of the lactoferrin), scheme 1 shows the Bates mechanism for Iron release from ferrilactoferrin that facilitates the determination of k_d. Therefore, supposing he steady-state concentrations of Fe-CO_3-Lf* and L-Fe-CO_3-Lf*, k_d in this case is given as:

$$k_d = k_{-1}(k_{-2}+k_3)/k_2k_3 \quad \text{..... (3)}$$

$$k_{obs} = k_{max}\,[L]/(k_d + [L]) + k'''\,[L] \quad \text{..... (4)}$$

$$k_{obs} = k_o + k'''\,[L] \quad \text{..... (5)}$$

In these equations, k''' is the rate constant for abstraction of iron from ferrilactoferrin through linear pathway, while k_o is the rate constant for deferration of iron from ferrilactoferrin at lower pH and is independent of the concentration of AHA. Under the pseudo first-order conditions, the simple saturation kinetics is governed by equation 2, while, equation 4 signifies the combination pathway, *i.e*. combination of linear and saturation pathway. Equation 5 works when there is a simultaneous, AHA concentration independent deferration of iron from ferrilactoferrin at lower pH.

Results and Discussion

Many studies have been undertaken to explore the kinetics of iron removal in monoferric and diferric transferrin by a variety of iron chelators. The presence of different behavior *i.e*., saturation, simple first-order and a combination of first-order and saturation pathway with varying nature of ligand and other applied conditions is well recognized (Brook *et al*., 2005). There are only a few studies exploring the kinetics of removal of iron from lactoferrin. In these studies, an increase in

the fluorescence intensity at 334 nm upon mixing AHA and protein solutions indicates the removal of Fe^{3+} and transformation of protein to either monoferric or apolactoferrin. The graphs of $|\ln F_\infty - F_t/F_\infty - F_o|$ versus time (t) show linearity with only a slight curvature. However, it is well established that the high ligand concentrations necessary for iron abstraction result in an artificially high ionic strength, which plays a significant role in iron removal (Baldwin *et al*., 1981). Consequently, due to the experimental limits imposed by the iron removal from lactoferrin during this research, ample accurateness in kinetic data to resolve the biphasic rates could not be achieved (Neilands, 1995; Dewan *et al*., 1993).

Moreover, the accurate, stable infinite time values for intensity were not obtained due to the long half-lives required for 99% completion of the reaction. Hence, the fluorescence intensity after ~48 h was considered as F_∞. The slopes of the graphs between $\ln|F_\infty - F_t/F_\infty - F_o|$ and t gave the values of rate constants through linear regression (R^2=0.98-0.999). The graphs were nearly linear for ~70-80% of the reaction.

The dependence of k_{obs} on [L] for all forms of lactoferrin, at respective pH values is shown in Fig. 2(a-c), 3(a-c) and their respective kinetic parameters, calculated through equations 2, 4 and 5 are given in Table 1.

In addition, other important considerable factors are (i) the change in the protein conformation before iron removal and (ii) the binding of AHA to KISAB (kinetically significant anion binding site). The studies show that conformational change is an important primary step in the process and has been confirmed through X-ray solution scattering and X-ray crystallographic studies (Moore *et al*., 1997). Bates and co-workers, first of all, proposed the mechanism for transferrin (Fu *et al*., 2024; Carmona *et al*., 2017; Cowart *et al*., 1982), which supports the saturation component of iron release from lactoferrin as well. Anion binding to KISAB site can also induce this conformational change. Saturation of k_{obs} at higher [L], often observed, is attributed to the conformational change during the course of iron abstraction reaction, as shown in equation 2.

Appearance of saturation kinetics. As stated earlier, most of the competing ligands follow simple saturation kinetics for iron release from transferrin. Transferrin shows a very high affinity for iron and dissociation constant of this interaction is approximately 10^{22}/M that makes the iron removal process complicated (Aisen and Listowsky, 1980). Iron exchange reaction between transferrin group of proteins and ligands cannot proceed spontaneously at measurable rates (Brook *et al*., 2005). It is well established that iron removal from ferri-transferrin to AHA follows a simple saturation pathway. In this study, similar pattern of saturation kinetics with respect to concentration of AHA (Shachar *et al*., 2004; Moore *et al*., 1997) at pH 7.5 were observed for all the three forms of lactoferrin. Nevertheless, there were some deviations when $[H^+]$ and other conditions of the reaction were varied.

Scheme 1 is equally important for the iron removal process from lactoferrin and its first step is consistent with the conformational change in the Fe^{3+} complex of lactoferrin from its native, inert, 'closed' conformation ($Fe\text{-}CO_3\text{-}Lf$)to a reactive, 'open' conformation ($Fe\text{-}CO_3\text{-}Lf^*$). This change causes a rapid removal of iron by the incoming ligand. Conformational change is considered

Table 1. Kinetic parameters for removal of iron from different forms of ferrilactoferrin by AHA

Form of protein	pH	k'''/(M/min)	k_{max} (min)/(10^2)	k_d (M)	k_o(M/min)/(10^3)	SEM
Fe_C- Lf	7.5	---	10.7[a]	1.53[a]	---	0.0016
	5.0	---	1.05[a]	0.267[a]	---	0.0003
	3.5	0.115[c]	---	---	8.2[c]	0.0001
Fe_N- Lf	7.5	---	2.94[a]	0.266[a]	---	0.0006
	5.0	---	1.82[a]	0.751[a]	---	0.0002
	3.5	0.315[c]	---	---	7.4[c]	0.0001
Fe_C- Lf-Fe_N	7.5	---	3.48 [a]	0.416 [a]	---	0.0007
	5.0	1.03E-2[b]	1.1[b]	0.0026[b]	---	0.0013
	3.5	---	5.76[a]	6.00[a]	----	0.0022

[a] = calculated through equation 2; [b] = calculated through equation 4; [c] = calculated through equation 5.

as rate-limiting in the iron removal process and its significance in the studies of kinetics of iron release is widely established (Brook *et al*., 2005). This fact was further confirmed by the crystal structure studies. Some modifications in the original Bates mechanism reported presence of an allosteric anion-binding that also affects the iron removal process (Brook *et al*., 2005; Shachar *et al*., 2004; Abdallah and Chahine, 2000; Moore *et al*., 1997; Feng *et al*., 1995; Neilands, 1995; Dewan *et al*., 1993; Baldwin *et al*., 1981).

Considering the Bates mechanism k_{max}, the rate constant under the saturation conditions corresponds to the one for the conformational change (Table 1). It is the characteristics of the incoming ligand not the protein. Bates suggested this mechanism to account for the presence of saturation kinetics without the accumulation of a significant concentration of the mixed ligand (Cowart *et al*., 1983). These interpretations were on implemented data because the plots of k_{obs} *vs* [AHA] (Moore *et al*., 1997) were apparently hyperbolic in many of the

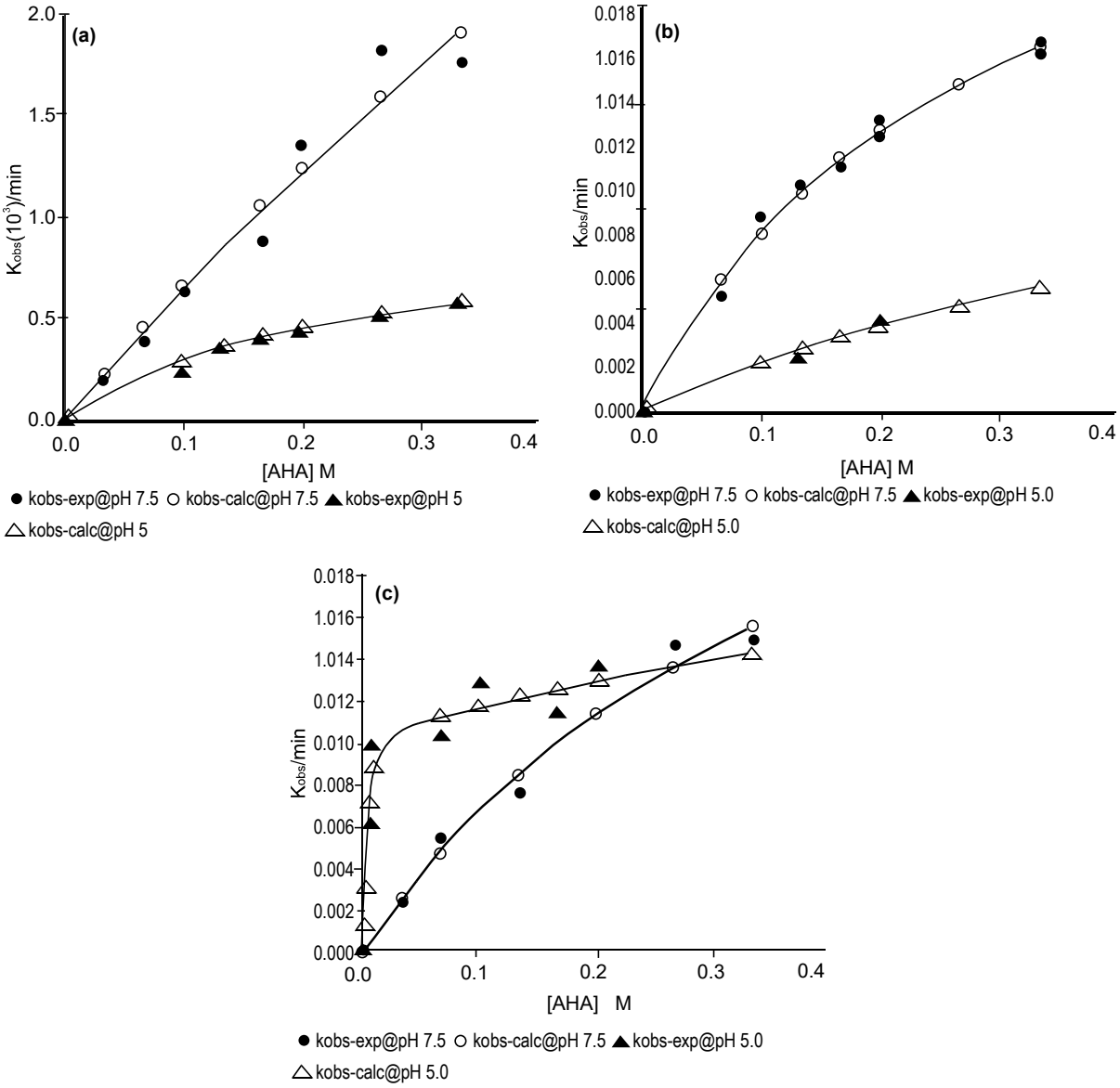

Fig. 2. Plots of the k_{obs}-calc and k_{obs}-exp at T = 25±0.5 °C, λ_{EX} = 280 nm, λ_{EM} = 334 nm for the removal of iron from **(a)** [Fe_C-Lf] = 6.41E-5 M, **(b)** [Lf-Fe_N] = 7.07E-5 M **(c)** [Fe_C-Lf-Fe_N] = 4.25E-5 M, by AHA. Symbols show experimental data while the two solid lines represent data fit to the best kinetic model.

reactions studied here. The R_2 values obtained in the range of 0.97-0.99 for the fits applied here. SEM values were also found out and are reported here in Table 1. Very small values of SEM are indicative of the good fits of the data. On the basis of the trends it is conclude in the values of k_{obs} with varying [AHA] (Shachar *et al*., 2004; Moore *et al*., 1997) that the conformational change is an essential step in the iron removal process from ferrilactoferrin (diferric and monoferric) to AHA at pH 7.5. Furthermore, some deviations are observed due to varying concentrations of H^+, the environment of the protein *i.e*., the nature of ions present in buffer solutions and are discussed further in this article.

First order component. During this study, a first order component of iron removal was also observed for few reactions. At pH 7.5, all the forms of protein *i.e*. Fe_C-Lf-Fe_N and monoferric forms (Fe_C-Lf and Lf-Fe_N) follow simple saturation kinetics with respect to [AHA] (Shachar *et al*., 2004) and no first-order path way was observed as shown in Fig. 2(a-c). At pH 5, both the monoferric forms (Fe_C-Lf and Lf-Fe_N) follow the same

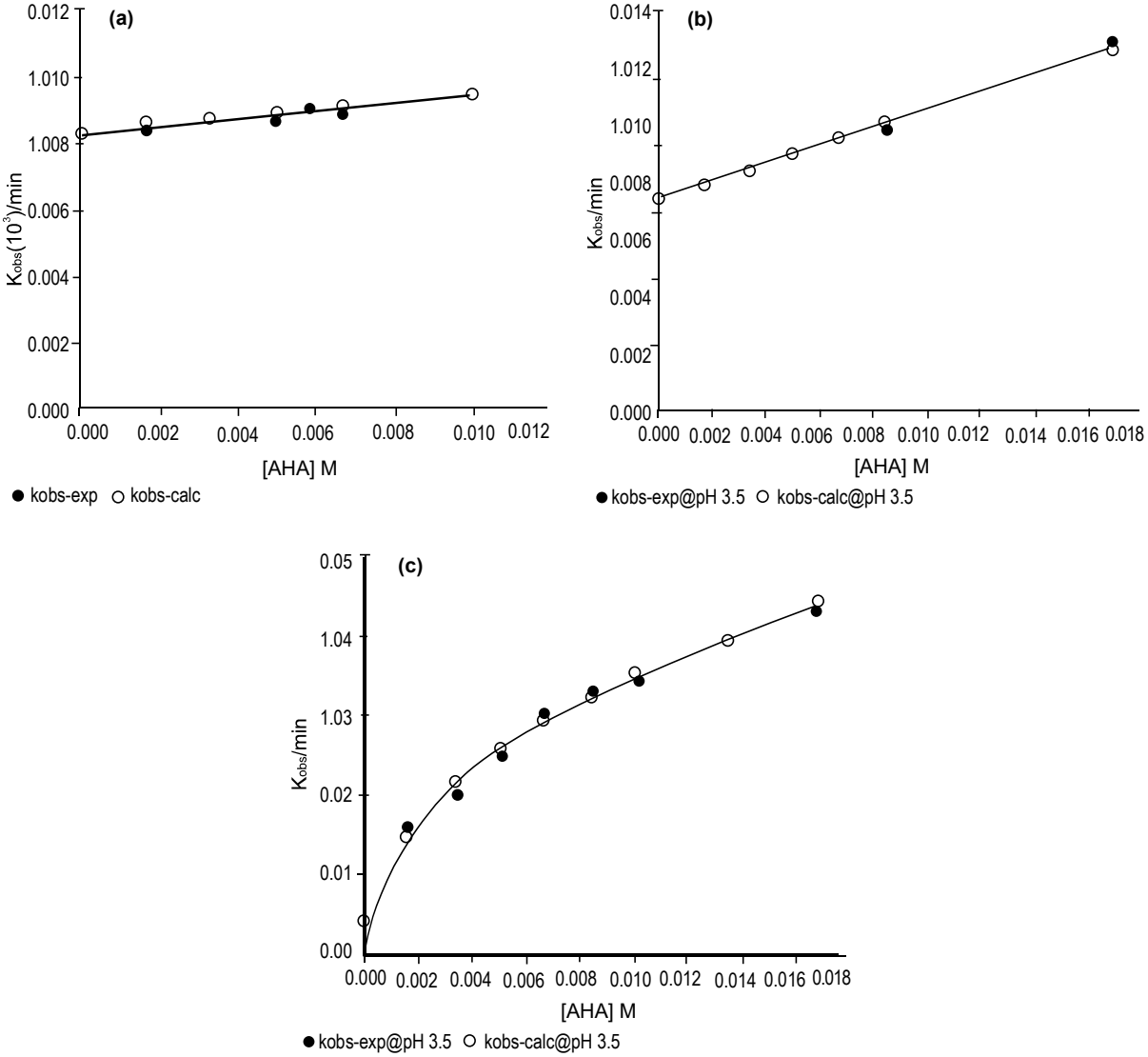

Fig. 3. Plots of the k_{obs}-calc and k_{obs}-exp at pH = 3.5, T = 25±0.5 °C, λ_{EX} = 280 nm, λ_{EM} = 334 nm for the removal of Iron from **(a)** [Fe_C-Lf] = 6.41E-5 M **(b)** [Lf-Fe_N] = 7.07E-5 M **(c)** [Fe_C-Lf- Fe_N] = 4.25E-5 M, by AHA. Symbols show experimental data while the solid line represents data fit to the best kinetic model.

pattern *i.e*. saturation pathway see Fig. 2a and 2b, while Fe_C-Lf-Fe_N shows a combination of both the first-order and saturation component for iron removal (Fig. 2c). A combination pathway, in case of Fe_C-Lf-Fe_N, describes best the fits of the obtained data.

Inclusion of 3rd parameter significantly improves the values of correlation co-efficient (R^2) from 0.77 to 0.99 and reduces the values of SEM to 0.0013. At pH 5.0 for Fe_C-Lf-Fe_N the apparent first-order rate constant k_{obs} cannot be described by simple saturation kinetics however a third parameter is introduced. Inclusion of 3rd parameter improves the R^2 value and hence confirms the presence of a first order component in the reaction according to equation 4. However, Bates mechanism does not account for the first order term. k'" is the rate parameter associated with the first-order component of iron removal and is noticeable at higher concentrations of some ligands.

One proposal about k'" is that it is correlated to the ligand's capability to displace carbonate anion from the inert, closed form of Ferrilactoferrin and avoids proceeding through the rate determining conformational change, occurring in the protein. So, some of the results at pH 5.0 (Fe_C-Lf-Fe_N, *vs* AHA) show a combination of complex kinetics while Lf-Fe_N and Fe_C-Lf show saturation behavior with respect to [AHA] (Moore *et al*., 1997).

Might expect that this linear pathway in case of Fe_C-Lf-Fe_N is due to AHA, but pKa of AHA is 9.36 at 25 °C and it is not likely to deprotonate at low pH and hence, cannot attach the metal ion as a synergistic anion. So, the possibility of involvement of AHA in first-order pathway is excluded. Another factor that needs to be considered is the $[H^+]$. That also plays an important role in release of iron from this protein.

Effect of pH. Saturation pathway is frequently observable at higher pH. Results at pH 7.5 show no metal ion release due to pH conditions of the medium. The removal of iron is totally a consequence of the interaction of the chelator with the protein. The saturation pathway signifies a major conformational change during the iron removal process as suggested by the Bates mechanism for transferrin. At pH 5.0 again the same path is followed but the values of rate constant are less than as expected due to the lowering of pH.

The iron removal process from lactoferrin is highly pH dependent and lowering the pH would cause a rapid release of iron (Feng *et al*., 1995). On the other hand, lactoferrin's ability of holding iron down to pH 2 is also known and well established (Abdallah and Chahine, 2000) pH 5.0 provides mild acidic conditions where spontaneous loss of iron due to H^+ is not very dominating and is practically too slow to observe. Hence, the removal of iron mainly takes place *via* the interaction with chelator. This fact is justified by a very low value of k_{obs} when there was no AHA present in the reaction mixture. This observation supports the idea that iron load of lactoferrin is not lost easily in mildly acidic media but takes place in acidic media where pH = 3.5 (Abdallah and Chahine, 2000). So, the bacteriostatic action of lactoferrin is highlighted here. Very small difference in the values of k_{obs} in the experimental concentration range of AHA from 0.00-0.334 M is suggestive of the fact that pH is the main trigger for iron removal from these protein forms. The data for Fe_C-Lf and Lf-Fe_N suggests that the Fe^{3+} removal process follows a linear pathway and the best R^2 values were obtained through linear fitting of the data to equation 5. Hence, it can be concluded at this stage that the linear pathway is dominating over the saturation. While, under the same conditions the saturation pathway is dominating over the linear pathway for the Fe^{3+} removal from Fe_C-Lf- Fe_N.

As stated earlier, ferrilactoferrin starts loosing Fe^{3+} quantitatively at and below pH 3.5 (Abdallah and Chahine, 2000). More $[H^+]$ triggers the weakening of Lf-Fe^{3+} bonds in the cleft due to the protonation of tyrosine and histidine ligands. Hence, the lower pH may boost the removal of Fe^{3+} from the protein through a first-order, linear pathway, which is dominating at pH 3.5.

As Histidine has a low pKa value *i.e*. 10.07 it is expected to protonate first. At mild acidic conditions such as pH

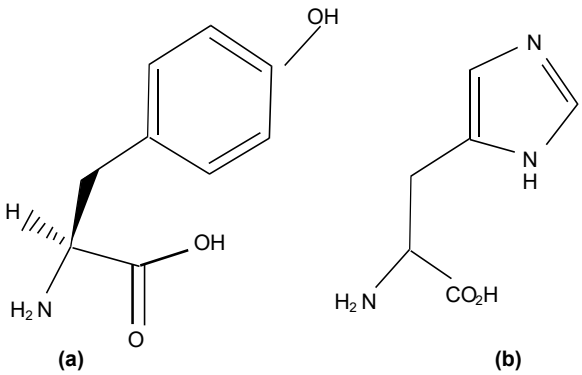

Fig. 4. Structures of **(a)** tyrosine and **(b)** histidine.

5 only this ligand is expected to protonate. At pH 3.5 both the tyrosine and histidine (pKa, 6) (Fig. 4) ligands are expected to protonate making iron removal process more facile. Hence, it is inferred here that deferration of the protein due to the pH of the medium is dominating over the ligand assisted removal of iron at low pH. The support for this proposal comes from the very small or negligible change in the values of k_{obs} within the experimental concentration range of AHA (Figs. 3a and 3b). So, it can be inferred at this stage that at pH 3.5 the deferration of holoprotein through the H^+ is more dominating and AHA has a little or no effect on iron removal. Moreover, Figs. 3a and 3b are showing the linear pathway for AHA assisted removal of Fe^{3+} and support for a linear pathway and its dominance over a saturation one comes from the decreasing values of kmax (for both the monoferric forms) with decreasing pH.

Conclusions

Lactoferrin, a well-known multifunctional glycoprotein which facilitates its several functions through its two lobes *i.e*. N-terminal and C-terminal. Although, both the lobes are structurally organized in a similar way but they comprise certain sections of unique sequences providing them significant functionalities (Sharma *et al*., 2013). The results of our study lead to conclude that under the same experimental conditions, all the three forms of ferrilactoferrin show different kinetic behavior towards the iron removal by AHA. Although, both the iron binding sites are identical, nevertheless both are chemically and thermodynamically distinct because they are located near different lobes of the molecule. Additionally, the results show that the C-terminal site is more labile towards iron removal by AHA than the N-terminal site. If AHA is given in combination with N-terminal monoferric lactoferrin to the patients with urinary tract infections, it will not disturb its iron load significantly and can be a better choice for boosting immunity. Very low values of rate constants obtained here strongly support this idea. However, as reported earlier (Baldwin *et al*., 1981) the lability of the two sites could be reversed depending upon the nature and concentration of cations and anions present. Moreover, Lactoferrin poses less sensitivity to changes in pH, and hence, is able to take up and retain iron even in the pH range of 3-4 enabling it to protect the host from pathogenic organisms in biological fluids having extremely low pH. Further studies need to done on both the monoferric forms of lactoferrin in order to apply them as a drug in future.

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Conflict of Interest. The authors declare that they have no conflict of interest.

References

Abdallah, F.B., Chahine, J.M.E.H. 2000. Transferrins: iron release from lactoferrin. *Journal of Molecular Biology*, **303:** 255-266.

Adlerova, L., Bartoskova, A., Faldyna, M.J.V.M. 2008. Lactoferrin: a review. *Veterinární Medicína*, **53:** 457-468.

Aisen, P., Listowsky, I. 1980. Iron transport and storage proteins. *Annual Review of Biochemistry*, **49:** 357-393.

Aisen, P., Leibman, A. 1972. Lactoferrin and transferrin: a comparative study. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, **257:** 314-323.

Anghel, L., Radulescu, A. Erhan, R.V. 2018. Structural aspects of human lactoferrin in the iron- binding process studied by molecular dynamics and small-angle neutron scattering. *The European Physical Journal E*, **41:** 1-7.

Baker, E.N., Baker, H.M. 2005. Lactoferrin: Molecular structure, binding properties and dynamics of lactoferrin. *Cellular and Molecular Life Sciences*, **62:** 2531-2539.

Baker, E.N., Anderson, B.F., Baker, H.M., Haridas, M., Jameson, G.B., Norris, G.E., Rumball, S.V., Smith, C.A. 1991. Structure, function and flexibility of human lactoferrin. *International Journal of Biological Macromolecules*, **13:** 122-129.

Baker, H.M., Baker, E.N. 2004. Lactoferrin and iron: structural and dynamic aspects of binding and release *Biometals*, **17:** 209-216.

Baker, H.M., Baker, C.J., Smith, C.A., Baker, E.N. 2000. Metal substitution in transferrins: specific binding of cerium (IV) revealed by the crystal structure of cerium-substituted human lactoferrin. *JBIC Journal of Biological Inorganic Chemistry*, **5:** 692-698.

Baldwin, D.A., de Sousa, D.M. 1981. The effect of salts

on the kinetics of iron release from N- Terminal and C-terminal monoferrictransferrins. *Biochemical and Biophysical Research Communications*, **99:** 1101-1107.

Bali, P.K., Harris, W.R., Nesset-Tollefson, D. 1991. Kinetics of iron removal from monoferric and cobalt-labeled monoferric human serum transferrin by nitrilotris (methylenephosphonic acid) and nitrilotriacetic acid. *Inorganic Chemistry*, **30:** 502-508.

Bali, P.K., Harris, W.R. 1990. Site-specific rate constants for iron removal from diferric transferrin by nitrilotris (methylenephosphonic acid) and pyrophosphate. *Archives of Biochemistry and Biophysics*, **281:** 251-256.

Bali, P.K., Harris, W.R. 1989. Cooperativity and heterogeneity between the two binding sites of diferric transferrin during iron removal by pyrophosphate. *Journal of American Chemical Society*, **111:** 4457-4461.

Bertini, I., Hirose, J., Luchinat, C., Messori, L., Piccioli, M., Scozzafava, A. 1998. Kinetic studies on metal removal from transferrins by pyrophosphate. Investigation on iron (III) and manganese (III) derivatives. A. *Inorganic Chemistry*, **27:** 2405-2409.

Brook, C.E., Harris, W.R., Spilling, C.D., Peng, W., Harburn, J.J., Srisung, S. 2005. Effect of ligand structure on the pathways for iron release from human serum transferrin. *Inorganic Chemistry*, **44:** 5183-5191.

Carmona, F., González, A., Sánchez, M., Gálvez, N., Cuesta, R., Capdevila, M., Dominguez-Vera, J.M. 2017. Varying iron release from transferrin and lactoferrin proteins. A laboratory experiment. *Biochemistry and Molecular Biology Education*, **45:** 521-527.

Chung, T.D., Raymond, K.N. 1993. Lactoferrin: the role of conformational changes in its iron binding and release. *Journal of the American Chemical Society*, **115:** 6765-6768.

Cowart, R.E., Swope, S., Loh, T.T., Chasteen, N.D., Bates, G.W. 1986. The exchange of Fe^{3+} between pyrophosphate and transferrin. Probing the nature of an intermediate complex with stopped flow kinetics, rapid multimixing and electron paramagnetic resonance spectroscopy. *Journal of Biological Chemistry*, **261:** 4607-4614.

Cowart, R., Kojima, N., Bates, G.W. 1982. The exchange of Fe^{3+} between acetohydroxamic acid and transferrin. Spectrophotometric evidence for a mixed ligand complex. *Journal of Biological Chemistry*, **257:** 7560-7565.

Day, C.L., Anderson, B.F., Tweedie, J.W., Baker, E.N. 1993. Structure of the recombinant N- Terminal lobe of human lactoferrin at 2· 0 Å resolution. *Journal of molecular biology*, **232:** 1084-1100.

Dewan, J.C., Mikami, B., Hirose, M., Sacchettini, J.C. 1993. Structural evidence for a pH-sensitive dilysine trigger in the hen ovotransferrin N-lobe: implications for transferrin iron release. *Biochemistry*, **32:** 11963-11968.

Feng, M., Van Der Does, L., Bantjes, A. 1995. Preparation of apolactoferrin with a very low iron saturation. *Journal of Dairy Science*, **78:** 2352-2357.

FU, J., Yang, L., TAN, D., Liu, L. 2023. Iron transport mechanism of lactoferrin and its application in food processing. *Food Science and Technology*, **43:** e121122.

Griffith, D., Musher, D., Campbell, J. 1973. Inhibition of bacterial Urease. *Investigative Urology*, **11:** 234-238.

Griffith, D., Musher, D. 1973. Prevention of infected urinary stones by urease inhibition. *Investigative Urology*, **11:** 228-233.

Haridas, M., Anderson, B.F., Baker, E.N. 1995. Structure of human diferric lactoferrin refined at 2.2 Å resolution. *Acta Crystallographica Section D: Biological Crystallography*, **51:** 629-646.

Harris, W.R., Wang, Z., Brook, C., Yang, B., Islam, A. 2003. Kinetics of metal ion exchange between citric acid and serum transferrin. *Inorganic Chemistry*, **42:** 5880-5889.

Harris, W.R., Bao, G. 1997. Kinetics of iron removal from monoferric and cobalt-labeled monoferric transferrins by ethylenediaminetetra (methylenephosphonic acid) and ethylenediaminetetraacetic acid. *Polyhedron*, **16:** 1069-1079.

Harris, W.R., Bali, P.K., Crowley, M.M. 1992. Kinetics of iron removal from monoferric and cobalt-labeled monoferric transferrins by diethylenetriaminepenta (methylenephosphonic acid) and diethylenetriaminepentaacetic acid. *Inorganic Chemistry*, **31:** 2700-2705.

Harris, W.R., Rezvani, A.B., Bali, P.K. 1987. Removal of iron from transferrin by pyrophosphate and tripodal phosphonate ligands. *Inorganic Chemistry*, **26:** 2711-2716.

He, Q-Y., Mason, A.B., Woodworth, R.C., Tam, B.M., Wadsworth, T., MacGillivray, R.T. 1997. Effects of mutations of aspartic acid 63 on the metal-

binding properties of the recombinant N-lobe of human serum transferrin. *Biochemistry*, **36:** 5522-5528.

Hong, R., Xie, A., Jiang, C., Guo, Y., Zhang, Y., Chen, J., Shen X., Li, M., Yue, X. 2024. A review of the biological activities of lactoferrin: mechanisms and potential applications. *Food and Function*, **15:** 8182-8199.

Kretchmar, S.A., Raymond, K.N. 1986. Biphasic kinetics and temperature dependence of iron removal from transferrin by 3,4-LICAMS. *Journal of the American Chemical Society*, **108:** 6212-6218.

Li, Y., Harris, W.R. 1998. Iron removal from monoferric human serum transferrins by 1,2-dimethyl-3-hydroxypyridin-4-one,1-hydroxypyridin-2-one and acetohydroxamic acid. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, **1387:** 89-102.

McCarthy, E.K., O'Callaghan, T.F. 2024. Bovine lactoferrin and its potential use as a functional ingredient for tackling the global challenge of iron deficiency. *Current Opinion in Food Science*, e101211.

Moore, S.A., Anderson, B.F., Groom, C.R., Haridas, M., Baker, E.N. 1997. Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. *Journal of Molecular Biology*, **274:** 222-236.

Neilands, J. 1995. Siderophores: structure and function of microbial iron transport compounds. *Journal of Biological Chemistry*, **270:** 26723-26726.

Nisar, S., Kazmi, S.A. 2010. Spectrofluorometric study of iron removal from bovine lactoferrin by ethylenediamminetetraacetic acid. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, **77:** 933-937.

Qian, Z.M., Li, H., Sun, H., Ho, K. 2002. Targeted drug delivery *via* the transferrin receptor-mediated endocytosis pathway. *Pharmacology Reviews*, **54:** 561-587.

Schwarzenbach, G., Schwarzenbach, K. 1963. Hydroxamatkomplexe I. Die Stabilität der Eisen (III)-Komplexe einfacher hydroxamsäuren und des ferrioxamins B. *Helvetica Chimica Acta*, **46:** 1390-1400.

Shachar, D.B., Kahana, N., Kampel, V., Warshawsky, A., Youdim, M.B. 2004. Neuroprotection by a novel brain permeable iron chelator, VK-28, against 6-hydroxydopamine lession in rats. *Neuropharmacology*, **46:** 254-263.

Sharma, S., Sinha, M., Kaushik, S., Kaur, P., Singh, T. P. 2013. C-Lobe of Lactoferrin: The Whole Story of the Half-Molecule. *Biochemistry Research International*, **2013:** 271641.

Shimazaki, K.I., Tsuda, H., Tomita, M., Kuwata, T., Perraudin, J.P. 2000. Lactoferrin: structure, function and applications. In: *Proceedings of the 4th International Conference on Lactoferrin: Structure, Function and Applications*, pp. XI:+465. Sapporo, Japan.

Turcot, I., Stintzi, A., Xu, J., Raymond, K.N. 2000. Fast biological iron chelators: kinetics of iron removal from human diferric transferrin by multidentate hydroxypyridonates. *JBIC Journal of Biological Inorganic Chemistry*, **5:** 634-641.

Vogel, H.J. 2012. Lactoferrin, a bird's eye view. *Biochemistry and Cell Biology*, **90:** 233-244.

Yajima, H., Sakajiri, T., Kikuchi, T., Morita, M. Ishii, T. 2000. Molecular modeling of serum transferrin for rationalizing the changes in its physicochemical properties induced by iron binding. Implication of the mechanism of binding to its receptor. *Journal of Protein Chemistry*, **19:** 215-223.