Implication of Methods and Techniques of Relaxation for Investigating the Iron release mechanism of Transferrin

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By

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Supervisor

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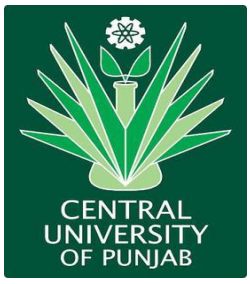

Department of Chemical Science

School of Basic and Applied Sciences

Central University of Punjab, Bathinda

May, 2018

CERTIFICATE

I declare that the project entitled "**Implication of Methods and Techniques of Relaxation for Investigating the Iron release mechanism of Transferrin"** has been prepared by me under the guidance of Dr. RAJESH KUMAR, Associate Professor, Department of Chemical Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this project has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that **Jitender Singh** has prepared his project entitled "**Implication of Methods and Techniques of Relaxation for Investigating the Iron release mechanism of Transferrin"**, for the award of M.Sc. Chemistry degree of the Central University of Punjab, Bathinda, under my guidance. He has carried out this work at the Department of Applied Agriculture, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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Abstract

“Implication of Methods and Techniques of Relaxation for Investigating the Iron release mechanism Of Transferrin’’

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Transferrins (Tfs, serum transferring (sTf), ovotransferrin (oTf), lactotransferrin(Lf)) are bilobal iron binding glycoprotein’s. In the absence of iron, the two lobs are in open conformation. Upon complex formation with iron and the protein is transformed to closed conformation and each lobs forms interdomains hydrogen bond and these are different from one transferrin to other. More over iron release from sTf and oTf take places in mildly acidic media while Lf releases iron in acidic conditions. This difference is likely due to proton assisted di-lysines triggers present in N-lobe of sTF and oTF but absent in Lf. This difference also reflected in kinetic and mechanism of iron release from TFs under mildly acidic pH conditions in the presence of physiological anions. This project discussing the implication of methods and techniques of relaxation for investigating the kinetics and mechanisms of iron release from Tfs” The comparison in kinetic and mechanistic steps of iron release from diferric-sTf, oTf and Lf showed that under mildly acidic conditions, the iron release from diferric-sTf and oTf occur in multiphasic manner while in diferric-sTf it occur it single phase.

Jitender Singh Dr. Rajesh Kumar

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Jitender Singh

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Chapter 1

Introduction

Human sTF, oTf, and Lf are bilobal glycoprotein's with very high affinity for iron. Due to high affinity for iron, these proteins not undergo iron catalyzed free radical formation. In this project, the main focus is to describe the implication of methods and techniques of relaxation for investigating the in-vitro mechanisms of iron release from TFs. This project mainly used the pH-jump method and chemical relaxation to differentiate the kinetic and mechanism of iron release from sTF, oTf, and Lf. The main focus here to identify the kinetic intermediate formed and the kinetic steps involves when diferric Tfs is submitted to rapid pH jump from physiological pH 7.4 to mildly acidic pH ($3 \leq pH \leq$ pH 5.4) in the presence of anions and absence of chelators. In addition, this project also differentiates the number of protons and anions interact with sTF, oTf, and Lf during iron release.

Chapter 2

Review of literature

At endosomal pH 5.4, sTF and oTF transport from biological fluids to cytosol by receptor-mediated endocytosis. However, due to absence of dilysines triggers in case of lactoferrin it is not able to such iron transport but it can work as iron scavenger. All the members of transferrin family (Tfs) consist of a single polypeptide chain of about 700 amino acid residues. Structurally, Tfs are bilobed (C and N) iron binding glycoproteins) and each lobe is linked by an inter-lobe chain of about ten to 12 residues. Each lobe has two domains containing four protein ligands (two tyrosines, one aspartic acid and one histidine) to which iron is coordinated.In addition, iron is also synergistically bound with carbonate anion without which the protein loses its affinity for the metal . In iron free state, the lobes are in open conformation while in iron-loaded state the two lobes in closed conformation. Upon complex formation with iron several interdomain hydrogen bonds formation occurs in each lobe.These hydrogen bonds may differ for transferrin family members . Due to differences in these interdomain hydrogen bonds and absence of proton assisted di-lysines triggers in Lf (as compared to sTf and oTf), the transferrin family members may show differences in uptake and release iron mechanism under mildly acidic condition in presence of philological anions.

Chapter 3

Results and Discussions

Several chemical reactions are occurring in ultrafast time so these can complete in less than a few seconds. This makes the measurement of rate of reaction very difficult. Under such cases, the relaxation methods can help to measure the correct rate of reaction. By using the methods and techniques of chemical relaxation, this project identifying the kinetic intermediate formed and the mechanistic kinetic steps involves when diferric Tfs is submitted to rapid pH jump from physiological pH 7.4 to mildly acidic pH (3≤ pH≤ pH 5.4) in the presence of anions and absence of chelators. In addition, this project also differentiates the number of protons and anions interact with sTF, oTf, and Lf during the iron release. When diferric Tfs solution is submitted to a fast pH jump from neutral to mildly acidic conditions pH (3≤ pH≤ pH 5.4) in the presence of physiological anions, the release of iron from diferric-Tf and oTf take place in multiphasic manner but for diferric-Lf it occurs in one phase (ref). Furthermore under these experimental conditions, iron release from diferric-Tf and oTf and Lf involve at least, five, six and three kinetically detectable steps.Earlier studies suggested that the first step is common in all member of transferrin family and which is a proton-mediated release of the synergistic carbonate anion under acidic media.This step is a necessary step for iron release and which proceed in less than 5 ms of burst phase.

$$Fe_2Tfs + nH \rightleftharpoons Fe_2sTf^* \text{ or } Fe_{NC}sTf^* + H_2CO_3$$

Since this cannot be determined accurately in stopped flow experiment, so arbitrarily *n* protons written in equation. Immediately, after synergistic carbonate anion release in acidic media, the involvement of single proton transfer occurs in case of diferric-oTf (in the range of 5 ms to 200 ms).

$$Fe_{NC}oTf^* + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe_{NC}oTf^*H$$

Under similar conditions, this step does not take in case of diferric-Tf and and Lf.

In case of iron release from diferric-Tf and oTf, the first fast phase is associated iron release from the N-lobe of diferric-Tf and oTf (≤ 100 ms) while the second phase is associated iron release from *C*-lobe of diferric-Tf and oTf. In first phase, the N-lobe of diferric-Tf and oTf involves protonation of Fe_2sTf^* or $Fe_{NC}oTf^*H$ followed by nonsynergistic anion binding (≤ 5 s).

$$Fe_{NC}sTf^*H + S \underset{k_{-1N}}{\overset{k_{1N}}{\rightleftharpoons}} Fe_{NC}sTf^*SH \quad (a)$$

$$Fe_{NC}sTf^*H + S \underset{k_{-1N}}{\overset{k_{1N}}{\rightleftharpoons}} Fe_{NC}sTf^*SH \quad (b)$$

$$Fe_{NC}oTf^*H + H^+ \rightleftharpoons Fe_{NC}oTf^*H_2 \quad (a)$$

$$Fe_{NC}oTf^*H_2 + nS \underset{k_{-1N}}{\overset{k_{1N}}{\rightleftharpoons}} Fe_{NC}oTf^*S_nH_2 \quad (b)$$

In case of diferric-Lf also, the protein involves protonation of Fe_2Lf^* followed by nonsynergistic anion binding (≤ 5 s).

$$Fe_{NC}Lf^* + 2H^+ \rightleftharpoons Fe_{NC}Lf^*H_2 \quad (a)$$

$$Fe_{NC}Lf^*H_2 + nS \underset{k_{-1N}}{\overset{k_{1N}}{\rightleftharpoons}} Fe_{NC}Lf^*S_nH_2 \quad (b)$$

In case of diferric-sTf, whether it is monoanion or dianions, one molecule of anion interacts with protein. However, in case of diferric-oTf and Lf, one molecule of dianion and two molecules of monoanions interact with proteins.Furthermore, in case of diferric-sTf and oTf one proton is gained while two protons are gained for diferric-Lf and Lf.When equation (b) is regarded as rate limiting, then the reciprocal relaxation time linked with equation (b) can be given as (refs)

$$\tau_{1N}^{-1} = k_{-1N} + \{k_{1N}\left([H^+]^l\,[S]^n\right)/K_{1H}\}$$

Here *l*= 1 for diferric-sTf and oTf and *l*= 2 for diferric-Lf. In this equation, n=1 for diferric-sTf. In case of diferric-oTf and Lf, n=1 for dianions and n=2 for monoanion.

After interactions with anions, iron is released from the $Fe_{NC}sTf^*SH$, $Fe_{NC}oTf^*S_nH_2$ and $Fe_2Lf^*S_nH_2$ intermediate by these mechanistic manner,

$$Fe_{NC}sTf^*S\,H \underset{k'_{-2N}}{\overset{k'_{2N}}{\rightleftharpoons}} Fe_{C}sTf^*S\,H + Fe \quad (c)$$

$$Fe_{C}sTf^*S\,H + 2H^+ \underset{k_{-2N}}{\overset{k_{2N}}{\rightleftharpoons}} Fe_{C}sTf^*S\,H_3 \quad (d)$$

$$Fe_{C}sTf^*SH_3 \underset{k''_{-2N}}{\overset{k''_{2N}}{\rightleftharpoons}} Fe_{C}sTf^*H_3 + S \quad (e)$$

$$Fe_{NC}oTf^{*}S_nH_2 \underset{k'_{-2N}}{\overset{k'_{2N}}{\rightleftharpoons}} Fe_{C}oTf^{*}S_nH_2 + Fe \qquad (c)$$

$$Fe_{C}oTf^{*}S_nH_2 + 2H^{+} \underset{k_{-2N}}{\overset{k_{2N}}{\rightleftharpoons}} Fe_{C}oTf^{*}S_nH_4 \qquad (d)$$

$$Fe_{C}oTf^{*}S_nH_4 \underset{k''_{-2N}}{\overset{k''_{2N}}{\rightleftharpoons}} Fe_{C}oTf^{*}H_4 + nS \qquad (e)$$

$$Fe_2Lf^{*}S_nH_2 \underset{k'_{-2N}}{\overset{k'_{2N}}{\rightleftharpoons}} Lf^{*}S_nH_2 + 2Fe \qquad (c)$$

$$Lf^{*}S_nH_2 + 2H^{+} \underset{k_{-2N}}{\overset{k_{2N}}{\rightleftharpoons}} Lf^{*}S_nH_4 \qquad (d)$$

$$Lf^{*}S_nH_4 \underset{k''_{-2N}}{\overset{k''_{2N}}{\rightleftharpoons}} Lf^{*}H_4 + nS \qquad (e)$$

If equation (d) is rate-limiting for diferric-sTf and oTf, then the reciprocal relaxation time associated with this mechanistic step can be expressed as equation (refs),

$$\tau_{2N}^{-1} = k_{-2N}([S]^n / K_{2S}) + k_{2N(obs)}[H^+]^2$$

In this equation, n=1 for diferric-sTf.In case of diferric-oTf, n=1 for dianions and n=2 for monoanion.If equation (d) is rate-limiting for diferric-Lf, then the equation that illustrates the reciprocal relaxation time for step can be expressed as equation,

$$\tau_{2}^{-1} = k_{-2}([S]^n / K_{2S}) + k_{2obs}[H^+]^2$$

These results reveal that iron release from N-lobe of diferric-sTf and oTf and Fe_2Lf*are controlled by a slow gain of two protons as shown by equation (c) to (e).

The kinetic intermediate generated after release of the iron from *N*-lobe of diferric-sTf and oTf is the intermediate that has iron only in *C*-lobe. Iron release from *C*-lobe of diferric-sTf and oTf also requires a sequence of processes that finally results in apo-protein. Iron release from the *C*-lobe of diferric-sTf and oTf take place in two kinetically detectable steps.The faster step can be treated as a relaxation process. In this step ,

the C-lobe of diferric-Tf and oTf involves protonation of $Fe_CsTf^*H_3$ or $Fe_CoTf^*H_4$ followed by nonsynergistic anion binding (≤ 15 s).

$$Fe_CsTf^*H_3 + H^+ \rightleftharpoons Fe_CsTf^*H_4 \quad (f)$$

$$Fe_CsTf^*H_4 + nS \underset{k_{-1C}}{\overset{k_{1C}}{\rightleftharpoons}} Fe_CsTf^*S_nH_4 \quad (g)$$

$$Fe_CoTf^*H_4 + H^+ \rightleftharpoons Fe_CoTf^*H_5 \quad (f)$$

$$Fe_CoTf^*H_5 + nS \underset{k_{-1C}}{\overset{k_{1C}}{\rightleftharpoons}} Fe_CoTf^*S_nH_5 \quad (g)$$

When equation (g) is regarded rate limiting, the reciprocal relaxation time linked with equation (g) can be given as (refs)

$$\tau_{3C}^{-1} = k_{-3C} + \{k_{3C}\left([H^+][S]^n\right)/K_{1H}\}$$

In final step, $Fe_CsTf^*S_nH_4$ and $Fe_CoTf^*S_nH_5$ releases iron in these manners (refs) (> 2hrs) (refs),

$$Fe_CsTf^*SH_4 \underset{k'_{-2C}}{\overset{k'_{2C}}{\rightleftharpoons}} sTf^*SH_4 + Fe \quad (h)$$

$$sTf^*SH_4 + 2H^+ \underset{k_{-2C}}{\overset{k_{2C}}{\rightleftharpoons}} oTf^*S\,H_6 \quad (i)$$

$$sTf^*SH_6 \underset{k''_{-2C}}{\overset{k''_{2C}}{\rightleftharpoons}} sTf^*H_6 + S \quad (j)$$

$$Fe_CoTf^*S_nH_5 \underset{k'_{-2C}}{\overset{k'_{2C}}{\rightleftharpoons}} oTf^*S_nH_5 + Fe \quad (h)$$

$$oTf^*S_nH_5 + 2H^+ \underset{k_{-2C}}{\overset{k_{2C}}{\rightleftharpoons}} oTf^*S_nH_7 \quad (i)$$

$$oTf^*S_nH_7 \underset{k''_{-2C}}{\overset{k''_{2C}}{\rightleftharpoons}} oTf^*H_7 + nS \quad (j)$$

When equation (i) is regarded as rate-limiting, the reciprocal relaxation time linked to equation (i) can be given as (21),

$$\tau_{2C}^{-1} = k_{-2C}([S]^n / K_{2S}) + k_{2C(obs)}[H^+]^2$$

Conclusion

This project discussed and compared the kinetic and mechanistic steps involve in iron release from diferric-sTf, oTf and Lf under mildly acidic condition in presence of philological anions by implication of method and techniques of chemical relaxation methods. Under these conditions, iron release from diferric-sTf, oTf and Lf involve at least five, six and three kinetically detectable steps. First step is the proton-assisted decarbonation of iron biding sites and which is necessary for iron release and common in all members of transferrin family. This step occurs in less than five milliseconds. After this step, the N-lobe of diferric-sTf gain one proton in less than 200 ms and this step does not occur in diferric-sTf and Lf. Under mildly acidic conditions and in presence of physiological anions, the release of iron from diferric-Tf and oTf take place in multiphasic manner but for diferric-Lf it occurs in single phase. After, proton-assisted decarbonation, the N-lobe of diferric-sTf and oTf gains a proton in a manner that is kinetically controlled by interaction with one monoanion/dianion for sTf and one dianion and two monoanions for oTf. In the similar step, the diferric-Lf gains two protons and it interacts with one dianions and two monoanion. After this step, N-lobe of diferric-sTf and oTf first release iron then it gains two protons and then release anions. In similar step, diferric-Lf first releases iron from both sites and it gains two protons and then release anions. This step is controlled by gains of two protons. After iron release from N-lobe of diferric-sTf and oTf, the kinetic intermediate generated is protein with iron loaded in *C*-site only. The C-site of sTf and oTf releases iron in similar way as the N-lobe released but relatively very slow.

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