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KALORIMETRIJSKO ODREĐIVANJE UTJECAJA SINERGIJSKOG ANIONA I SIJALINIZACIJE NA TERMODINAMIKU VEZANJA ŽELJEZA NA LJUDSKI SERUMSKI TRANSFERIN

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CALORIMETRIC STUDY OF THE EFFECT OF SYNERGISTIC ANION AND SIALYLATION ON THE THERMODYNAMICS OF THE IRON BINDING TO HUMAN SERUM TRANSFERRIN

DOCTORAL DISSERTATION

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Sažetak

Ljudski serumski transferin glavni je prenosioc željeza u organizmu. Željezo vezano na transferin doprema se iz probavnog sustava do stanica kojima je ono potrebno i otpušta s transferina promjenom pH. Transferin ima dva mjesta za vezanje željeza u kojem sudjeluje i sinergijski anion. Kod zdravih pojedinaca najčešća glikoforma transferina je glikoforma s dva biantenarna oligosaharidna lanca s ukupno četiri sijalinske kiseline, a sinergijski anion je karbonat. U *in vitro* uvjetima pokazano je kako drugi strukturno slični karboksilatni ioni poput oksalata mogu biti sinergijski anioni. Desijalinizacija transferina primijećena je kod pacijenata s dijagnozom kroničnog alkoholizma, ali i raznih upalnih bolesti te sepse, dok su povećane serumske koncentracije oksalata često rezultat hiperoksalurije ili gastrointestinalnih bolesti. Cilj ovog doktorskog rada bio je odrediti termodinamičke parametre vezanja željeza u formi FeNTA na transferin s različitim indeksom sijalinizacije, u prisutnosti karbonata i oksalata kao sinergijskih aniona. U mjerenjima sa sijaliniziranim transferinom korišten je komercijalno dostupan nativni serumski transferin, dok je desijalinizirani transferin pripremljen djelovanjem enzima neuraminidaze u *in vitro* uvjetima. Nakon što je desijalinizacija proteina potvrđena i udio sijalinskih kiselina je smanjen za oko 99%, termodinamički parametri određeni su korištenjem izotermne titracijske kalorimetrije. Potvrđeno je kako se dva vezna mjesta razlikuju kinetički i termodinamički te je otkriveno kako desijalinizacija utječe na termodinamičke parametre vezanja željeza u formi FeNTA na transferin tako da efektivne entalpije vezanja postaju egzotermije, a u prisutnosti karbonata pri koncentracijama koje odgovaraj onima u serumu, konstanta vezanja se povećava. Navedeno ide u prilog mogućoj povezanosti desijalinizacije i sekvestracijskog odgovora organizma na infekcije patogenom. Osim desijalinizacije, i oksalat kao sinergijski anion u *in vitro* uvjetima povećava konstantu vezanja željeza na transferin, a vezanje je egzergonije. Uz jače vezanje oksalata na transferin, navedeno daje osnovu za mogući nastanak ternarnog kompleksa Fe-oksalat-Tf u *in vivo* uvjetima, pogotovo u stanjima s povećanim koncentracijama oksalata.

Ključne riječi: ljudski serumski transferin, sijalinska kiselina, sinergijski anion, glikoproteomika, izotermna titracijska kalorimetrija, termodinamika

Summary

Introduction

Human serum transferrin is the major iron transport protein in human organisms. Iron bound to transferrin is transported from the digestive system to cells that require iron. A change in the pH causes the release of iron from transferrin. Transferrin has two iron binding sites that require the presence of the synergistic anion located on the C- and N-lobe of the protein. It is a highly glycosylated protein with two potential sites for N-glycans located on Asn-413 and Asn-611 residues of the C-lobe. Different combinations of the two N-glycans result in a range of asialo to hexasialo variants of human serum transferrin. The most common glycoform of transferrin in healthy humans consists of two biantennary oligosaccharide chains that together contain four sialic acids. Many pathological conditions are correlated with the glycan composition of glycoproteins. Diseases such as chronic alcoholic disease, inflammatory diseases and sepsis can increase the amount of desialylated transferrin isoforms. Also, the sequestration of the free iron is an antimicrobial response of the organism, and human serum transferrin has the main role in sequestration of the iron in serum. There is an assumption for a linkage between the sialylation and iron sequestration in pathological conditions. Sialic acids are negatively charged at physiological pH and may affect protein conformation and oligomerization, as well as protein interactions with other proteins and the extracellular matrix.

The usual synergistic anion in serum is carbonate, but other structurally similar carboxylic ions such as oxalate have shown to act as synergistic anions *in vitro*. Diseases such as hyperoxaluria or gastrointestinal diseases can lead to higher serum oxalate levels. In recent decades, isothermal titration calorimetry (ITC) has emerged as the most direct method for measuring the heat change in complexation reactions at constant temperature. ITC is applicable to many biological systems, and calorimetric studies have been performed on the binding of iron in the form of an iron nitrilotriacetic acid complex (FeNTA) to transferrin. It is shown that the two sites are thermodynamically and kinetically distinct and exhibit domain interactions. The objective of this study was to determine possible differences in the thermodynamics of iron binding to native sialylated transferrin (Tf+s) and enzymatically desialylated transferrin (Tf-s) both in the presence of carbonate and oxalate as synergistic anions using ITC.

Materials and methods

Sialylated human serum apotransferrin used in this study was commercially available, and desialylated apotransferrin (Tf-s) was prepared by incubating the buffered solution of native human serum apotransferrin in SialEXO® columns containing immobilized sialidase enzyme at room temperature. To verify the results of enzymatic desialylation, the native and desialylated hTf samples were analyzed using chromatofocusing. The separation of transferrin sialoforms was performed using pH gradient ion exchange chromatography buffers. After separation, the pH of each fraction containing the eluted protein was measured, corresponding to the approximate isoelectric point (pI) of the protein. To fully describe the glycan composition of the samples, the Tf+s and Tf-s samples were analyzed by UHPLC. N-glycans were assigned based on known retention times and results of the previous UHPLC-ESI-qTOF-MS analysis. Finally, the content of individual glycans was determined.

Once the desialylation was confirmed and resulted with satisfactory amount of reduced sialic acid content, working solutions for the ITC experiments were prepared independently for each ITC titration. Each test experiment required 0.2 mM apoTf and 4 mM FeNTA working solutions, while control experiments also required a working buffer solution. The buffer used in the titrations was either 0.1 M HEPES, pH 7.4, with 25 mM $NaHCO_3$ or 0.1 M HEPES, pH 7.4, with 25 mM $K_2C_2O_4$. ITC titrations of Tf+s and Tf-s with FeNTA in the presence of carbonate or oxalate were performed on the PEAQ-ITC MicroCal calorimeter. Titrations were performed at 25 °C in 30 injections with an added volume of 0.9 µL per injection. The interval between injections was 300 s. Titrations for all four combinations with two different transferrin sialoforms (Tf+s and Tf-s) and two synergistic anions were repeated three or more times. Only the control experiment with FeNTA added to the cell containing buffer without the protein resulted in a significant heat change. The injection peaks in all titrations and their control experiments were integrated and the *point-to-point* method was used to subtract the control experiments from the test experiments.

Data were analyzed using a model for two non-identical binding sites available in MicroCal PEAQ-ITC Analysis Software (version 1.30) and by refining a total of 7 parameters. The statistical significance of the differences was evaluated using two-way and one-way ANOVA. To account for the possible effects of the observed heterogeneous variances, an additional analysis was performed based on the more robust Welch-James statistic with approximate degrees of freedom.

Results

The most abundant glycoforms in Tf+s were biantennary sialylated glycoforms, which accounted for more than 70% of the total glycans. Conversely, Tf-s contained more than 90% biantennary glycoforms without sialic acid. The sialylation index values for Tf+s and Tf-s confirmed the 99% reduction in sialic acid content for the enzymatically desialylated protein. The results of pH chromatofocusing also confirmed successful desialylation, resulting in the expected difference in pI values of Tf+s (pI ≈ 5.4) and Tf-s (pI ≈ 6.8).

Calorimetric titrations of different transferrin sialoforms and different synergistic anions yielded peak profiles similar to those previously reported. It has been shown that there are 2 phases in the binding of FeNTA to transferrin: (i) a fast phase called contact binding caused by the weak and reversible binding of FeNTA to the binding site, and (ii) a slow phase due to the incorporation of the synergistic anion into the binding site and the replacement of NTA. The first series of injections corresponds to the saturation of the C-site of transferrin and shows a fast exothermic process followed by a slow exothermic process, while the second series of injections corresponds to the saturation of the N-site and shows a fast exothermic process followed by a slow endothermic process. The binding enthalpies in this study refer to the apparent binding enthalpies because the experiments were performed in one buffer only and the ionization enthalpy of the buffer was not subtracted from the apparent binding enthalpies. Therefore, the calculated thermodynamic parameters reflect not only the binding of the iron(III) ion to apoTf, but the overall process: the contact binding of FeNTA to apoTf, the removal of NTA from the FeNTA complex and its protonation, the binding of the synergistic anion, and the exchange of protons with the buffer.

Desialylation resulted in more exothermic binding of iron(III) to both sites, regardless of the synergistic anion. The binding entropies indicate more positive values for the N-lobe, regardless of sialylation or synergistic anion. Desialylation apparently has no effect on the binding entropy, but the effect of the synergistic anion is twofold: in the presence of oxalate, more positive values were observed for the C-site, while the same difference is much less pronounced for the N-site. Desialylation was found to increase the binding of the first iron(III) ion by about 10-fold in the presence of carbonate, while the effect on the binding of the second iron(III) ion was about 20-fold. Conversely, desialylation had virtually no effect on the overall saturation of transferrin in the presence of oxalate. Binding to the C-site is driven primarily by the enthalpy contribution ($\Delta_r H°$), whereas binding to the N-site is driven primarily by the entropy contribution ($-T\Delta_r S°$). The analysis of heat change rates using the proposed parameter

weighted width (l_x) confirmed a stronger prebinding of oxalate to the C-site of hTf compared to carbonate. This translates into the faster contact binding on the C-site in the presence of oxalate. Conversely, the reaction for the N-site was faster in the presence of carbonate for both Tf+s and Tf-s, which could explain the observed preferential loading of the transferrin N-site *in vivo*, despite the higher iron-binding affinity of the C-site.

Conclusion

Native or desialylated human serum transferrin binds iron(III) in the form of FeNTA in a consecutive manner with the C-site being saturated first and both carbonate and oxalate acting as synergistic anions. Lower sialylation index of transferrin (i.e., a lower sialic acid content) leads to more exothermic apparent binding enthalpies for both lobes, regardless of the synergistic anion. Furthermore, in the presence of carbonate at concentrations corresponding to those in human serum, desialylation increases the binding constant. These findings are in support of the possible connection between desialylation and sequestration as an organism's response to pathogen infections. In addition to desialylation, oxalate as a synergistic anion in *in vitro* conditions also increases the binding constant of iron to transferrin, and the binding becomes more exergonic. Together with the stronger prebinding constants of oxalate to human serum transferrin, the above provides the basis for the possible formation of the Fe-oxalate-Tf ternary complex in *in vivo* conditions, especially in conditions with increased concentration of oxalate.

Keywords

human serum transferrin, sialic acid, synergistic anion, glycoproteomics, isothermal titration calorimetry, thermodynamics