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PREPARATION AND MODELLING OF THE STRUCTURE OF TRANSFERRIN-Fe^{3+}-AZIRIDINE-CARBOXYLATE COMPLEX

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ABSTRACT. The absorption of the iron ions by the cells is realised through a transport protein, the transferrin, via receptor mediated endocytosis. The presence of a synergistic anion (which *in vivo* is the bicarbonate) is crucial for stabilizing the iron-binding site of human serum-transferrin. This efficient cellular uptake pathway has been exploited for the site-specific delivery of anticancer drugs. We substituted bicarbonate with another synergistic anion, the aziridine carboxylate, which possesses cytotoxic effect against the cancer cells. Using in silico methods we determined, that this substitution has no significant change in the polypeptide folding or domain orientation in the structure of human serum transferrin.

Keywords: *human serum transferrin, iron uptake, synergistic anion, in silico method*

INTRODUCTION

Human serum transferrin is a glycoprotein, member of the transferrins family, which are an important class of iron-binding proteins that are widely distributed in the physiological fluid of vertebrates. The primary role of serum transferrin is therefore to bind, transport and release of the iron to supply the growing cells. The most important role of the iron is in the newly synthesised hemoglobin synthesis in new blood cells [1]. Serum transferrin is mainly synthesized by hepatocytes in liver in a concentration of 2.5 mg/ml, but only 30% is saturated with iron in the plasma [2].

The human serum transferrin contains 678 amino acid residues and, including the two asparagine-linked glycans, has an overall molecular weight of ~79,550 kDa. This protein is divided into two evolutionary related lobes, in

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which 40% of the residues are identical. The N-lobe consist from amino acid residues ranging from1 to 336 and C-lobe consist from residues ranging from 337 to 678 [3], being linked by a short spacer sequence (figure 1).

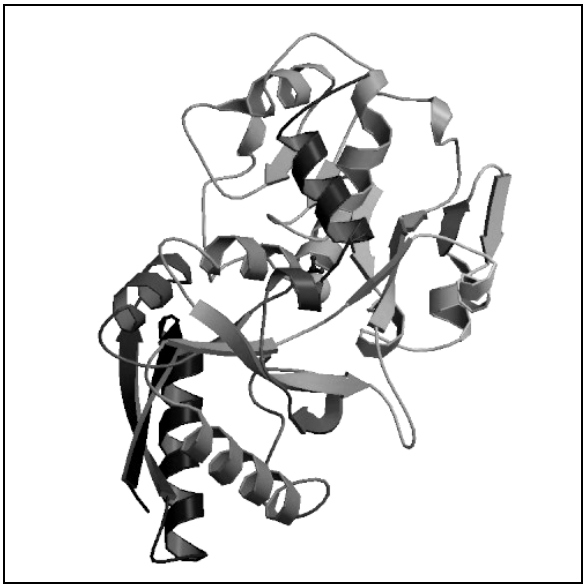


Figure 1. The X-ray cristal structure of N-termianl lobe of human serum transferrin [Protein Data Bank-1A8E (http://www.rcsb.org/pdb/)]

Each lobe contains two domains comprising a series of α-helices, which overlay a central β-sheet backbone, forming a deep, hydrophilic metal ion-binding site (Figure 2). The binding site in both N- and C-terminal lobes has four conserved amino acids including two tyrosine, one aspartic acid and one histidine (N-terminal lobe – Asp-63, Tyr-95, Tyr-188 and His-249) [4]. Iron is bound in a distorted octahedral coordination involving four amino acid ligands and two oxygen atoms donated by a carbonate molecule to stabilize the iron atom [5]. The synergistic relationship of metal and anion refers to the fact that neither binds tightly in the absence of the other.

When iron is released, the two domains of each lobe, termed the NI- and NII domains and the CI- and CII-domains, rotate around a hinge to change the protein conformation from „closed" to "open". While the iron ligands play a primary role in iron binding, other residues (including Gly-65, Glu-83, Tyr-85, Arg-124, Lys-206, Ser-248 and Lys-296 in the transferrin N-lobe) make up a second shell network that also contributes to the stabilization of the iron binding site [4, 6].

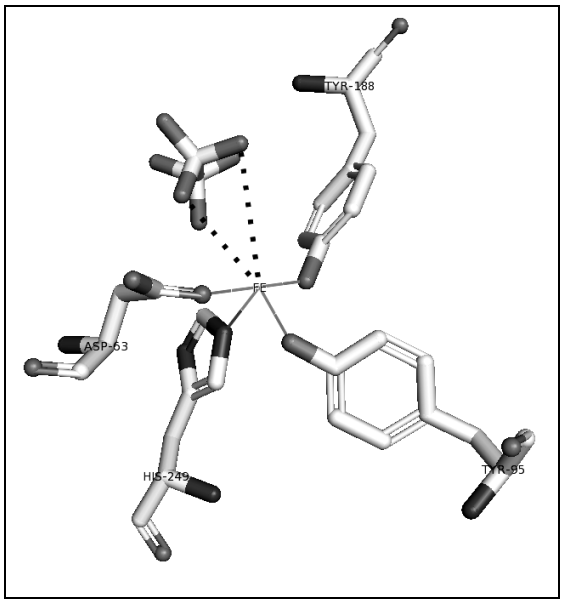


Figure 2. The iron binding site of N-lobe of human serum transferrin

The absorption of transferrin-coupled iron ions by the cells is through the transferrin-transferrin receptor system, via receptor mediated endocytosis. Transferrin has capability to bind *in vitro* several other metals (ex. Ga^{3+} In^{3+}, Bi^{3+}, Ti^{4+}, Ru^{3+}) expect iron ions [7-13] or it have possibility to replace bicarbonate synergistic anions to another.

In the literature are described many transferrin complexes formatted with different type of anions: inorganic anions (sulphate, sulphite, nitrate, nitrite, phosphate, borate), aldehydes and ketones (dihydroxyacetone, glyceraldehyde), monocarboxylic acids (acetate, propionate) and several monocarboxylic acids with proximal aldehyde, ketone, alcohol- (glycolate, glioxylate), amino- (glycine, phenylalanine), or thiol functional groups (thioglycolate), dicarbxylic acids (oxalate, malonate) [2, 14-17].

Our aim is to replace the bicarbonate anion with aziridine-carboxylate at the iron binding of human apo-Tf.

Aziridine is a three-membered heterocycle with one amine group and two methylene groups. This functional group is content of a series natural and synthetic organic compound. The simple natural alkaloid, aziridine also called ethyleneimine was detected in various foodstuffs including baker's yeast (*Sacharomices cerevisiae*). Aziridine-2-carboxylic acid (Figure 3.), a metabolite, is isolated from scaly wood mushroom (*Agaricus silvaticus*) [18, 19].

In the literature there are described the crystal structures of the transferrin complexes formed with different anions, ex. oxalate or bicarbonate, but there is no information about binding geometry of aziridine-carboxylate to transferrin's binding site, this anion was newer complexed or conjugated with Tf. Therefore, we realized a molecular model using a docking program (AutoDock Vina) to predict the possibility of using aziridine-carboxylate as synergistic anion at iron binding of transferrin.

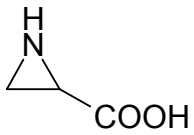


Figure 3. Chemical structure of aziridine-2-carboxilic acid

Based on these findings the Tf-Fe(III)-aziridine-carboxylate complex was prepared. The complexation reaction was analysed using high performance capillary electrophoresis techniques: capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF).

RESULTS AND DISCUSSION

There are data in the literature regarding to the binding of oxalate and bicarbonate to the transferrin, however there is no information about the binding of aziridine-carboxilate to the protein. Therefore, in order to verify the ability of transferrin to bind the aziridine-carboxilate anion we used molecular docking technique. For this purpose we used AutoDock Vina. The results were visualised in PyMol.

In Figure 4 is presented the obtained bond lengths and binding geometry. The distances obtained from the model are compared with crystallographic data from the literature regarding the oxalate and bicarbonate anions binding to the transferrin and are presented in Table 1.

Table 1. Bond distances in metal and anion sites.

Anion bond	Distance (Å)
Fe-carbonate1 O1	2.06
Fe-carbonate1 O2	2.24
Fe-carbonate2 O1	1.99
Fe-carbonate2 O2	2.42
Fe-oxalate O1	2.05
Fe-oxalate O2	2.16
Fe-aziridine-carboxylate O1	2.6
Fe-aziridine-carboxylate O2	2.7

The crystallographic data and kinetic analysis show that the oxalate in the oxalate complex to the iron is bond more tightly than in the case of bicarbonate complex [16]. Our results show that the aziridine-carboxylate is the most loosely bound by transferrin from the anions. This result was however expected taking into account the slightly larger size of the anion. This loose bond can have the advantage of ease of release of the anion and the iron once reached inside the cell.

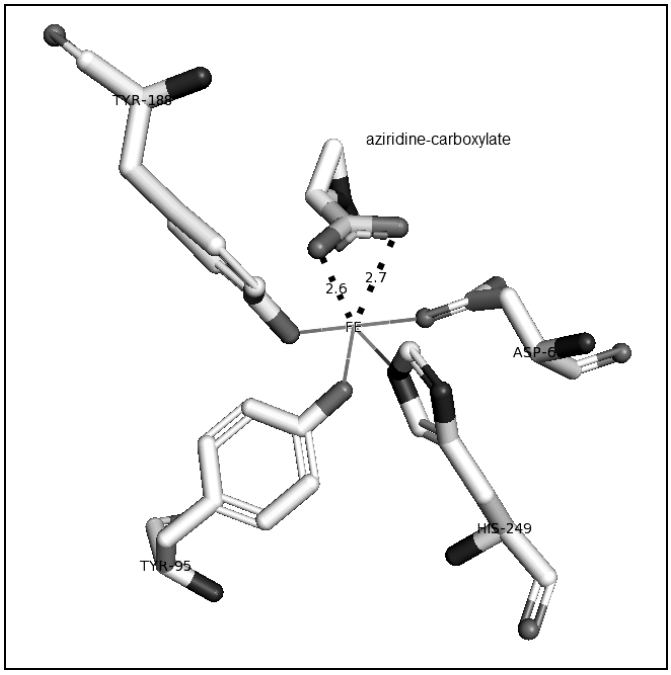
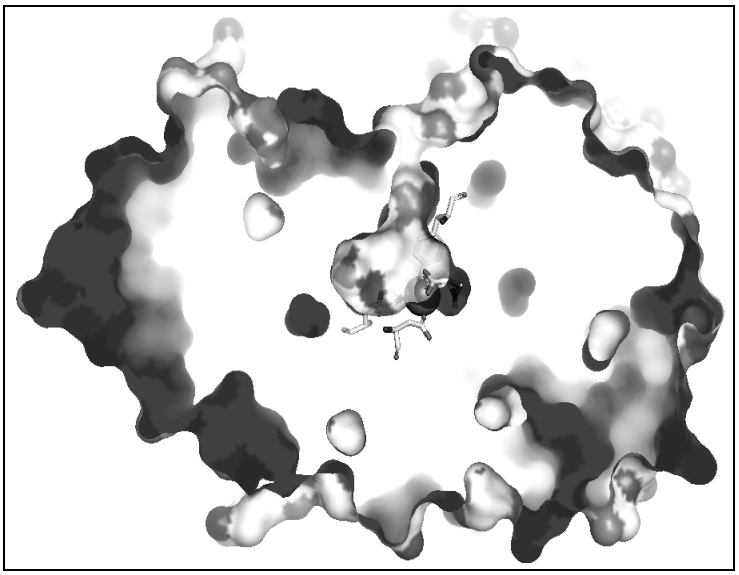


Figure 4. The geometry of the binding of the aziridine-carboxylate to the holo-transferrin's N-lobe (1A8E) trough iron (theoretical model). The distances between the anion and the iron is expressed in Å. The model was realized with AutoDock Vina, and visualized by PyMol, represented as sticks.

In Figure 5-a, b are shown the 3D structure of the iron complexed aziridine-carboxylate human serum transferrin. Substitution of carbonate to another synergistic anion, such as aziridine-carboxylate produces no significant changes in polypeptide folding or domain orientation in human serum transferrin as revealed by our structure.

Based on these findings we propose that aziridine-carboxylate binds in a symmetrical bidentate fashion, in the same manner as the oxalate. The overall results of this molecular docking study and the data from the literature support the synthesis of transferrin-iron-aziridine-carboxylate complex.



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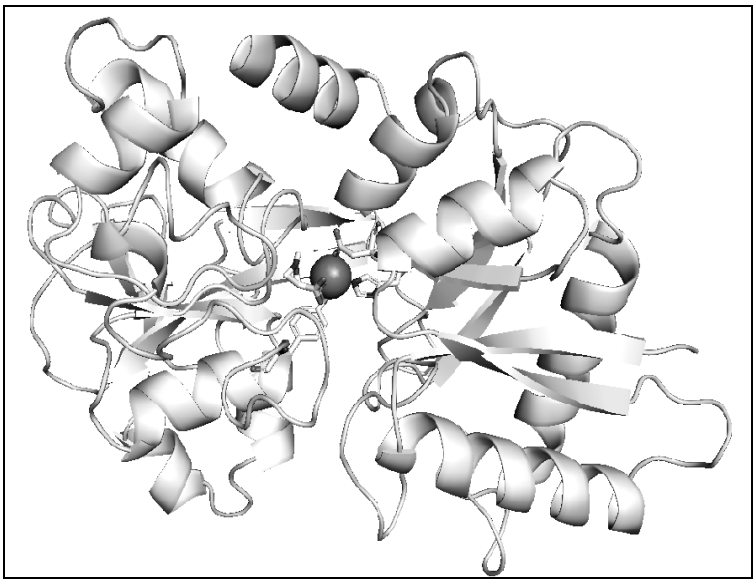

B

Figure 5. Theoretical model of the binding of aziridine-carboxylate to the active site of the N-lobe holo-transferrin (1A8E) trough iron.
A: surface representation of the N-lobe, the residues from the active site and the aziridine-carboxylate are represented as sticks, iron as ball.
B: ribbons representation of the N-lobe, residues from the active site and the aziridine-carboxylate represented as sticks, iron as ball.
Models realized with AutoDock Vina, and visualized by PyMol.

CONCLUSIONS

During experiments we realized a molecular model using a docking program (AutoDock Vina) to predict the possibility of using aziridine-carboxylate as synergistic anion at iron binding of transferrin. The obtained data of binding geometry predict that aziridine-carboxylate has loosely, but realisable bound by transferrin. Based on these findings we prepared the transferrin complex using the aziridine-carboxylate as synergistic anions at human serum transferrin's iron binding. The complexation reaction and its analysis using CZE and cIEF techniques [17] demonstrate that the substitution of carbonate to another synergistic anion, such as aziridine-carboxylate produces no significant changes in polypeptide folding or domain orientation in human serum transferrin as revealed by our structure.

EXPERIMENTAL SECTION

Material and methods

Iron-saturated human serum transferrin, the ferric chloride ($FeCl_3$) powder, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Li-aziridine-2-carboxylate was purchased from Sigma-Aldrich, and the iron-free transferrin from Behring Werke AG. The chelating agent, nitrilotriacetic acid (NTA) was a Fluka product. Other used chemical reagents, sodium chloride (NaCl), hydrochloric acid (HCl) were Reanal product.

Complexation of transferrin with synergistic anions

The complexation reactions were realized conform to the protocol determined by Kilár et. al [18]. At the complexation reactions we used aziridine-carboxylate as synergistic anions.

Samples containing 15 mg/ml holo-Tf were prepared in a HEPES buffer (20 mM, pH=8.1) which including also 20 mM Li-aziridine-2-carboxylate. Solid $FeCl_3$ was dissolved in 6 M HCl and was added to NTA solution (100 mg/ml, prepared in 1 M NaOH) to achieve Fe^{3+}:NTA ratio 1:2. This iron-NTA solution was added to the samples containing the anion. To get the desired iron-saturation the ratio of apo-Tf:Fe^{3+} was 1:2. All steps of Tf-Fe^{3+}-aziridine-carboxylate complex formation were realized under nitrogen atmosphere. After 30 minutes of incubation the iron-saturated Tf samples were dialysed in HEPES buffer (20 mM. pH 7.5) on 4°C for 16 h.

Modelling of transferrin-Fe3+-anion ternary complexes

For modelling of transferrin-complexes we used the AutoDock Vina molecular docking program and PyMol v.1.3 for visualizing and construction of the ligand [19].

The human serum transferring (1A8E) macromolecule was prepared using AutoDockTools-1.5.2. An AutoDock-specific coordinate file (PDBQT) was created in which the partial charge of the iron was set to 3.00. A configuration file was written for the AutoDock Vina software, in which the search space was defined and the maximum number of binding nodes to generate was set to 50.

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