The unsuspected "bodyguards" of red blood cells

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Transferrin is the main intrabody carrier of iron. An in-depth analysis of the structure, conformation distribution and functions of this protein allowed us to predict its unsuspected "non-canonical" function in the metabolism of mature red blood cells. In this work we propose a theory of the energy landscape with static and dynamic macromolecular simulations, performed with an unconventional statistical calculus, which analyze the conformation transformations of Transferrin from a closed to an open and activated structure ("open jaws") during the follow-up of mature red blood cells. The result is a finite set of N states of low entropy, of which a subset N-p corresponds to conformations of minimum entropy where Transferrin binds to a specialized receptor, surviving in mature red blood cells.

I. SIGNIFICANCE STATEMENT

In this paper we propose a theory of the energy landscape with simulations that illustrates a new "non-canonical" function of Transferrin, the most important intrabody carrier of iron.

II. INTRODUCTION

Transferrins belong to a family of 80 kDA monomer glycoproteins (Baker and Lindley [1]) composed of single chain of 670-690 residues and structurally organized in two homologous lobes (identified as "N" and "C") fatherly divided in four sub domains identified respectively as "N1" and "N2" in "N" lobe and as "C1" and "C2" in "C" lobe. The bilobate characteristic topology of transferrin is generally referred to the evolution from a common monolobate precursor that could have given origin to a phylogenetic line common to the PBS bacteria that also transfer Fe^{3+} ions to the gram-negative bacteria and have a semi-transferrin structure, now completely identified. The complex structural-functional relationship of transferrin is clearly outlined in figure 1, courtesy of Heather M. Baker et Al. [2]

Transferrin is the main intrabody carrier of iron (Aisen et al. 1989 [3]), whether absorbed by food or released by the monocyte-macrophagic cells due to the degradation process of hemoglobin released by elderly RBCs. The processes of iron absorption are different according to whether the metal is contained in food under haeminic form (hemoglobin or myoglobin) of which meat is rich or under non-haeminic form (ferritin and hemosiderin) present in vegetables. In the first case, the absorption of metal by the intestinal mucous cells occurs directly, whereas in the second case the metal should be linked to a chelate such as ascorbic acid. In all cases, iron meddled through food after having being absorbed by the intestinal epithelial cells and realized in plasma, becomes linked to transferrin and carried out to the various organs and tissues. Among these, the main iron receiver is the bone marrow inside which the metal is utilized by the erythron

for the hemoglobin synthesis.

Then, whilst the transferrin's role of "carrier" in the initial and final life cycle of the erythrocyte is well-known, its function has not yet been connected with the metabolism of the mature erythrocyte, notwithstanding some evidences of its direct involvement with it.

III. MATERIALS AND METHODS

No organic materials or clinical trials on humans or animals were used for the present research. In this work we propose an energy landscape theory based on a free-scale macromolecular simulation of the role of transferrin in the metabolism of mature red blood cells. We developed this theory and simulations as part of the research of macro-molecular mathematical models based on REM energy landscapes for the fight against the COVID-19 pandemic within the new Coronavirus disease research community - COVID-19 at the European Organization for Nuclear Research -CERN (Geneva, CH) [4, 5] .

The simulations were generated on an autocad (static) by AutodeskTM and dynamic platforms by means of the category calculus "SHT", patented by the present author in 1997-2011 for the study of complex systems [6] .

From a practical point of view, the SHT analysis does not work with invasive operations on the sample, that is, it does not reduce the system nor simplify it, nor subtract the contribution of the environmental background or noise: SHT algorithms analyzed the system *sic rebus stantibus*, also considering the "junk". SHT considers the sample as a kind of "dynamic system". It studies the maps, bifurcations, attractors. If SHT finds an attractor, it will become a "category" of the experiment. But only about that particular experiment. Once the possible categories have been identified, SHT defines the "morphisms" and transforms them into probability functions. In this case, SHT defines these morphisms as "profiles of maximum congruence". In this way, systematic errors can be easily identified. From a programming point of view, SHT analysis is performed through algorithms written in the machine codes of the operating system

FIG. 1. Courtesy of Heather M. Baker et Al. [2] (A) Ribbon diagram showing the characteristic bilobal structure of transferrins. Shown here is the iron-bound form of human Lf, with the N lobe on the left and the C lobe on the right. In each lobe, domain1 is gold and domain2 is green, with a single Fe^{3+} ion (red sphere) and CO_3^{2-} (orange) bound in the interdomain cleft. An α -helix (magenta, top) joins the two lobes; in Tf this is non helical. The C-terminal α -helix (pink) may play a role in communication between the lobes (57). (B) The conformation change that accompanies iron binding, shown here for the N lobe of human Tf (21). A hinge in the two β -strands that run behind the iron site allows one domain to move relative to the other. Two helices (blue) act as a fulcrum; one pivots on the other. (C) The canonical iron binding site of transferrins, shown here for the N lobe of human Lf, involves two tyrosine ligands, one aspartate, one histidine, and a bidentate CO_3^{2-} ion in a pocket formed by an arginine side chain and the N terminus of an α -helix. In the N lobe of sTfs, a pair of lysine residues forms a pH-sensitive interaction that assists iron release; these replace Arg-210 and Lys-301 shown here for Lf. (D) Comparison of the iron binding site found in transferrins (Left) with that in the bacterial periplasmic ferric binding protein (Right). In both cases a coordinating anion (carbonate and phosphate, respectively) is at the N terminus of a structurally homologous α -helix and a carboxylate ligand is contributed from a homologous loop. The histidine and two tyrosine ligands come from quite different parts of the structure, yet generate a binding site that is spatially and chemically almost identical.

or integrated into commercial electronic tables such as Matlab[®], Origin[®], Mathematica[®], Kaleidagraph[®], etc. The analysis is generally conducted on many logic levels. The last levels are generally made of time series, and maximum congruence regressions. The first and intermediate levels are dedicated to the declaration of variables, labeling ("tagging"), and, if necessary, to the reconstruction of time intervals and delays ("lagging"), or to the temporal calibration of the devices (detectors or transducers).

In the present case, the analysis was performed by arranging the structural and protein folding conformation dataset from literature into an entropic oriented REM energy landscape (Hans Frauenfelder's "rugged funnel" [7–10]), which we used to call "data-funnel". It is a section

of a real plane (2D) or real space (3D) of a hyper-space map representing the configuration entropies as a function of the configurations, the relative energies and a set of control parameters. This procedure thus avoid the subjective introduction of selection criteria or parametric or other tools of subjective tuning. The ultimate goal of the SHT first level processes is to create a "cladistics" of the data (configuration) sample.

We chose Transferrin because the configurations of this protein lend themselves well to simulations. The environmental boundary conditions and control parameters are based on the structural and functional configurations of the protein. The result is a probabilistic frame that reveals a possible "non-canonical" function of Transferrin, which has a definite conformation probability, much

higher than the other simulations conducted with the proteins studied in the COVID-19 pandemic. If this new function is confirmed, we may also have a valid model suitable to be used in the fight against the COVID-19 Pandemic. Further technical clarifications are available, if of interest.

IV. RESULTS

The first point which is directly brought about to our attention is the common query of all researchers about the so-called "synergistic" anion (bi)carbonate which starts and drives the suitable conformation transformation of folding (i.e. the stiff rotation of the two lobes around the hinge) of the apo-protein Transferrin from the "closed" (C) form to the "open" (O) form and coordinates the union of 4 residuals in order to build-up the linkage site of trivalent iron. Numerous spectroscopic and kinetic studies [2, 11–18] have clearly demonstrated that carbonate anion links firstly with the apo-protein, starting the folding transformation. The first step in the complex process of iron capture is hence given by the interaction with carbonate anion, without which the protein does not have affinity for iron in the range 10^{20}M^{-1} (Aisen 1989 [3], Bellounis et al. 1996 [19]). Generally, the process enfolds with a chain of fast reactions starting with the linkage with carbonate. After this starting process -and in fast succession- 13 four of the six iron linking items, two oxygen carbonates and two tyrosine join in one item in order to create the linking site (fig.1).

We note that this starting process is almost general and useful, depending on the chemical peculiarity of the native anion and the topology of the linkage site (i.e. "mono" or "bi-dentate" [20, 21]): indeed for transferrin the native anion is a carbonate, while for the nFBP (the periplasmic proteins carriers of iron in some pathogenic bacteria like *Neisseria meningitidis*, *N. Gonorrhoeae*, etc) the native anion is a phosphate [21]). Consequently, for transferrin family we find a relevant dependence of the concentration of the synergistic native anion carbonate, as displayed by FB Abdallah et al. (1998), see fig. 3 [12].

We could now guess why the native synergistic anion for transferrins is just a carbonate and not phosphate and try to look for a local answer through topological, physical, and chemical features of the linkage itself. However, if we try to consider the problem through the point of view of metabolism we may be immediately driven to the carbon cycle in the aerobic metabolism. As known indeed, the main final product of aerobic metabolism is carbon dioxide. In organisms with complex structures, carbon dioxide is released in the blood and carried to the lungs by the erythrocytes to be afterwards exhaled. Notwithstanding the CO_2 hydration and the HCO_3^- dehydration occur at a "reasonable" speed even in the absence of a catalyst [22–25], mostly all the organisms contain enzymes (i.e. "carbonic anhydrase") which catalyze

these processes. These enzymes are clearly necessary because the CO_2 hydration and the HCO_3^- dehydration in the blood are blended with fast transportation processes [22–25] (See figure 2).

Therefore, here we may find the key to understand the role of transferrins in the metabolism of mature erythrocytes: indeed the protein is "forced" (by the strong affinity we have just described) to follow the flux of the synergistic carbonate anions issued by the erythrocytes in order to establish almost a linkage with the RBC cell mediated by the folding "starting" transformations. At this purpose, I suggest the following model of "follow-up", as displayed in the following figure 3.

During the "follow-up" along the anionic wake of the erythrocyte, the apo-protein is almost linked with the erythrocyte and "forced" to activate itself displaying an extremely precise conformation dynamic [26]. Many researches have indeed suggested several models of this structural dynamic which we used to study and describe by the means of Frauenfelder's rugged funnel energy landscape model [7–10]. That's why we decided to use the SHT entropy oriented landscape frame for the simulation (see materials and methods). Some of them [11, 17, 20, 27, 28] suggest a sequence of four main conformation states, respectively:

- 1- Apo C (closed);
- 2- Apo O (open);
- 3- Holo O (open);
- 4- Holo C (closed)

Direct measurements and theoretical previsions about the radius of gyration [1, 2, 28–31] show that both the two closed conformations ("apo" and "holo") offer a considerable fluído-dynamical advantage in the motion of the protein (whether charged or discharged) within the plasma and even through the haematic barriers of the organisms [32, 33]. The other two open conformations ("apo" and "holo") that can be activated by the linkage with the synergistic anion are often called "open-jaws" [18] because they offer a major active surface and therefore a major radius of gyration that is suitable to capture the metal and other important functions that we will try to explain later.

Finally, we are supposed to ask ourselves why does the protein be "forced" (by anionic affinity) to "follow-up" the erythrocyte's anionic wake within the plasma and then to start the folding transformation very closely (and almost linked) to the erythrocyte itself. At this purpose, we start trying to find a possible answer from two simple considerations.

1. The first consideration come from some results [34, 35] that have just demonstrated that adding (either *in vivo* or *in vitro*) doses of transferrin or transferrin-complexes in several haematic pathologies has sensibly reduced -and even inhibited in some cases- the erythrocytic lysis and prevented the macro-aggregates formation.

2. The second consideration is in direct connection with the erythrocytic metabolism.

We know in fact that the metabolism of the RBC de-

FIG. 2. Aerobic metabolism and transferrin follow-up along the anion trail of mature erythrocytes. Labeled snapshot taken from a free scale dynamic and static simulation with category calculus (Zangari 1997-2011 [6]) with autocad-AutodeskTM

FIG. 3. Follow-up along the anion wake of mature erythrocytes. Free scale dynamic and static simulation with category calculus (Zangari 1997-2011 [6]) with autocad-AutodeskTM

velops at least according to three fixed points:

2.1 To safeguard the integrity of the membrane and the osmotic gradient Na^+/K^+ respect to the plasma environment: for this purpose it needs energy (ATP) which is obtained by glucose through anaerobic glycolysis;

2.2 Keeping the heme iron in its ferrous state (Fe^{2+}) preventing O_2 to oxidize it to the ferric state (Fe^{3+}). Indeed, a specific enzyme ("methemoglobin reductase" that needs NADH which is furnished by anaerobic glycolysis) provides at this purpose reducing the "ferric" hemoglobin ("methemoglobin") into "ferrous" hemoglobin;

2.3 To protect hemoglobin from oxidative denaturation and maintaining it into solution.

Those that are provided by catalases and peroxidases that neutralize H_2O_2 produced by oxidation and glutathione (GSH) which is the "master antioxidant" and safeguard the groups SH of hemoglobin from oxidation. Indeed, as the lysis of human erythrocytes is stimulated by carbonate anions, we know also clearly that methemoglobin formation, glutathione depletion and conversion of oxyhemoglobin to methemoglobin are associated with super oxide anion production and lead to the formation of ferryl hemoglobin, hydrogen peroxide or hydroxyl radicals [36, 37].

Finally, we are therefore led to answer our question by noting that transferrin must follow the anion trail of the erythrocyte to participate directly in mature erythrocyte metabolism. We may try to think to transferrin as a sort of erythrocyte's "bodyguard", following the erythrocyte with an ever-increasing closeness clearly regulated by the magnitude of the anionic flux and so denaturation and/or oxidation damages when the other defenses (ie. peroxidases, catalases and methemoglobin reductase) fail and the anionic flux increases in magnitude. As the anionic flux overcomes a critical value, the protein must link with the erythrocyte itself in order to intervene directly into the critical erythrocytic metabolism. We can call it a "non-canonical action" of transferrin [38]

Clearly, the natural site for transferrin to bind is the transferrin receptor (TfR) on the cell membrane, and we expect the protein to form a complex with TfR. But it is generally accepted that after maturation the reticulocyte expels the obsolete membrane proteins through the formation of exosomes (Johnstone 1991 and Harding 1983 [39]). We therefore ask ourselves: "Is the transferrin receptor really obsolete in the mature red blood cell?". However, the binding of transferrin to mature red blood cells must occur through the formation of a complex with the transferrin receptor (TfR), which are not obsolete and must survive on the red blood cell membrane in order to enter the cell through the process of "receptor mediated endocytosis". Let's consider this hypothesis and see how it can be articulated with our energy simulation. From the point of view of our energy landscape model, we find a non-zero probability for the survival of a Tf receptor in the low entropy states of the funnel, as represented in the two-dimensional funnel of the following figure.

FIG. 4. Real plane section of an entropy-oriented REM landscape ("rugged funnel") The N low-entropy funnel states that survive beyond the glass transition are significant for the survival of a transferrin receptor on mature RBCs. Of these only $N-p$ states are good candidates. In the example represented by the figure we have two iso-energetic probability configurations $<50\%$ for the receptor-ligand system ($Q \sim 1.0$), which allow us to estimate the minimum energy of the configuration.

Henceforth, we must necessarily suggest that a specialized and active receptor for transferrin (that we may call TfRx) will survive on the membrane of the mature erythrocyte, as can happen, under certain conditions, in the case of chickens [40]. Today we have several generations of well-known Tf receptors (TfR2 is the most known) with some specifications still unclear in relation to the different transformations they can undergo, see i.e. Hiroshi Kawabata's comprehensive studies which we recall among the others [39, 41–52]

The TfRx family might be a progeny of the TfR "ancestors" coordinated for the hemoglobin synthesis upon the surface of the stem cells (i.e. CFU-GEMM, BFU-E, and CFU-E) and upon the erythroid precursors (erythroblasts). Therefore, as for erythroblasts we just need to look for a linkage process able to let the TfRx complex coordinate for the hemoglobin synthesis upon the membrane of the mature erythrocyte and thus intervene directly in its critical metabolism at the beginning of the erythrocytic membrane structural rearrangement that triggers the collapse of the entire cell. This rearrangement is clearly one of the causes of the arise of antigenic activities identified by immune globulin fractions normally present in plasma. The complex antigen-antibody, similar to the one that arise in the haemolytic self-immune anemia, could therefore expose the erythrocyte to the capture by macrophages ("catching"). Now we know that the membrane rearrangement is due to the globin deposition on the erythrocytic membrane. Indeed, some evidences [47] show an increase in membrane bound globin, starting from monomers up to dimers, as

displayed in the following figure 5.

Therefore, we suggest that TfRx can complex with monomers or even dimers of globin and then start to coordinate the synthesis of Hemoglobin that could permit the erythrocyte to reach its half-life of 120 days (See next figure 6).

V. CONCLUSIONS AND DISCUSSION

Together with the role of iron carrier, we suggest that transferrin plays a very important role in the metabolism of mature erythrocytes, coming into action when the main defenses (peroxidase, catalase, GSH and so on) fail to fight the denaturation and oxidative damage of the main erythrocyte and hemoglobin pathologies. To this end, we developed an energy landscape theory of the role of transferrin in the metabolism of mature red blood cells and carried out macromolecular simulations using the advanced calculation system SHT, representing an entropic oriented REM energy landscape (Frauenfelder's rugged funnel). We studied the probability densities that Transferrin undergoes conformation transformations from a compact structure to an open and activated one ("open jaws") during the follow-up of the anion wake and binds to a surviving Tfr receptor in mature red blood cells.

We can discuss the goodness of the entropy-oriented REM representation and the parameters that were used to compose this representation. As we know, the choice of control parameters is a very delicate operation. In this case we have decided to comply with the choice of

a control parameter for each set of open configurations and another for each set of closed configurations. In the case of the linked configuration we have studied a subset of the compact configurations that form complexes. For each of these, the configuration space was left free to vary, minimizing entropy.

The result, as pictorially represented in the example of Figure 4 (section of the real plane), is a finite set of N states of minimum entropy, of which N-p states correspond to the structures that admit receptor ligand binding with TfRx, which can help us in finding the structural and phylogenetic peculiarities of TfRx.

If this role is confirmed by specific experiments, important progress can be made in blood diseases.

In addition, if the qualitative result of the simulation were confirmed by experiments, we could say that we have achieved an algorithmic functional model also for research in the fight against the COVID-19 Pandemic.

VI. AUTHORS' CONTRIBUTIONS

GZ coordinated the research, simulations and wrote the manuscript.

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Declaration of interests. The author declares that he has no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIG. 5. Transferrin follow-up along the anion wake leads to mature erythrocytes (catching). Note that in preparation for the link with the TfRx receptor, transferrin undergoes a conformational transformation to an open configuration ("open jaws"). Free scale dynamic simulation with category calculus (Zangari 1997-2011 [6]) and Autocad -AutodeskTM

FIG. 6. Openjaws transferrin link with the Tfrx receptor. Free scale dynamic simulation with category calculus (Zangari 1997-2011 [6]) and Autocad -AutodeskTM

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