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The unsuspected "bodyguards" of red blood cells

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Transferrin is a glycoprotein universally described as a "carrier" in the "iron cycle" between the organic deposits (ferritin and hemosiderin) and the sites responsible for hematopoiesis (bone marrow). An in-depth analysis of the structure and function of transferrin, accompanied by macromolecular simulations carried out by means of an advanced calculation system dedicated to the study of complex systems, has allowed us to understand an unsuspected "non-canonical" function of this protein in the metabolism of mature red blood cells . In this work we offer a dynamic scenario showing this new function of the protein according to three steps related to its known conformational transitions.

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1 Transferrins

Transferrins belong to a family of 80 kDA monomer glycoproteins (Baker&Lindley,1992 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-transferrinic 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 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 hemosiderrin) 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.

As known erythron is the anatomical- functional basic unit of erythropoiesis and it includes the entire cellular population which goes from the erythroid-oriented stem cells to the circulating erythrocytes, which are cells with no nucleus, concave disk shaped, from 6 to 8 μm of diameter, 2.5 μm thick and from 6 to 8 mm of diameter, 2.5 mm thick and from 80 to 100 mm^3 of volume, carrying a considerable quantity of hemoglobin (about 27-32 pg for each erythrocytic cell) and protect it from oxidation during the tissue respiratory cycle.

2 The role of transferrin in the metabolism of the mature erythrocyte

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. Hence, 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 conformational transformation of folding (i.e. the stiff rotation of the two lobes around the hinge) of the apo-protein 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 (^{2,4-11}) 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 ¹²). Generally, the process enfolds with a chain of fast reactions starting with the linkage with carbonate and described by the following reaction

Where $T'cH_2$ indicates the lobe "C" of the apo-protein without carbonate in a neutral medium, while TcH_3 indicates the apo-protein's lobe "C" linked with the carbonate (deprotonated formula). After this starting process and in fast succession ¹³ four of the six iron linking items, two oxygen carbonates and two tyrosine united 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"; ^{13,14}): 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 (¹⁴). Consequently, for transferrin family we find a relevant dependence of the concentration of the synergistic native anion carbonate, as displayed in fig. 3 (⁴).

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 "global" 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 (^{15,16}), 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 (^{15,16}).

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 fig.2.

During this "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 conformational dynamic (¹⁷). Many researches have indeed suggested several models of this structural dynamic that one may describe in the Frauenfelder's rugged funnel energy landscape model (¹⁸⁻²¹) of protein's sub-conformational dynamic (not first order transitions): some of them (^{139,102223}) suggest a sequence of four main sub-conformational 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 (^{24,23,25,26}) show that both the two closed conformations ("apo" and "holo") offer a considerable fluid dynamical advantage in the motion of the protein (whether charged or discharged) within the plasma and even through the haematic barriers of the organisms (^{27,28}). The other two open conformations ("apo" and "holo") that can be activated by the linkage with the synergistic anion are often called "open-jaws" (¹¹) 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 recent results (^{29,30}) 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 erythrocytary lysis and prevented the macro-aggregates formation.

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

We know in fact that the metabolism of the GR develops 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 (³¹³²).

Finally, we are therefore well 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 trans-

ferrin ³³).

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.

Even if it is generally accepted that after maturation the reticulocyte expels the obsolete membrane proteins through the formation of exosomes (Johnstone 1991 and Harding 1983; ³⁴), the linkage of transferrin with the mature RBC must occur through the formation of a complex transferrin-receptor (TfR) on the RBC membrane in order to enter the cell by the endocytosis process (i.e. "Receptor Mediated Endocytosis"). Therefore, TfR might not be considered an "obsolete membrane protein" to be expelled by the mature reticulocyte. Henceforth, we must necessarily suggest that a specialized and active receptor for transferrin (that we may call TfRx) it will survive on the membrane of the mature erythrocyte, as can happen, under certain conditions, in the case of chickens ³⁵. We should not be surprised because today we have several generations of well-known Tf receptors (TfR2 and so on) with specificities still little studied in relation to the different transformations they can undergo, see Hiroshi Kawabata's very complete and comprehensive studies which we recall among the others (^{36,3738394041424334373044454647}).

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 globulinic 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. Now we know that the membrane rearrangement is due to the globin deposition on the erythrocytic membrane. Indeed some evidences ⁴⁸ show an increase in membrane bound globin, starting from monomers up to dimers, as displayed in the following figure 3.

Therefore we suggest that TfRx might complex with monomers or even dimers of globin (see figure 4) and then start to coordinate the synthesis of Hemoglobin that could permit the erythrocyte to reach its half-life of 120 days.

3 Conclusions.

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 carried out macro-molecular simulations that divide the action of transferrin into three steps (follow-up, catching and linkage) during which the protein undergoes conformational transformations from a compact structure to an open and activated one (" open jaws ") during the follow-up of the anion wake. If this role is confirmed by specific

experiments, important progress will then be made in blood diseases.

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Figure 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, domain 1 is gold and domain 2 is green, with a single Fe^{3+} ion (red sphere) and CO (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 conformational 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 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.

Figure 2: Follow up along the anion wake of mature erythrocytes. Dynamic simulation with category calculus (Zangari 1997-2011 ⁴⁹) and Autocad -Autodesk (R) ⁵⁰)

Figure 3: 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"). Dynamic simulation with category calculus (Zangari 1997-2011 ⁴⁹) and Autocad -Autodesk (R) ⁵⁰)

Figure 4: Open jaws transferrin link with the TfRx receptor. Dynamic simulation with category calculus (Zangari 1997-2011 ⁴⁹) and Autocad -Autodesk (R) ⁵⁰)