pH Induced Active ("Uphill") Liquid Membrane Transport of Ferrioxamine B by the Ionizable Ionophore Lasalocid

Ivan Spasojević and Alvin L. Crumbliss*

Department of Chemistry, Duke University, Box 90346, Durham, North Carolina, 27708-0346

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Introduction

Iron uptake mechanisms vary among living organisms.[1] Microbes are able to acquire iron as ferrous as ion, ferric ion, or by excreting siderophores, potent and highly selective iron chelators.[2,3] The iron(III)−siderophore complex is recognized by cell receptors, and iron is liberated from the siderophore during transport through the membrane or inside the cell.[4−7] Two X-ray crystal structures have been reported recently for receptor−siderophore complexes.[8,9] Fe(III) reduction has been shown to be a chemically reasonable mechanism for iron release from a siderophore,[2,10−12] and in some cases this mechanism has been clearly shown to be operative in a cellular system.[13,14]

Iron acquisition in humans occurs through the food chain. Excess iron intake that is not immediately needed for metabolism is stored in iron transport (transferrin) and storage (ferritin) proteins. In the case of acute iron poisoning, or disorders in iron metabolism in which frequent blood transfusions are necessary, iron storage proteins may become saturated, resulting in uncontrollable iron deposition and tissue damage.[15,16] In addition, increased blood serum iron concentration increases the risk of opportunistic infections by pathogenic bacteria.[17]

Iron-starved *Streptomyces pilosus* produces large quantities of the siderophore desferrioxamine B, which is presently the only approved drug (Desferal−Novartis) for iron overload therapy.[16] However, oral bioavailability and absorption of desferrioxamine B from the gastrointestinal tract is poor. Considerable effort has been made to improve therapeutic efficiency by chemical modification of desferrioxamine B, but with little success.[15,16,18] One alternative approach to alter the high hydrophilicity of desferrioxamine B, or its Fe(III) complex, ferrioxamine B, may be to bind it noncovalently to a lipophilic molecule, thereby increasing the overall lipophilicity of the assembly without affecting the chelation efficiency of the ligand. We have established that ferrioxamine B can be recognized in two-phase (aqueous/organic) extractions[19−26] and transported in a three-phase (aqueous/organic/aqueous) bulk liquid membrane (BLM) experiment[27] by neutral (18C6 family)[19−23] and ionizable (benzo-18C6 carboxylic acid lariat)[26] synthetic crown ethers and neutral (valinomycin, nonactin)[22,23] and ionizable (lasalocid A)[25] natural antibiotic ionophores.

Ionizable ionophore carriers have increased transport properties relative to their neutral analogues and also add new characteristics to the transport mechanism. Namely, an anionic ionophore (e.g. lasalocid anion) excludes the need for a counteranion to be transported across the membrane along with a cationic guest, as in the case of neutral ionophores. Thus an unnecessary and energetically expensive process is avoided. However, the release of ferrioxamine B from the {ferrioxamine B−lasalocid} transport assembly (**I**) is then dependent on the existence of both a counteranion to accompany ferrioxamine B in the receiving aqueous phase and a countercation to accompany lasalocid anion in the membrane. We have shown in our previous work that ferrioxamine B/desferrioxamine B can be transported in a synergistic fashion in a lasalocid anion facilitated BLM transport experiment.[27]

* To whom correspondence should be addressed. FAX: 919-660-1605. E-mail: ALC@chem.duke.edu.

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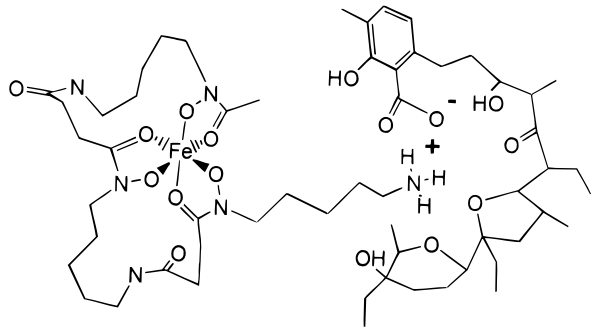

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As previously demonstrated, at low pH lasalocid will remain undissociated and neutral ($pK_a = 3.7$),[28] greatly diminishing its ability to form a host−guest complex with ferrioxamine B.[25,27] Thus, the proton is able to liberate ferrioxamine B from a ferrioxamine B−lasalocid assembly at the membrane/receiving phase interface. If, however, the source phase is at a higher pH, the lasalocid will be deprotonated at the membrane/source phase interface and capable of binding ferrioxamine B again. Here we report our most recent results for such a system, whereby we observe the active ("uphill") transport of ferrioxamine B driven by a concentration gradient of protons transported in the opposite direction as illustrated in Figure 1. In principle, this serves as a simplified model for proton motive force (pmf) driven siderophore mediated active iron transport in a single cell organism.[4]

Experimental Section

Materials. Ferrioxamine B ($FeHDFB^+$) aqueous stock solution at pH 3.2 was prepared as described previously.[27] The sodium salt of lasalocid (Na^+lasalocid$^-$) and dicyclohexano-18-crown-6 (DC18C6) crown ether were purchased from Aldrich and used as received. Lasalocid acid (Hlasalocid) was prepared as described previously.[25]

Instrumentation. Bulk liquid membrane transport experiments were carried out in a specially designed U-shaped, 1 cm path length spectrophotometer cell equipped with a stirring mechanism, as described elsewhere.[27]

Methods. The three-phase BLM transport experiments were performed as follows. An appropriate amount of ionophore (carrier: lasalocid) (e.g. 20−40 μM) was dissolved in chloroform, and 4 mL of this solution was added to the bottom of the U-cell. To both side-arm compartments, 2 mL of "source" aqueous solution (pH 9.5, 400 μM ferrioxamine B) and 2 mL of "receiving" aqueous solution (pH 3.0, 400 μM ferrioxamine B) were simultaneously added. These solutions were prepared by dilution of the ferrioxamine B stock solution with deionized H_2O and the pH adjusted by addition of small amounts of 0.1 M $HClO_4$ or 0.1 M NaOH. The assembly was then carefully placed in a spectrophotometer and the absorbance in either the receiving or source phase recorded as a function of time. After 6 h, the slope of the absorbance vs time trace (excluding the nonlinear portion recorded during the "lag-time" period; i.e., the first 60 min when presumably "loading" of the membrane phase occurs[27]) was calculated as a *flux* (mol cm^{-2} s^{-1}). The molar absorptivity of ferrioxamine B was taken as 2600 M^{-1} cm^{-1} at 428 nm.[29] The chloroform/aqueous phase interface surface area was taken as 1 cm^2, and it was not corrected for surface deformation due to the presence of 1 mm^2 shafts and the "meniscus" formed due to the water/glass-wall interaction.

Results and Discussion

We have shown previously that, in its deprotonated form, lasalocid anion is able to recognize ferrioxamine B through the second-sphere complexation of the positively charged amine side arm and is able to transport the siderophore complex across an artificial lipophilic membrane.[23,25,27] The dependence of the transport process on the presence of other species (co-transported cations and anions) in each of the phases was investigated as well.

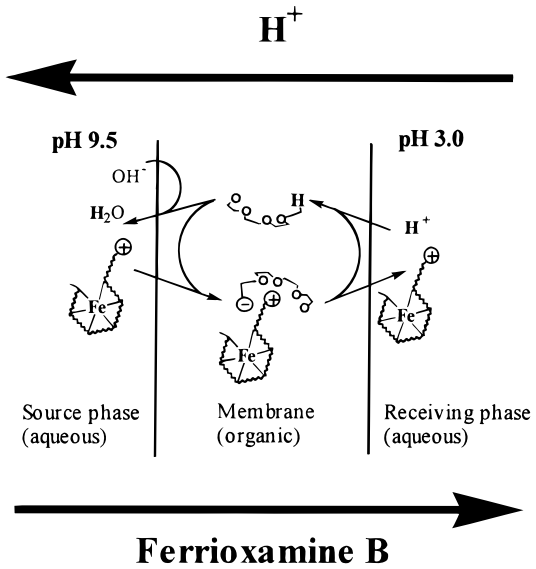

Figure 1. Schematic representation of the *active* ("uphill") transport of ferrioxamine B by lasalocid, facilitated by the *passive* transport of H^+ in the opposite direction.

Here we present the results of an *active* ("uphill") transport of ferrioxamine B by lasalocid anion, facilitated by a *passive* transport of H^+ that is maintained due to a pH difference between the source and receiving aqueous phases. The process may also be viewed as a "switching" mechanism in which the affinity of lasalocid anion for ferrioxamine B diminishes dramatically upon protonation of the lasalocid anion. If the overall transport is viewed as the sum of the transport of ferrioxamine B in both directions, then the transport toward the phase at lower pH is favored over the transport toward the phase at higher pH due to a competition between proton and ferrioxamine B.

To demonstrate that the transport of ferrioxamine B indeed goes against its concentration gradient, the BLM experiment was started with the same concentration (400 μM) of ferrioxamine B in both source (pH 9.5) and receiving phase (pH 3). To keep the number of different species transported as low as possible, no buffer was used. The experiment was started with the membrane carrier in its protonated form (Hlasalocid) so that the transport of ferrioxamine B was controlled by the deprotonation of Hlasalocid to lasalocid anion at the source/membrane interface from the very beginning of the experiment.

Figure 2 shows the results of a typical BLM experiment. The concentration of the ferrioxamine B was continuously monitored over the course of 6 h in both the receiving phase and (in a separate but identical experiment) in the source phase. Both curves show the same effect—an active (i.e. "uphill", against the concentration gradient) transport of ferrioxamine B from the solution of higher pH toward the solution of lower pH, as illustrated in Figure 1. In 6 h, about 40% of the ferrioxamine B was transported against the concentration gradient. Since the concentration of Hlasalocid was 10% of the ferrioxamine B concentration, in 6 h every lasalocid carrier molecule has transported four molecules of ferrioxamine B (i.e. each carrier molecule participated in at least four protonation/deprotonation

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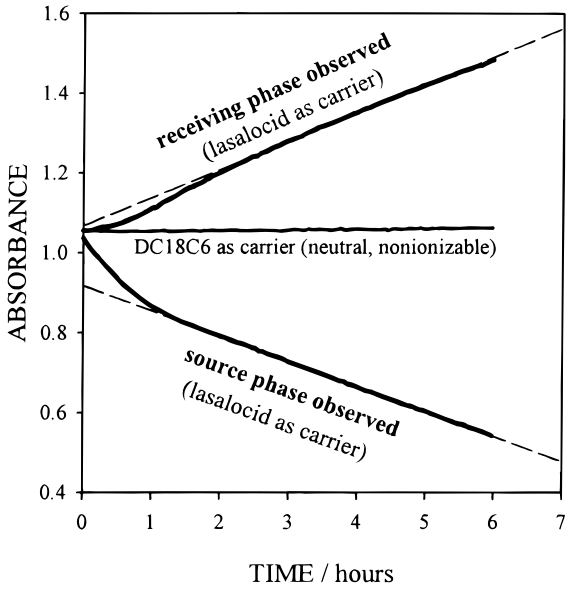

Figure 2. Ferrioxamine B absorbance vs time traces recorded during BLM transport experiments. Dashed lines represent the linear regression fit of the linear portion (last 4 h) of the receiving phase trace (r^2 = 0.998) and source phase trace (r^2 = 0.999). Conditions: (a) source phase, 0.4 mM $FeHDFB^+$, 2.0 mM ClO_4^-, 0.8 mM Mg^{2+}, pH 9.5; (b) membrane, 0.04 mM Hlasalocid or 0.02 M DC18C6 in $CHCl_3$; (c) receiving phase, 0.4 mM $FeHDFB^+$, 2.0 mM ClO_4^-, 0.8 mM Mg^{2+}, pH 3.0.

cycles shown in Figure 1). The slope of the absorbance in the receiving phase vs time trace (Figure 2), between 2 and 6 h, gives an average flux of ferrioxamine B of 1.52×10^{-11} mol s^{-1} cm^{-2}. Very similar results were obtained from the source phase trace (i.e. 1.35×10^{-11} mol s^{-1} cm^{-2}). No measurable flux was observed in the "control" experiments that were performed under identical conditions: (1) with neutral crown ether DC18C6 as carrier (Figure 2) and (2) with no carrier in the membrane.

Other authors have reported the transport of group I and II aqueous cations against their concentration gradients coupled with counter directional H^+ transport using polyethers with ionizable carboxylic acid groups.[30−32] Similar transport experiments using lasalocid as a carrier were performed previously using metal−amine complexes coupled to an ammonium ion counter gradient[33] and a proton driven active transport of dopamine.[34] Our system may be viewed as a combination of these two systems. Namely, the metal−siderophore guest, ferrioxamine B, is recognized through its positively charged amine placed on a pendant carbon chain. In that way the first coordination shell of the metal−siderophore complex remains intact, while the cationic site of the ligand may fully be involved in a host−guest interaction with an ionophore to form an ionophore−siderophore complex (**I**).

Conclusions

We have demonstrated that ferrioxamine B can be transported across a hydrophobic membrane against its concentration gradient by the ionizable ionophore lasalocid and that such transport may be triggered by pH changes above and below physiological (pH 7.4) conditions. On the basis of our previous studies[27] such *active* transport is expected to be operative for the iron-free desferrioxamine B (Desferal) as well. This result may be important both in terms of iron-transport mechanisms operative in microorganisms and studies on iron acquisition and release in humans. The ferrioxamine B−lasalocid assembly also represents a *reversible* (labile) siderophore−antibiotic system that may be recognized, due to the preserved structure of the first coordination shell of the iron−siderophore, by the natural iron−siderophore receptors and thus potentially useful in antibiotic administration studies.[35]

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