Extremely High Avidity Association of Fe(III) With the Sickle Red Cell Membrane

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Red blood cells (RBCs) from patients with sickle cell anemia and thalassemia carry abnormal accumulations of molecular Fe(III) at the cytosol/membrane interface. The avidity of the red cell membrane for this iron has not been defined. Using open ghost membranes prepared from sickle RBC, we examined the ability of membrane-associated Fe(III) to resist removal by 15 chelators representing a 40-log range of affinities for Fe(III). Efficacy of chelators was compared with literature values for their idealized affinity for iron as represented by the cummulative stability constant (β_n), their effective stability constant reflecting affinity under biologic conditions (K_{eff}), and an indicator of their ability to chelate Fe(III) in the presence of an insoluble phase of iron (K_{sol}). Deferoxamine, a very high affinity chelator having log β_n = 30.6, was found to be the lowest affinity chelator able to remove RBC membrane Fe(III). Regardless of chelator β_n, only those agents able to preserve log $K_{eff} \geq 12$ were able to do so, indicating that the membrane's effective avidity for Fe(III) is on the order of 10^{12}. Additional confirmation that membrane avidity for Fe(III) is extremely high is found in the observation that only chelators having log $K_{sol} > 0$ were effective. Potential physiologic iron chelators in cytoplasm of pathologic red cells are unable to prevent or reverse iron accumulation on the membrane because they do not have sufficiently high affinity for iron. These data argue that RBC membrane Fe(III) is truly pathologic.

THE CYTOPLASMIC ASPECT of sickle and thalassemic red blood cell (RBC) membranes contains increased amounts of iron, which may contribute to disease pathobiology.[1] Although these iron deposits include a substantial amount of denatured hemoglobin, there is an even greater amount of non-heme/non-ferritin iron,[2,3] which we will refer to as "molecular iron." This membrane-associated molecular iron seems to be largely, if not exclusively, Fe(III) in nature,[2] and it is capable of valence cycling between ferric and ferrous states.[4,5] Two mechanisms have been identified thus far to explain this association of Fe(III) with the red cell membrane. Some appears to exist in the form of a chelate structure involving the polar head groups of aminophospholipids[2]; and some is found bound to the denatured hemoglobin deposited at the cytoplasm/membrane interface.[6]

The avidity of the membrane for this Fe(III) iron is unknown. Indeed, it is not understood why membrane free iron deposits would even exist in the intact red cell, since the cytoplasm contains multiple small physiologic chelators of iron (ie, citrate,[7] adenosine triphosphate [ATP], adenosine diphosphate [ADP]). Thus it is somewhat disconcerting that evidence for the presence of free iron on pathologic red cell membranes is derived from studies of ghost membranes prepared by hypotonic lysis, with only a single study providing a hint that bioavailable free iron is located at the cytosolic/membrane interface in intact sickle red cells.[8] To clarify this, we have used open ghost membranes prepared from sickle red cells to examine the ability of membrane-associated molecular Fe(III) to resist removal by 15 iron chelators selected from various structural categories to provide a 40-log range of affinities for Fe(III). Our results indicate that pathologic Fe(III) is associated with the red cell membrane with extremely high avidity.

MATERIALS AND METHODS

Reagents. Fifteen iron chelators were used for these studies: the trihydroxamic acid, deferoxamine; two catechols, 2,3-dihydroxynaphthalene-6-sulfonic acid (2,3-DHNS) and pyrocatechol (1,2-benzenediol); a phenol, 8-hydroxyquinoline (8OHQ); three aminocarboxylic acids, EDTA, diethylenetriaminepentaacetic acid (DTPA), and nitriolotriacetic acid (NTA), as well as a phenolate derivative of EDTA, NN′-bis(o-hydroxybenzyl)ethylenediamine-NN′-diacetic acid (HBED); one of the hydroxypyridones, 1,2-dimethyl-3-hydroxypyrid-4-one ("L1"); two nucleotides, ATP and ADP; a tricarboxylic acid, citrate; apo-transferrin, the major iron binding/carrying protein from plasma; salicylic acid; and tropolone. These were obtained from Aldrich or Sigma Chemical Co (St Louis, MO), except for L1 (provided by Robert McClelland and Nancy Olivieri, University of Toronto, Ontario, Canada) and HBED (provided by Robert Grady, Cornell Medical Center).

Experimental procedure. Open ghost membranes were prepared from sickle cell anemia red cells by hypotonic lysis in ice-cold 5 mmol/L sodium phosphate buffer at pH 8.0 containing 0.5 mmol/L EDTA. These conditions were used because they minimize association of hemoglobin with the membrane during hypotonic lysis; we previously established that neither presence of EDTA nor pH 8.0 artifactually affects amount or nature of membrane-associated free iron. After washing three times in this buffer, membranes were washed an additional three times in 5 mmol/L sodium phosphate buffer at pH 7.4 without EDTA. Buffers were rendered iron-free with Sigma chelating resin before use. Ghost membranes at 1 mg/mL were incubated without versus with chelators at 0.5 mmol/L for 30 minutes at 37°C, conditions chosen to indicate quickly yet definitively whether the chelators would be able to remove any membrane iron.

After incubation, ghost membrane molecular iron content was monitored by measuring the phase of rapid color development in the presence of ferrozine, sodium dodecyl sulfate (SDS) and reducing agents, as described.[2] The sickle RBC ghost membranes used for these measurements contained 16 nmol of molecular iron per milligram membrane protein at zero time and after control incubation with buffer in absence of any chelator. Data are reported as the

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Submitted October 30, 1995; accepted March 5, 1996.

Supported by the National Institutes of Health Grant No. HL37528.

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0006-4971/96/8801-0035$3.00/0

Table 1. Iron Chelators Used to Assess Binding of Fe(III) to Sickle RBC Membranes

Chelator	log β_n	log K_{eff}	log K_{sol}	Membrane Molecular Fe(III) Removed (%)
2,3 DHNS[9]	44	~26	0.89	24 ± 7
Pyrocatechol[9]	43.8	12.2	0.2	32 ± 1
HBED[9]	39.7	20.8	8.9	13 ± 6
8OHQ[9]	36.9	20.9	2.9	45 ± 5
Apo-transferrin[10]		~20		28 ± 1
L1[11]	35.9	19.2	9.6	97 ± 4
Salicylic acid[9]	35.3	8.01	−9.9	None
Tropolone[12]	32.3	21.9	9.9	90 ± 1
Deferoxamine[9]	30.6	16.3	4.4	91 ± 4
DTPA[9]	28.0	11.0	−0.95	None
EDTA[9]	25.0	8.1	−3.81	None
NTA[9]	24.3	8.17	−6.74	None
Citrate[13]	~11.5			None
ATP[14]	~6			None
ADP[14]	~4			None

For each chelator used, columns 2 to 4 indicate data obtained from the literature for three descriptors of chelator affinity for Fe(III): log β_n, log K_{eff}, and log K_{sol}. Column 5 indicates the percentage (mean ± SD for 3 to 5 experiments) of iron removed in 30 minutes by 0.5 mmol/L chelator from the open ghost membranes prepared from sickle RBC (starting with 16 nmol/mg free iron). Chelators found to be unable to remove any iron are indicated by "None" in column 5.

percentage of iron removed by the various chelators; all experiments were performed three to five times.

Dose-response study. These same analytical methods were also used to examine the dose- and time-response of the removal of membrane iron by deferoxamine, with dose varying from zero to 0.5 mmol/L and time varying from 0 to 11 hours.

DEFINITIONS

Table 1 indicates values obtained from the literature[9-14] for three relevant properties of the chelators used for this study: log β_n, log K_{eff}, and log K_{sol}.

The log of the stability constant, log β_n, indicates chelator affinity for iron in an idealized system without competing ions and with chelator fully dissociated and deprotonated and the metal not hydrolyzed.[9] Here the subscript n refers to the number of ligands per iron in the fully complexed chelate, so for $n > 1$ (ie, for all but the true hexadentate chelators) the value indicated is the cummulative stability constant.

The log of the effective stability constant, log K_{eff}, is more pertinent to physiologic conditions. It adjusts log β_n for the equilibrium constants for competing reactions[9] and thereby takes into account the reduction in affinity caused by such factors as chelator protonation, metal hydrolysis, and lack of chelator specificity for binding iron in the presence of calcium or other competing metal ions. Despite this, log K_{eff} still only adjusts for the interferences relevant to homogeneous aqueous solutions.

Table 1 also indicates the value of log K_{sol}. This parameter is similar to log K_{eff}, but it additionally takes into account the presence of an insoluble phase of iron and is influenced by chelator concentration and stoichiometry. In this situation, the chelator/metal equilibrium condition is complicated by the fact that the free metal concentration actually is governed and kept constant by the equilibrium between iron in the solution versus solid phases, as defined by its solubility product.[9] The specific values shown for log K_{sol} are for presence of ferric hydroxide as the insoluble phase, assuming a solubility product $[Fe][OH]^3 = 10^{-41}$ and a chelator concentration of the same order of magnitude that we have used in our study. Those chelators shown as having log $K_{sol} > 0$ are able to dissolve insoluble ferric hydroxide.[9] Essentially, this requires that the chelator at low concentration be able to keep the free iron concentration lower than that dictated by the solubility product for iron and the other solid phase component; for ferric hydroxide this limiting free $[Fe^{3+}]$ is about 10^{-20} mol/L.

RESULTS AND DISCUSSION

The 15 iron chelators used for this study (Table 1) were selected from a variety of structural categories (noted in Materials and Methods) to encompass a 40-log range of affinities for Fe(III). To indirectly assess the avidity of the red cell membrane for molecular Fe(III), we examined their ability to remove Fe(III) associated with the open ghost membranes prepared from sickle red cells. Results show that chelators may be divided into those that can versus those that cannot remove red cell membrane iron, as shown in the far right column of Table 1. With the informative exception of salicylic acid, chelators at the upper end of the affinity spectrum are able to remove membrane Fe(III): 2,3DNHS, pyrocatechol, HBED, 8OHQ, L1, tropolone, apo-transferrin, and deferoxamine. Indeed, the high-affinity chelator deferoxamine (log $\beta_n = 30.6$) actually is the lowest affinity agent capable of removing Fe(III). In contrast, all chelators at the lower end of the affinity spectrum are unable to remove membrane iron: the three unmodified aminocarboxylic acids (DTPA, EDTA, and NTA) and all three potential cytosolic chelators (citrate, ATP, ADP).

Significantly, idealized chelator affinity (as stated by the stability constant, β_n) is an imperfect predictor of the ability of chelators to remove Fe(III) from the red cell membrane. This is illustrated by the fact that salicylic acid, despite having a β_n that is nearly five logs greater than that of deferoxamine, was unable to remove membrane Fe(III). Nor is there any obvious relationship between chelator charge or hydrophobicity and ability to remove membrane Fe(III), because the effective chelators include some that are extremely hydrophilic (eg, deferoxamine) as well as some that are extremely hydrophobic (eg, 8OHQ and HBED). On the other hand, our results do indicate that the value of log K_{eff} predicts ability to strip Fe(III) from the red cell membrane. That this demarcation between effective and ineffective chelators occurs at log K_{eff} ~12 (between DTPA and pyrocatechol) allows us to estimate that the effective avidity of the membrane for molecular Fe(III) is on the order of 10^{12}. This conclusion is consistent with our earlier binding data[2] that would estimate the Fe(III) chelate with liposomal phosphatidylserine to have an association constant on the order of

10^{12}, assuming a stoichiometry of three phosphatidylserines per iron molecule.

Notably, this demarcation between ineffective and effective chelators also occurs at the transition between those having, respectively, negative versus positive values of log K_{sol}. Because our studies included chelators having only slightly positive and slightly negative values for log K_{sol}, this suggests that the solubility product for the insoluble iron phase in our experiments (ie, ferric iron on its membrane binding sites) is on the same order of magnitude as the solubility product for $Fe(OH)_3$. In turn, this predicts that the red cell membrane, like a precipitate of insoluble ferric hydroxide, can keep the free Fe(III) below the 10^{-20} mol/L threshold predicted by the $Fe(OH)_3$ solubility product, thus additionally arguing for the existence of extremely high membrane avidity for Fe(III). This conclusion is consistent with our earlier observation that molecular iron cannot be detected by atomic absorption spectrophotometry (limit of detection = 2.5 nmol/mL) in hemoglobin-free cytosolic preparations from sickle and thalassemic red cells (Shalev and Hebbel, unpublished data, 1995).

We originally had hoped to use these chelators to establish a simple equilibrium condition that would allow us to formally assign an apparent association constant describing the avidity of the red cell membrane for molecular Fe(III). However, the membrane/chelator system does not achieve a simple equilibrium that leaves some iron on the membrane, as is evident in the time/dose response experiment shown in Fig 1. This situation, in fact, is consistent with log K_{sol} being an appropriate indicator of iron removability. In such a system, if sufficient quantity of chelator is present to be able to bind all iron present in the insoluble phase, then at equilibrium the chelator will do so, completely dissolving rust or, in the present case, completely removing membrane iron. To avoid confusion, it should be specifically noted that membrane-associated Fe(III) is not actually rust (insoluble ferric hydroxide), since earlier studies have documented its biochemical bioavailability.[4,5] Rather, our studies simply indicate that molecular Fe(III) is associated with very high affinity to the membrane, itself an insoluble particulate.

That the effective chelators removed varying amounts of Fe(III) in this study was not unexpected since we report data only at a single time point, our goal being to simply indicate amenability to chelation rather than other factors influencing rate of removal such as reaction kinetics, steric factors, hydrophobicity, and so on. For example, 8OHQ and HBED very probably do chelate membrane Fe(III) very effectively, but they are so hydrophobic that they tend to remain within the membrane bilayer as a chelator/metal complex and thus are observed to remove little iron from it. The chelators indicated in Table 1 as being unable to remove membrane iron remain unable to do so even at longer time points (data not shown), so differences depicted in the table do reflect true thermodynamic ineffectiveness rather than just kinetic sluggishness.

In summary, our results reveal that potential physiologic cytosolic chelators (eg, citrate and nucleotides) are unable to prevent or reverse iron accumulation on the membrane of pathologic red cells because of the extremely high avidity of the membrane for Fe(III). While pathologic red cell membranes have Fe(III) associated with both phospholipid[2] and denatured hemoglobin,[6] our studies to date neither exclude nor confirm existence of a two-component system having free Fe(III) associated with two different affinities. In aggregate our past and present studies thus argue that membrane-associated molecular Fe(III) truly is pathologic since it not only is biochemically active but also cannot be removed by physiologic chelators.

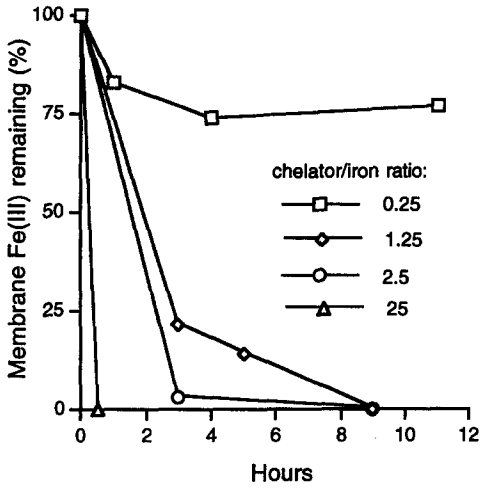

Fig 1. Dose- and time-response of membrane molecular iron removal by deferoxamine. Open ghost membranes prepared from sickle RBC (starting with 20 nmol/mg molecular iron) were incubated for varying times at various deferoxamine concentrations. As long as chelator quantity in the system is sufficient to bind all membrane iron, it all is removed if enough time is allowed (eg, at chelator: iron molar ratios greater than 1). A plateau is reached only if the amount of chelator is insufficient in quantity to bind all iron present (ie, at chelator: iron molar ratios <1). In that case, the amount of iron removed corresponds precisely to that predicted from the chelator: iron molar ratio, so the plateau is only an artifact of this fact and does not represent establishment of an equilibrium condition.

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