# Exchange of Iron by Gallium in Siderophores†

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ABSTRACT: Siderophores are iron transport compounds produced by numerous microorganisms and which strongly chelate Fe(III), but not Fe(II). Other trivalent metals, such as Al(III), Cr(III), or Ga(III), are not capable of significantly displacing iron from siderophores. However, I demonstrate here that Ga(III) can effectively displace iron under reducing conditions. With ascorbate as reductant and ferrozine as Fe(II) trapping agent, the kinetics of reductive displacement of iron by Ga(III) were followed spectroscopically by the increase of absorbance at 562 nm due to formation of the Fe(II)–ferrozine complex. No significant reduction of siderophore occurred in the absence of Ga(III). With excess Ga(III), the displacement was quantitative and very rapid. The rate of metal exchange was pseudo first order with respect to Ga(III) concentration and highly pH dependent, suggesting that siderophore ligands are displaced from the iron in a concerted mechanism by Ga(III) and protonation to expose the Fe(III) to reduction by ascorbate. Reaction rates were dependent upon the structure of the siderophore, being greatest for ferric rhodotorulic acid and slowest for ferrichrome A at pH 5.4. The pH profile for ferric rhodotorulic acid was unusual in that it showed a maximum at pH 6.5, while all other siderophores examined showed an increase in rate as pH was lowered from 7.0. The physiological significance of this reaction to the clinical use of gallium is discussed.

When salts of Ga(III) are injected into the bloodstream of animals, the metal is concentrated in pathological tissues, such as abscesses and tumors (Edwards & Hayes, 1969). Gallium-67 scans are now used routinely in diagnostic radiology clinics to detect these abnormal tissues. However, the mechanism of concentration of the metal under physiological conditions is still unknown. In normal individuals, injection of gallium leads to its accumulation in tears and in the milk of lactating women (Hoffer et al., 1977). Since these tissues contain the iron binding protein lactoferrrin, it was suggested that the gallium ion may mimic the ferric ion in vivo (Emery & Hoffer, 1980). Both ions have three positive charges, have nearly identical ionic radii, and form octahedral complexes with oxygen ligands, suggesting complexation of gallium by iron binding proteins. The concentration of gallium in tumor and microbial cells may thus be due to the increased iron requirement during the rapid proliferation of these tissues, gallium being mistaken for iron.

Little is known about the mechanism of iron uptake by tumor tissue, but microbial iron transport has been studied in detail for a number of years. Microorganisms utilize low molecular weight Fe(III) chelates, called siderophores, to sequester iron by specific transport mechanisms (Raymond et al., 1984). It has long been known that the hydroxamic acid functionality of siderophores can chelate aluminum and gallium in addition to iron (Francis et al., 1953; Emery, 1971). Detailed nuclear magnetic resonance studies of the gallium chelate with tris(hydroxamato)-type siderophore ligands have demonstrated that there is little conformational difference between the iron and gallium chelates (Llinâs et al., 1970). We have shown that if Fe(III) of ferrichrome is chemically replaced with Ga(III), the ferrichrome uptake system of the fungus *Ustilago sphaerogena* is tricked and takes up the gallium derivative at a rate identical with that of ferrichrome itself (Emery & Hoffer, 1980). This observation supports the hypothesis that Ga(III) minics Fe(III) in vivo. Recently, Tufano and Raymond (1981) have shown that Fe(III) can exchange between siderophore ligands, or between siderophores and a competing ligand, such as ethylenediaminetetraacetate (EDTA), but the rate is too slow to be of physiological significance at near-neutral pH. We wished to examine the possibility of displacement of Fe(III) by Ga(III) in siderophores by similar exchange mechanisms because of its relevance to the clinical use of $^{67}$Ga described above. Our previous work demonstrated that Fe(III) is bound by siderophore ligands much more strongly than Ga(III), so it would seem unlikely that concentrations of gallium would ever be sufficient in a whole animal to bring about any significant metal replacement. However, it is now recognized that iron can be reductively removed from siderophores in vivo (Ecker et al., 1982). Ferrous ion is believed to be important for iron transport in both plants and animals (Castingnetti & Smarrelli, 1984; Topham et al., 1982), as well as the substrate for heme synthesis catalyzed by ferrochelatase (Lascelles, 1964). Since no divalent oxidation state of gallium is known, the displacement of iron by gallium in siderophores could be driven to completion by trapping of Fe(II), as might occur by heme biosynthesis. In this paper, we demonstrate that under reducing conditions, Ga(III) can rapidly displace Fe(III) from siderophores, whereas without concerted reduction of the iron there is no significant exchange. The physiological significance is discussed.

## Experimental Procedures

*Materials.* Metallic gold-label 99.9999% gallium was obtained from Aldrich. A carefully weighed amount was dissolved in reagent-grade 3 N $HNO_3$ at 50 °C and taken to dryness several times to remove excess acid. The residue was dissolved in deionized water to give a 1.00 M stock solution of $Ga(NO_3)_3$. Siderophores were isolated and purified from the appropriate organism according to literature procedures:

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ferrichrome and ferrichrome A from *Ustilago sphaerogena* (Garibaldi & Neilands, 1955), rhodotorulic acid from *Rhodotorula pilimanae* (Atkin & Neilands, 1968), triacetylfusarinine C from *Penicillium sp.* (Moore & Emery, 1976), malonichrome from *Fusarium roseum* (Emery, 1980). Deferriferrioxamine B was obtained from the local hospital pharmacy as the methanesulfonate salt under the trade name of Desferal. Ferrioxamine B was prepared by addition of a standard solution of $FeCl_3$ to deferrioxamine B, maintaining a 10% excess of ligand to metal to ensure that no uncomplexed metal remained in solution. The ferric rhodotorulate complex was prepared similarly to produce $Fe_2$(rhodotorulate)$_3$, again maintaining a 10% ligand to metal excess. All iron complexes gave a single spot on thin-layer chromatography and paper electrophoresis. Stock solutions, typically 4.0 mM, of these siderophores were prepared in the appropriate buffer. Concentrations were determined spectroscopically by using published extinction coefficients (see above references). Ferrozine was obtained from Aldrich. All other chemicals were from commercial sources and of the highest purity available.

*Procedures.* The reductive displacement of iron from siderophores by gallium was followed spectroscopically. Stock solutions of the following compounds were prepared: siderophore (4.0 mM), ascorbate (200 mM), $Ga(NO_3)_3$ (10 mM), and ferrozine (20 mM). Ascorbate was made fresh immediately before use, and ferrozine was kept in the cold out of light. Ascorbate and ferrozine solutions were adjusted to the appropriate pH with sodium hydroxide before use. Buffers were acetate at pH 4.0, 4.7, and 5.4 and 4-morpholinepropanesulfonic acid (MOPS) at pH 6.5, 7.0, and 7.4.

The reaction mixture contained the following in a final volume of 3.0 mL: buffer (0.5 M), ascorbate (20 mM), ferrozine (2 mM), and siderophore (0.05 mM). The reaction was initiated by addition of the gallium nitrate solution to the desired concentration. After rapid mixing, the absorbance at 562 nm was followed at 22 ± 0.5 °C on a Varian 624 dual-beam spectrophotometer. Gallium was omitted from the reference cell. Kinetic constants were obtained from the slope of the rate curves, log $[s/(s-x)]$ vs. time, $s$ being the initial siderophore concentration, typically 0.05 mM, and $x$ the concentration of the Fe(II)–ferrozine complex determined from absorbance at 562 nm.

## Results

Displacement of Fe(III) by Ga(III) in hydroxamate-type siderophores can be conveniently followed spectroscopically. Tris(hydroxamato)-type siderophores have absorption maxima in the 420–440-nm range with extinction coefficients of approximately 3 $mM^{-1}$ $cm^{-1}$. Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid] forms a complex with Fe(II) with an absorption maximum at 562 nm and an extinction coefficient of 29.1 $mM^{-1}$ $cm^{-1}$ (Stookey, 1970). At the concentrations of siderophores used in this study, there is negligible contribution to the higher wavelength, and the increase of absorbance at 562 nm could be used as a direct measure of the amount of iron reductively displaced from the siderophore. Because of its physiological role in iron metabolism, ascorbate was used a reductant. In the absence of gallium, there is no significant reductive displacement of Fe(III) from siderophores at pH 5.4, a pH chosen for direct comparison with published iron exchange data (Tufano & Raymond, (1981). The standard redox potential of hydroxamate-type siderophores is approximately −400 mV, and a powerful reductant such as hydrosulfite is used in the laboratory to reduce siderophore iron. Upon addition of a 10-fold excess of Ga(III), immediate and rapid reduction was observed

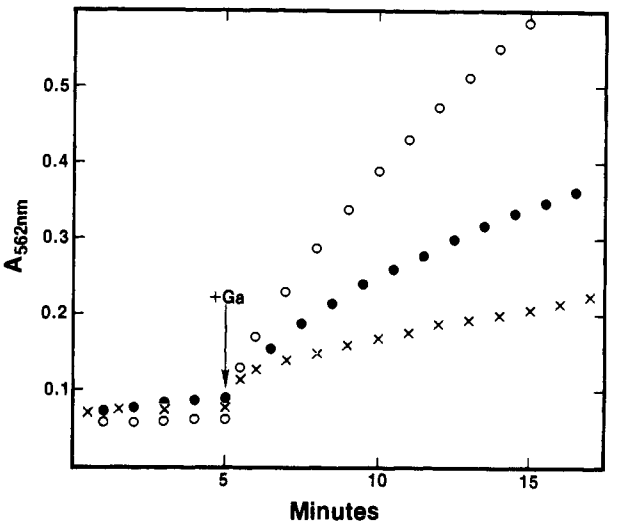


FIGURE 1: Reductive displacement of iron from siderophores by gallium at pH 5.4. The reaction mixture contained the following in a final volume of 3.0 mL: acetate buffer, 0.5 M; ascorbate, 20 mM; ferrozine, 2 mM; siderophore, 0.05 mM. At 5 min, $Ga(NO_3)_3$ was added to a final concentration of 0.5 mM. Siderophores were ferrichrome A (×), ferrioxamine B (●), and ferrichrome (○).

as evidenced by the increase of absorption at 562 nm due to formation of the ferrous–ferrozine complex (Figure 1). Spectral scans showed concomitant disappearance of the siderophore peak at 420–440 nm (not shown). The dramatic difference in rates exhibited for ferrichrome, ferrioxamine B, and ferrichrome A demonstrates that the course of the reaction is highly dependent upon siderophore structure. With the exception of the ferric rhodotorulate chelate (see below), rates of reduction were very low at all pH values examined in the absence of added gallium. All rates were corrected for these low background rates.

At a lower pH and with a large excess of Ga(III), the reductive displacement of Fe(III) by Ga(III) was quantitative. At pH 4, addition of 17 mM gallium nitrate to 0.039 mM ferrichrome under the same conditions described for Figure 1 caused greater than 95% reductive displacment of iron within 10 min. Similar results were observed with ferrichrome A and ferrioxamine B. This offers a convenient means of preparing the gallium chelates of siderophores without the necessity of prior removal of the iron. Following exchange, the gallium deferrisiderophore chelate can be quantitatively extracted into an appropriate organic solvent and purified by the same methods used for siderophores themselves (Garibaldi & Neilands, 1955). Because of the charged sulfonic acid groups of ferrozine, the ferrous–ferrozine complex remains in the aqueous phase. In the absence of reducing agent, gallium is quite ineffective in displacing Fe(III) from most siderophores, with the exception of ferrichrome which binds iron relatively weakly (Emery & Hoffer, 1980). Even in the case of ferrichrome, however, equilibration is slow and proceeds to only a limited extent (see Discussion).

The data of Figure 2 show that over a 4-fold concentration range the rate of the gallium–iron exchange reaction is psuedo first order with respect to gallium. Ferrozine, as ferrous trapping agent, and ascorbate, as reductant, were present in 40- and 400-fold excess, respectively, over siderophore concentration. A 10-fold decrease in the concentration of either ferrozine or ascorbate had no effect on the exchange reaction rate. The reaction was not sensitive to ionic strength. Addition of 0.2 M sodium chloride did not affect the reaction rate.

To compare quantitatively reaction rates for several siderophores, a 1:1 gallium to siderophore concentration ratio was

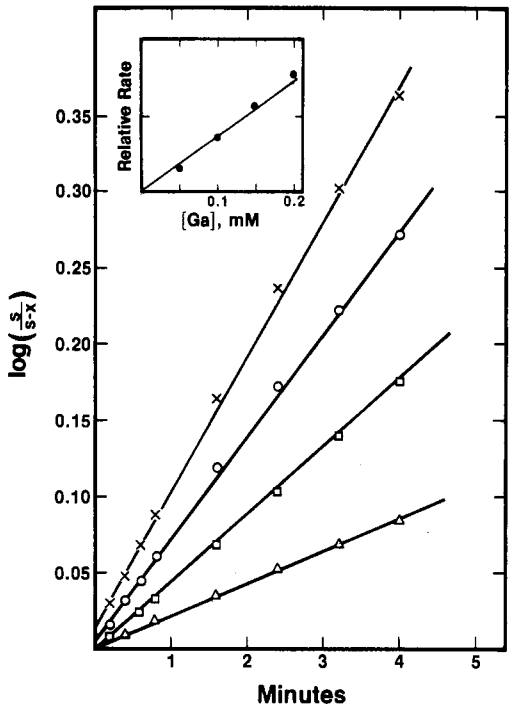


FIGURE 2: Effect of Ga(III) concentration on rate of iron displacement from ferrioxamine B. Conditions were as for Figure 1 except that gallium concentrations were 0.05 (△), 0.10 (□), 0.15 (○), and 0.2 mM (×). The concentration of ferrous–ferrozine complex formed [ferrisiderophore reacted (×)] was determined by the increase of absorbance at 562 nm (see text). Initial concentration of ferrisiderophore, *s*, was 0.05 mM.

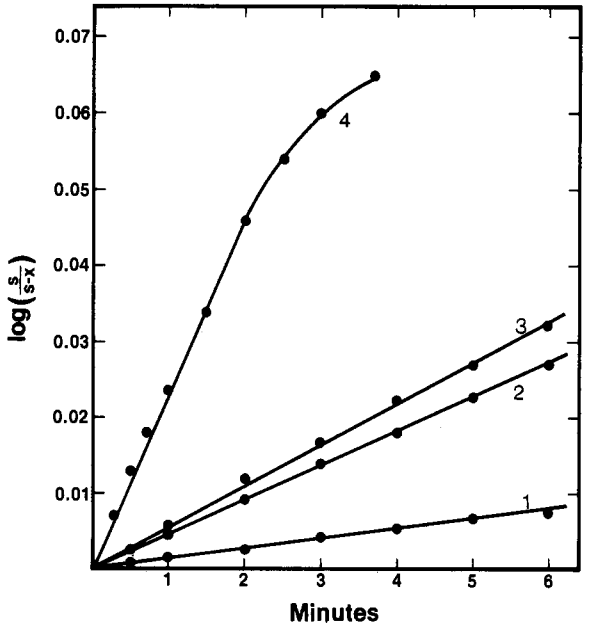


FIGURE 3: Gallium–iron exchange rates for siderophores. Conditions were as for Figure 1 except the concentration of Ga(III) was 0.05 mM. Siderophores were (1) ferrichrome A, (2) ferric triactylfusarinine C, (3) ferrichrome, and (4) ferric rhodotorulic acid.

used at pH 5.4. Semilog plots of reaction progress with time showed that the reaction was pseudo first order for several siderophores during the initial phase of the reaction (Figure 3). Calculated pseudo-first-order rate constants for rhodotorulic acid, ferrichrome, triacetylfusarinine C, and ferrichrome A are $8.4 \times 10^{-4}$, $2.1 \times 10^{-4}$, $1.7 \times 10^{-4}$, and $0.48 \times 10^{-4}$ $s^{-1}$, respectively. In a separate experiment, the rate constant for malonichrome was found to be $3.1 \times 10^{-4}$ $s^{-1}$. At this pH,

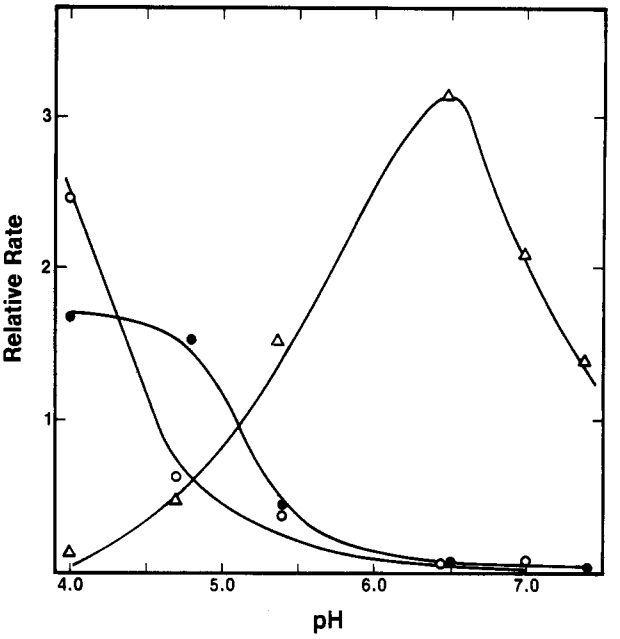


FIGURE 4: Rate–pH profiles for ferrichrome (○), ferrioxamine B (●), and ferric rhodotorulic acid (△). Conditions were as for Figure 1 except that the gallium concentration was 0.05 mM.

ferrioxamine B gave results virtually identical with ferrichrome. Note that this was not the case when an excess of gallium was used (Figure 1).

If ferrozine is omitted from the reaction mixture, the reaction may still be followed by monitoring the decrease in absorbance in the 420–440-nm region due to reductive removal of iron from the siderophore itself. In this case, the Fe(II) produced is undoubtedly immediately complexed by the excess ascorbate present. Thus, the reaction does not depend upon trapping of Fe(II) with ferrozine so long as reoxidation of the iron is prevented. However, the extinction coefficient of Fe(II)–ferrozine is about 10 times greater than that of siderophores, so a 10-fold increase in sensitivity is gained by utilizing ferrozine as trapping agent. Therefore, no detailed studies were done in the absence of ferrozine; however, the initial rate of reduction of ferrichrome with or without added ferrozine was the same within experimental error.

Chromium is know to form octahedral chelates with hydroxamate-type siderophores when vigorously reacted with the free siderophore ligand. However, Cr(III) is quite exchange inert and is not capable of significantly displacing Fe(III) from its complexes in aqueous solution. We found that addition of a 10-fold excess of chromic chloride under the same conditions as used for the gallium experiments resulted in no significant exchange. Measurable reaction was observed, however, with a 100-fold excess of the metal. At pH 5.4 and 5.0 mM Cr(III), the initial rate was 16% that observed with 0.05 mM Ga(III).

Addition of sodium citrate at a concentration equal to that of the Ga(III) completely inhibited the reductive exchange reaction, probably because of complex formation with the gallium (see Discussion).

The exchange of Fe(III) between siderophore ligands or between a siderophore and EDTA is highly pH dependent (Tufano & Raymond, 1981). A mechanism was proposed involving initial protonation of one of the hydroxamate ligands followed by a bimolecular reaction of the ferric siderophore with the competing ligand. Rates were found to be very slow above pH 5. The effect of pH on reductive iron displacement by gallium from several siderophores is shown (Figure 4). No detectable rate was observed at pH 7.0 over this time scale.

The precipitous decrease of the rate in the region of pH 5 is in agreement with the results cited above on iron exchange.

In general, lowering the pH greatly increased the reaction rate for all siderophores examined in a manner similar to that observed for ferrioxamine B. However, for a fixed Ga:siderophore ratio of 1, each siderophore exhibited its own peculiar pH profile. The rapid decrease in rate above pH 5 that was observed for ferrioxamine B was also found for ferrichrome. Most unusual, however, was the pH reaction profile for ferric rhodotorulic acid, which exhibited a maximum rate of exchange at a pH of approximately 6.5 and decreased, rather than increased, at lower pH. It is noteworthy that the rate of exchange of iron from ferrioxamine B to the catechol-type iron chelate MECAM also showed a maximum at around pH 6.5, although the rate did again increase below pH 5 (Raymond et al., 1984).

## Discussion

We previously showed that Ga(III) is the only known metal whose kinetic lability and thermodynamic stability allow it to significantly displace Fe(III) from siderophores (Emery & Hoffer, 1980). Nevertheless, the much greater stability of the ferric siderophore chelate allows metal substitution to proceed to only a limited extent. For instance, a 10-fold excess of Ga(III) only displaces about 35% of the iron from ferrichrome A, and displacement of iron from triacetylfusarinine C is negligible even at pH 4. A typical dose of Ga(III) used clinically is 25 pmol, and it is unlikely that this low concentration would suffice to cause any direct displacement of iron from siderophores resulting from microbial infection, or from a mammalian protein such as transferrin.

Thus, the question of how iron can be displaced in vivo from extremely stable siderophore chelates is still open. Tufano and Raymond (1981) proposed that non-reductive exchange of Fe(III) between siderophore ligands, or competing ligands such as EDTA, involves protonation of one of the three hydroxamate groups, causing its displacement from the metal and thus exposing the inner coordination sphere of the metal to complexation by the second chelator. Monzyk and Crumbliss (1983) found that simple hydroxamic acids could also catalyze the exchange of iron from ferrioxamine B into EDTA, and their data support a mechanism involving ternary and tris(hydroxamato) complexes.

We now demonstrate an alternate mechanism for iron displacement from ferrisiderophores involving reduction of the metal. In the presence of an appropriate reducing agent, such as ascorbate, Ga(III) can readily displace Fe(III) from ferrisiderophores with concomitant reduction of the iron. Under these conditions, even a stoichiometric concentration of Ga(III) can effectively displace iron. In the absence of gallium, no significant reduction occurs, which demonstrates that reduction is concerted with metal ion exchange. Our results are in agreement with those cited above wherein nonreductive exchange of Fe(III) from siderophore ligands involves exposure of the inner coordination sphere of the iron. In our case, exposure of the Fe(III) would lead to its reduction by ascorbate, a reaction prevented when the metal is in strong hexacoordinate chelation. Gallium ions and protons can compete for the hydroxamate ligand in a concerted manner; at pH 5.4, neither one alone is sufficient to cause significant reaction.

Gallium and hydrogen ion are not equivalent in their efficacy in displacing a hydroxamate ligand from iron, as demonstrated by the unusual pH dependency of reductive gallium–iron exchange for different siderophores (Figure 4). The rate of exchange of iron from ferrioxamine B into EDTA is directly proportional to the hydrogen ion concentration (Tufano & Raymond, 1981). In the gallium reaction, each siderophore shows its own unique pH dependency for which no obvious explanation can be given based on the known chemistry of the compounds. Especially unusual is the pH dependency of the reductive gallium–iron displacement for rhodotorulic acid. Rhodotorulic acid forms a considerably weaker chelate with Fe(III) than most siderophores, having a p$M$ value of 21.9 as compared to 25.2 for ferrichrome and 26.6 for ferrioxamine B (Raymond et al., 1984). Therefore, the greater reaction rate for the ferric rhodotorulate chelate above pH 5 as compared to other siderophores was not unexpected. Surprising was its low reactivity below pH 5. Below pH 4, rates could not be compared because of the spontaneous reduction of rhodotorulate iron by ascorbate even in the absence of gallium. This unusual behavior is similar to that observed for iron exchange from ferrioxamine B into MECAM (Raymond et al., 1984).

Citric acid has a strong inhibitory effect on the ability of Ga(III) to reductively displace Fe(III) from siderophores. In the pH range of 2–6, gallium citrate is known to form stable polymers which are kinetically quite inert (Glickson et al., 1975). Thus, in the presence of citrate, the coordination sphere of the gallium is not available for competition with the hydroxamate ligand, and the exchange reaction is prevented.

Like siderophores, transferrin strongly binds Fe(III) but has a very low affinity for Fe(II) (Bates et al., 1973). Lactoferrin is similar. The precise mechanism by which Fe (III) is removed from transferrin in vivo is still unknown. Johnston and Cottingham (1969) described an in vitro system in which ascorbate reduced the iron of transferrin in the presence of the Fe(II) trapping agent 1,10-phenanthroline. Our system is very analogous but demonstrates that the presence of Ga(III) during such a reaction would greatly enhance the rate and enable the incorporation of gallium into the protein or siderophore, should the latter be present in the course of microbial infection. The inhibitory effect of citrate that we have observed might argue against this mechanism of gallium incorporation; clinically, the gallium is injected with a large excess of citrate. However, excess citrate would probably be rapidly diluted and metabolized in the body.

## Acknowledgments

I greatly appreciate the technical assistance of Dr. Jeffrey Lodge and Liliane Emery.

**Registry No.** Fe, 7439-89-6; Ga, 7440-55-3; ascorbic acid, 50-81-7; ferrichrome A, 15258-80-7; ferrioxamine B, 14836-73-8; ferrichrome, 15630-64-5; ferric triacetylfusarinine C, 59200-35-0; ferric rhodotorulic acid, 67740-26-5.

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# Amino Acid Sequence of the b Subunit of Human Factor XIII, a Protein Composed of Ten Repetitive Segments†

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ABSTRACT: Factor XIII is a plasma protein that participates in the final stages of blood coagulation. The complete amino acid sequence of the b subunit of human factor XIII was determined by a combination of cDNA cloning and amino acid sequence analysis. A λgt11 cDNA library prepared from human liver mRNA was screened with an affinity-purified antibody against the b subunit of human factor XIII. Nine positive clones were isolated from $2 \times 10^6$ phage and plaque-purified. The largest cDNA insert was sequenced and shown to contain 2180 base pairs coding for a portion of the leader sequence (19 amino acids), the mature protein (641 amino acids), a stop codon (TGA), a 3′ noncoding region (187 nucleotides), and a poly(A) tail. When the b subunit of human factor XIII was digested with cyanogen bromide, nine peptides were isolated by gel filtration and reverse-phase high-performance liquid chromatography. Amino acid sequence analyses of these peptides were performed with an automated sequenator, and 299 amino acid residues were identified. These amino acid sequences were in complete agreement with the amino acid sequence predicted from the cDNA. The b subunit of factor XIII contained 10 repetitive homologous segments, each composed of about 60 amino acids and 4 half-cystine residues. Each of these repeated segments is a member of a family of repeats present in human $\beta_2$-glycoprotein I, complement factor B, and haptoglobin $\alpha^1$ chain. Three potential Asn-linked carbohydrate attachment sites were also identified in the b subunit of factor XIII.

Factor XIII (fibrin stabilizing factor, fibrinoligase, or plasma transglutaminase) is a plasma glycoprotein that circulates in blood as a proenzyme. During the final stages of blood coagulation, thrombin converts the proenzyme to an active form called factor $XIII_a$. Factor $XIII_a$ is a transglutaminase that catalyzes the polymerization of fibrin monomers through the formation of intermolecular ε-(γ-glutamyl)lysine bonds. This reaction occurs in the presence of $Ca^{2+}$ [see Folk and Finlayson (1977) and Lorand et al. (1980) for reviews].

The cross-linking reactions catalyzed by factor $XIII_a$ lead to dimerization of the γ chains of fibrin (γ-dimerization) (Chen & Doolittle, 1970) followed by polymerization of the α chains of fibrin (α-polymerization) (Pisano et al., 1972). The γ-dimerization and α-polymerization reactions result in a fibrin with considerable mechanical strength (Lorand, 1972; Roberts et al., 1973; Mockros et al., 1974; Shen & Lorand, 1973; Shen et al., 1975) and an increase in its resistance to proteolytic degradation by plasmin (Lorand & Jacobsen, 1962; Gaffney & Whitaker, 1979). Factor $XIII_a$ also catalyzes a cross-linking between the α chain of fibrin with fibronectin (Mosher, 1975; Tamaki & Aoki, 1981) and the α chain of fibrin with $\alpha_2$-plasmin inhibitor (Sakata & Aoki, 1980; Tamaki & Aoki, 1981). The cross-linking of collagen and fibronectin is also catalyzed by factor $XIII_a$ (Mosher & Schad, 1979), and this reaction appears to be related to wound healing (Duckert, 1972; Folk & Finlayson, 1977; Lorand et al., 1980).

The cross-linking of $\alpha_2$-plasmin inhibitor to fibrin (Tamaki & Aoki, 1981) or fibrinogen (Ichinose & Aoki, 1982) in the presence of factor $XIII_a$ occurs at a faster rate than with other proteins. Accordingly, in plasma, $\alpha_2$-plasmin inhibitor and fibrin are considered to be the best amino acceptor and amino donor, respectively, for factor $XIII_a$ (Tamaki & Aoki, 1982; Carmassi & Chung, 1983). Deficiencies of either factor XIII or $\alpha_2$-plasmin inhibitor result in "delayed bleeding", while primary hemostasis in individuals with these traits is normal (Folk & Finlayson, 1977; Aoki et al., 1979; Lorand et al., 1980). This suggests that these proteins also play an important role in the protection of the fibrin clot from digestion by plasmin (Sakata & Aoki, 1980, 1982).

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