Siderophore-Mediated Mechanism of Gallium Uptake Demonstrated in the
Microorganism Ustilago sphaerogena

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The radioactive gallium analog of ferrichrome, Ga-67 deferriferichrome, has been prepared and compared with ferrichrome in the specific siderophore-transport system of Ustilago sphaerogena. The gallium analog is taken up by the cells in an active transport process indistinguishable from that of ferrichrome. Ga(III) effectively competes with Fe(III) for siderophore ligands both in vitro and in vivo at a rate that is highly dependent upon the chemical nature of the ligand. The findings may explain how Ga(III) mimics Fe(III) in clinical use.


The extreme insolubility of ferric ion at neutral pH is a formidable problem faced by all living organisms. Microorganisms have adapted to this problem by the excretion of iron-chelating agents of low molecular weight that are capable of solubilizing extracellular oxides of iron and even metallic iron (1). The resulting chelates (siderophores) act as specific ferric ionophores to transport the metal into the cell. In animals, the role of transferrin for the specific transport of iron in a soluble form is well known. Metals similar to ferric ion in size and charge may substitute chemically for iron in these compounds. For example, it has been shown that trivalent chromium forms a stable chelate with siderophore ligands, and the resulting chelates are taken into the cells through the normal iron-transport system (2). However, no physiological importance has ever been attributed to this phenomenon.

Radioactive Ga-67 citrate has been used during the past decade for tumor and abscess detection (3,4). The injected gallium may also be localized in secretions, including tears and human milk, and this observation has been traced to chelation of the metal by the iron-binding protein lactoferrin (5,6). A gallium-lactoferrin complex has also been demonstrated within human leukocytes following in vitro incubation of the leukocytes in medium containing Ga-67 citrate (7). These observations that gallium mimics iron in mammalian storage and transport systems, and their potential clinical relevance, have prompted us to investigate this phenomenon in a simple microbial system. The results described here demonstrate that gallium is an effective competitor of iron in siderophore-transport systems, and they also suggest a direct mechanism by which Ga-67 may be incorporated into microorganisms contained in inflammatory lesions.

**MATERIALS AND METHODS**

Ferrichrome and ferrichrome A were isolated from cultures of *Ustilago sphaerogena* and the deferri derivatives prepared as previously described (8). Ferrioxamine B was prepared from deferrioxamine mesylate. N,N',N''-triacetylfusarinine C (triacetylfusigen) was obtained from cultures of *Pencillium sp.*, and its iron chelate prepared as previously described (10). All siderophore solutions were assayed spectrophotometrically using the following millimolar extinction coefficients: ferrichrome, 2.9 at 425 nm; ferrichrome A, 3.4 at 440 nm; triacetylfusarinine C, 3.0 at 440 nm; ferrioxamine B, 2.5 at 430 nm. Deferrisiderophore solutions were assayed by the addition of 0.1 ml of a solution of 0.01 M ferric perchlorate in 0.1 N nitritotriacetic acid to 2.9 ml of the deferrisiderophore solution, and reading the absorbance at the appropriate wavelength (above) against
EMERY AND HOFFER

a blank containing the ferric nitrilotriacetic acid. No increase in absorbance upon addition of an additional 0.05 ml of the ferric nitrilotriacetic acid ensured that all ligand was chelated to iron.

A stock solution of Ga(NO₃)₃ was prepared by dissolving Gold Label metallic gallium* in 3 N HNO₃ at 50° and repeatedly taking the solution to dryness to remove excess acid. The salt was then dissolved in deionized water to give a 0.50 M stock solution. Gallium-67 deferriferrichrome was prepared as follows. To 0.5 ml of 0.01 M Ga(NO₃)₃ was added about 10 μCi of carrier-free ⁶⁷Ga(Cl) followed by 1.0 ml of 5.9 mM deferriferrichrome. After it had stood at room temperature for 30 min, 3 mg of citric acid was added and the solution allowed to stand an additional 10 min. The pH was then adjusted to 6–7 with 0.3 N KOH, and the solution saturated with ammonium sulfate. The gallium chelate was extracted into benzyl alcohol and purified as previously described for ferrichrome (8). The final aqueous solution of Ga-67 deferriferrichrome had a specific activity of approximately 1 μCi/μmole. Control experiments showed that negligible radioactivity extracted into benzyl alcohol in the absence of a siderophore ligand. Gallium chelates with other siderophore ligands were prepared similarly, but phenol:chloroform (1:1 by volume) was used to extract the chelates of deferrioxamine B and triacetylfusarinine C.

Uptake experiments using 24-hr washed-cell suspensions of U. sphaerogena (ATCC 12421), 17 mg/ml dry wt., and 0.036 mM substrate, were performed exactly as previously described (9), except that a centrifuge† was used to separate cells rapidly from the uptake medium. Uptake runs with Ga-67 deferriferrichrome and [⁵⁹Fe(III)] ferrichrome were repeated eight times. The same batch of 24-hr cells was always used in the comparison of uptake rates. Individual batches of cells could vary as much as 20% in the absolute rate of uptake; however, with a given batch of cells the uptakes of the two chelates always fell within the statistical accuracy of the counting data over the entire time period, and were almost quantitative by the end of 90 min.

Ligand-exchange experiments were performed by following the increase of absorbance upon addition of ferric nitrilotriacetic acid to the gallium chelates, or the decrease in absorbance upon addition of gallium citrate to solutions of the siderophores, at the wavelength of maximum absorption of the siderophore. The gallium chelates have no absorption in the visible region of the spectrum. Each exchange experiment was repeated at least three times, and the results were reproducible to better than ±10%. Typical exchange curves are shown in Fig. 2.

RESULT AND DISCUSSION

Figure 1 shows typical uptake results for the iron-labeled ferrichrome compared with uptake of the gallium-labeled derivative. The data also show the complete inhibition of uptake of the gallium derivative by 1 mM sodium azide. Inhibition by azide has also been shown for ferrichrome uptake (9), and this is indicative of an active transport process. Heating the cell suspension to 100° for 10 min also completely abolishes uptake of both ferrichrome and Ga-67 deferriferrichrome. We have previously shown by the use of C-14-labeled ligand that ferrichrome ligand is released from the cells during the late stages of ferrichrome uptake, and this was attributed to cellular removal of the metal from the siderophore by reduction to the ferrous state by ferrichrome reductase (11). We did not observe this phenomenon in the case of the C-14-labeled gallium derivative, nor would it be expected because of the inability of Ga(III) to be reduced to the divalent state.

Because gallium citrate is the form of the metal used clinically, we were interested in determining how actively growing cells would handle this chelate in contrast to Ga-67 deferriferrichrome. Fifteen μmoles of Ga-67 citrate containing about 50 μCi of Ga-67 were added to 400 ml of an actively growing 24-hr culture of U. sphaerogena at 25°C. At the end of 1 hr, 96% of the gallium had disappeared from the growth medium. The cells were collected by centrifugation, washed twice with cold water, and resuspended in 10 ml of water at 100°C. Heat treatment has been shown to release intracellular siderophores (9). After centrifugation of cell debris, over 90% of the counts taken up by the cells were found in the

FIG. 1. Percent radioactivity (Fe-59 or Ga-67) taken up by cell suspension of Ustilago sphaerogena. Cell concentration was 17 mg/ml, dry wt., and either [⁵⁹Fe] ferrichrome (-O-) or Ga-67 deferriferrichrome (-□-) at a final concentration of 0.036 mM was added at zero time. Uptake was followed at 30° as previously described (9). To a control flask of the gallium-labeled derivative was added 1.0 mM sodium azide.

936 THE JOURNAL OF NUCLEAR MEDICINE
FIG. 2. Exchange of Ga(III) and Fe(III) in siderophores. Decrease of visible absorption occurs when Fe(III) is displaced by Ga(III), increase of absorption when Ga(III) is displaced by Fe(III), (-O-): 0.25 mM ferrichrome A plus 2.5 mM gallium citrate; (-•-): 0.25 mM Ga deferriferrichrome A plus 2.5 mM Fe(III) nitritotriacetate; (-△-): 0.25 mM ferrichrome A plus 2.5 mM AlCl₃ or CrCl₃. All solutions buffered at pH 4.0 in 0.05 M acetate, 25°C. Insert: Dependence of Ga-Fe exchange rates upon siderochrome ligand. Tenfold excess of gallium citrate was added to ferrichrome (-•-), ferrioxamine B (-O-), or triacetylfusarinine C (-△-), all at a final concentration of 0.2 mM. All solutions buffered at pH 4.0 with 0.05 acetate, 25°C. Note contrasting time scales.

Ga-67 deferriferrichrome and 55% as Ga-67 deferriferrichrome A. Although ferrichrome A is produced by U. sphaerogena, this chelate has been shown not to act as a ferric ionophore (9).

The above results suggested that although gallium is transported into the cells as the gallium deferriferrichrome chelate through the ferrichrome transport system, trivalent gallium is capable of rapid intracellular exchange between siderophore ligands. Because of the possible clinical significance of ligand exchange, we investigated exchange of Ga(III) and siderophores, making use of the visible absorption of siderophores and absence of such absorption of the gallium analogs. Figure 2 shows the rapid decrease in visible absorption when a tenfold excess of Ga(III) is added to a solution of ferrichrome A. The rapid approach to equilibrium not only demonstrates the kinetic lability of the Ga-Fe system, but also shows that gallium is thermodynamically competitive with iron for the ferrichrome A ligand. The kinetic lability is also demonstrated in the reverse experiment, in which Fe(III) is added to gallium deferriferrichrome A. Although exact binding constants are difficult to calculate because of competing hydrolysis reactions, the spectral data allowed us to estimate that iron is only soluble supernatant. These counts were quantitatively extractable into benzyl alcohol. Controls showed that gallium citrate does not extract into benzyl alcohol, strongly suggesting that all of the cellular gallium was in the form of siderophore-type chelates. To confirm this, the radioactive material was re-extracted into water by addition of ether to the benzyl alcohol, and the aqueous solution was examined by paper chromatography and electrophoresis. The gallium derivatives of siderophore ligands behave exactly as the siderophores on electrophoresis and chromatography. Paper electrophoresis at pH 5.1 followed by radioactive scanning revealed only two spots, which agreed exactly with those of ferrichrome and ferrichrome A. Paper chromatography likewise revealed just two peaks of radioactivity with the following Rₐ values: 0.94 and 0.73 on 65% aqueous pyridine; 0.32 and 0.44 on n-butanol: acetic acid: water (4:1:5); 0.67 and 0.32 on 80% methanol on paper buffered with 0.1 M phosphate, pH 7. On all three systems the radioactive peaks agreed exactly with the Rₐ values of ferrichrome and ferrichrome A, respectively. Intracellular gallium is thus distributed entirely between ferrichrome and ferrichrome A ligands. Integration of the areas under the two peaks showed about 45% as
about 50–100 times more strongly bound than gallium. By comparison, the stability constant of Al(III) for hydroxamate ligands is less than a millionth of that of Fe(III), and the difference for other trivalent metals is even more extreme (12). The inability of Al(III) and Cr(III) to compete with iron is shown in Fig. 2. Even after 24 hr, there is little change in absorbance. The kinetic inertness of Cr(III) with siderophore ligands is well known (2).

The siderophore ligand itself also has a dramatic effect on the rate of Ga-Fe exchange. Figure 2 (insert) shows the displacement of iron by gallium for three different siderophores. When the two time scales are compared, it is seen that ferrichrome is kinetically much more labile than ferrichrome A. This cannot be due to the three negative carboxylate groups of ferrichrome A—which are absent from the electrically neutral ferrichrome—because triacetylsufarurine C, which is also electrically neutral, is kinetically very inert. Ferroxamine B, whose ligand is used clinically to relieve iron stress, lies between these extremes of lability with respect to gallium.

The exchange results shown in Fig. 2 were performed at pH 4 in order to compare the lability of different ligands at rates rapid enough for convenient spectroscopic study. Note, however, that both the rates of exchange and the equilibrium values are pH-dependent. Rates of exchange are about an order of magnitude slower at pH 6 (t1/2 ≈ 1 hr), possibly due to hydrolysis of gallium citrate at the higher pH. Rates at pH 2.5 were very similar to those at pH 4. In these metal-exchange experiments, the metal of the siderophore chelate must be completely displaced from the ligand by the competing metal. Regardless of mechanism, this type of exchange necessitates the complete release of the metal from three strong bidentate ligands of the siderophore. This is not the case for the in vivo exchange described earlier, in which gallium citrate added to the microbial culture was found rapidly chelated by intracellular siderophores. This latter exchange of gallium from citrate into an uncomplexed siderophore ligand obviously proceeds very rapidly even at pH 6.8. Thus, the in vivo experiment more closely resembles the clinical situation, in which gallium may be picked up by uncomplexed lactoferrin or siderophore ligands after injection of gallium citrate. Nevertheless, our in vitro exchange studies suggest that researchers in this field must be aware of the possibility of actual displacement of iron from chelates, such as lactoferrin, by gallium over the longer time periods typical of the use of gallium citrate in clinical diagnosis.

Studies of proton magnetic resonance have shown that the solution conformations of gallium chelates with siderophore ligands are little different from those of the iron chelates (13). Our results show that the specific ferrichrome transport system of U. sphaerogena cannot discriminate against the gallium analog, and, furthermore, our exchange experiments demonstrate that gallium effectively competes with iron for siderophore ligands. Gallium is the only metal thus far known whose kinetic lability and thermodynamic stability allow it to displace ferric iron from siderophores to any significant degree. Most interesting is the specificity of gallium exchange with respect to different siderophore ligands in spite of the demonstration by X-ray crystallography that the coordination of hydroxamate groups about the metal is extremely similar (14,15). The use of deferoxamine B to alleviate iron overload and iron poisoning in humans depends upon its ability to mobilize the metal rapidly. The rate of exchange of a given iron chelate with Ga(III) may be a useful indicator of the potential efficacy of the ligand to remove iron from the body.

From a broader point of view, these observations provide additional evidence that gallium is treated by various biological systems as an analog of ferric ion. Although Ga(III) is incapable of in vivo reduction, it is easily incorporated into the Fe(III) transport system of both mammals and microorganisms. It is interesting that Ga(III) has a distinctly lower affinity than Fe(III) for both transferrin and lactoferrin (16), and yet it has only slightly lower affinity for the ferrichrome ligand. These results provide specific insight into possible mechanisms of gallium localization in inflammatory lesions. It has been speculated that 67Ga(III) localizes in such lesions by leukocyte-mediated deposition of lactoferrin or Ga-67 lactoferrin complex (7), and by direct uptake of Ga-67 by pathogenic bacteria (17,18). While the latter process has been observed (18), the mechanism of uptake has not been previously explored. It is likely that most pathologic bacteria produce siderophores, and the siderophore-mediated uptake of gallium described in our current study provides a likely mechanism of incorporation. Our results reinforce the concept of gallium activity in biological systems as a ferric-ion analog, and provide a potential method for evaluating the efficacy of siderophore-type compounds as clinical iron chelators.

FOOTNOTES

* Aldrich Chemical Co.
† Eppendorf Model 5412.

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