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A MODEL FOR CARRIER-MEDIATED IRON TRANSPORT

THOMAS EMERY

Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322 (U.S.A.)

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SUMMARY

A model transport system is described in which iron can be quantitatively transported from an outside compartment to an inside compartment separated by an organic phase. Transport is dependent upon the presence of a ferric ionophore, ferrichrome or ferrichrome A. The metal is reduced to the ferrous state by ascorbic acid in the inner compartment and trapped by the water-soluble ferrous chelator, ferrozine. The iron-trapping mechanism provides a thermodynamic drive analogous to trapping by heme synthesis in vivo. Conditions are described which allow ferrichrome, but not ferrichrome A, to act as an ionophore. It is shown that transfer of iron from ferrichrome A to deferriferrichrome is kinetically possible, suggesting a role for ferrichrome A in vivo.

INTRODUCTION

The extreme insolubility of ferric ion at physiological pH makes the assimilation of this element especially difficult for living cells. Many aerobic microorganisms have solved this problem by the synthesis and excretion of specific iron transport agents, called siderochromes [1]. We have shown that the fungus, *Ustilago sphaerogena*, possesses a specific active transport system for ferrichrome [2]. Similar iron transport systems utilizing ferrichrome or other siderochromes have been demonstrated in several microorganisms [3].

The binding constant of siderochromes for ferric ion is of the order of 10^{30}, and it is therefore obvious that there can be no "free" iron available to the cells. It has frequently been suggested that release of the iron may involve reduction to the ferrous state, for which siderochromes have little affinity. If the reduction of iron is coupled to heme synthesis, heme could serve as a thermodynamic trap to force the reduction to completion. This paper describes a model system in which iron transport is driven to completion by such a thermodynamic trap.

EXPERIMENTAL

Materials

Ferrichrome and ferrichrome A were crystallized from the culture fluid of a

U. sphaerogena fermentation by previously described methods [4]. Labeled ferrichrome A ($2.2 \cdot 10^5$ cpm/μmole) was prepared by allowing 4 mg of ferrichrome A in 0.5 ml water containing $^{59}FeCl_3$ (Amersham/Searle) to stand for 48 h at room temperature and removing unchelated iron by passage of the solution through a 0.8 cm $\times$ 4 cm column of Chelex-100 (BioRad). Ferrozine (the disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) was purchased from Hach Chemical Co., Ames, Iowa.

Methods

The transport model employed was similar to the one described by Ashton and Steinrauf [5]. Into one side of a U-tube with inside diameter 1.2 cm was placed 2.2 ml of a solution of 5.0 mM ferrozine and 100 mM ascorbic acid. The other side contained 2.2 ml of 0.2 mM deferriferrichrome [6]. The two sides were separated by a lower phase of 2.5 ml benzyl alcohol–chloroform (1 : 1, by vol.) stirred by a glass-covered magnetic stirring bar. In order to minimize the volume of the lower phase and yet ensure that no mixing of the two compartments would occur, the bottom of the U-tube was flattened to a diameter of 5 mm. To initiate an experiment, approximately 1 μCi of $^{59}FeCl_3$ (0.15 μg iron) was added to the outside compartment. At appropriate intervals, 50-μl aliquots were removed and counted as previously described [7]. A temperature of 55 °C was maintained by immersion of the U-tube in a constant temperature bath. All counting data were normalized to a constant specific activity of ^{59}Fe.

RESULTS

The transport system

The left arm of the U-tube contains the hydroxamic acid ligand, deferriferrichrome. Addition of $^{59}FeCl_3$ forms the ferric chelate, ferrichrome, which then diffuses through the lower, immiscible, organic phase. The right side of the U-tube contains the specific ferrous chelator, ferrozine, and ascorbic acid as reducing agent. When ferrichrome reaches this side the ferrichrome is reduced by the ascorbate to the ferrous state and the metal is immediately chelated by the water-soluble ferrozine. A unidirectional flow of iron thus occurs. Ferrozine represents a water-soluble analog of the natural trapping agent, protoporphyrin.

In order for such a model system to work, it is necessary that the ionophore be soluble in both the aqueous and organic phases. This is in contrast to the valinomycin model transport system in which the ionophore remains in the organic phase and chelate formation and dissociation must occur at the organic–aqueous interface [5]. The partitioning of ferrichrome between benzyl alcohol–chloroform (1 : 1, by vol.) and the aqueous phase is shown in Table I. The partitioning of uncharged ferrichrome is little affected by buffer. Ferrichrome A, on the other hand, has three carboxylic acid functions, and phosphate buffer prevents ferrichrome A from dissolving in the organic phase.

Fig. 1 shows the reduction of ferrichrome in the presence of a large excess of ascorbate and ferrozine. Under identical conditions, ferrichrome A reduction is extremely slow, reflecting the greater affinity of this siderochrome for ferric ion [1]. Nevertheless, under conditions of higher temperature and lower pH, a rapid reduction

TABLE I

PARTITION OF FERRICHROME AND FERRICHROME A BETWEEN AQUEOUS PHASE AND ORGANIC PHASE

Approximately 2 mg of ferrichrome or ferrichrome A was equilibrated between 3 ml of the aqueous phase and 3 ml of benzyl alcohol–chloroform (1 : 1, by vol.) overnight at 55 °C. The concentration of siderochrome in each phase was determined spectrophotometrically with a Beckman DU spectrophotometer [7].

Aqueous phase		Ferrichrome concn (mM)	Ferrichrome A concn (mM)
Water	upper	0.279	0.330
	lower	0.607	0.328
0.01 M phosphate pH 7.0	upper	0.266	0.700
	lower	0.750	< 0.007

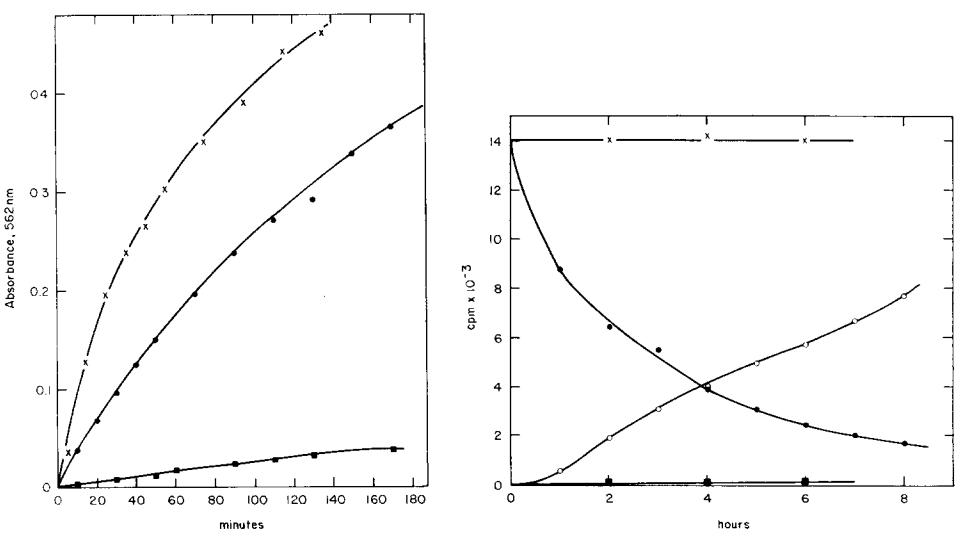

Fig. 1. Comparison of the rate of reduction of ferrichrome (●) and ferrichrome A (■) by ascorbate in the presence of ferrozine. 50 μl of a 4.1 mM solution of the siderochrome was added to 3.0 ml of a solution of 10 mM ferrozine and 0.2 M ascorbic acid, pH 4.5. Temperature was maintained at 27.5 °C. Siderochrome reduction was followed by formation of the blue colored Fe^{II}–ferrozine complex. The blank contained no siderochrome. Ferrichrome A reduction at 55 °C and pH 3.2 is also shown (·).

Fig. 2. Ferrichrome as a ferric inophore. The outer compartment of the U-tube contained 2.2 ml of 0.2 mM deferriferrichrome. The inner compartment contained 2.2 ml of 5.0 mM ferrozine and 100 mM ascorbate, pH 4.5. After temperature equilibration at 55 °C, $^{59}FeCl_3$ was added to the outer compartment. (●) counts in outer compartment. (○) counts in inner compartment. The experiment was repeated with 0.2 mM citrate in place of the deferriferrichrome. (×) counts in outer compartment. (■) counts in inner compartment.

of ferrichrome A can also be achieved (Fig. 1). For this reason, our transport experiments were performed at 55 °C.

Ferrichrome transport

To demonstrate the transport mechanism of the model, $^{59}FeCl_3$ was added to a dilute aqueous solution of deferriferrichrome in the outside compartment and the course of iron transfer followed. The results are shown in Fig. 2. By the end of 4 h, the concentrations of iron in the two compartments were equal. Iron continued to be transported and trapped on the inside until at the end of 24 h (not shown) over 90 % of the iron was found in the inside compartment. As can be seen from the data of Fig. 2, between 30 and 40 % of the iron is not accounted for in the two compartments during the run but is located in the organic phase. At the end of 24 h, however, this iron has also reached the inside compartment. The dissolution of the ferrichrome in the relatively large volume of organic phase also accounts for the more rapid initial decrease of counts from the outside compartment as compared to the increase of counts in the inside compartment.

Citrate is ineffective as a ferric ionophore for *U. sphaerogena* in vivo compared to ferrichrome [2]. Similarly, when deferriferrichrome was replaced by citrate in our model system, all of the counts remained in the outside compartment after addition of $^{59}FeCl_3$ (Fig. 2). This is understandable in view of the insolubility of the charged iron–citrate chelate in the organic phase. Since ferrichrome has no ionizable groups, it would not be expected that the pH of the outside compartment should influence transport. No significant difference was found in the transport of ferrichrome at pH 4.5 and 7.0.

In the absence of a trapping mechanism, the concentrations of carrier should equalize in the inside and outside compartments. Fig. 3 shows the slow equilibration of iron (ferrichrome) concentrations in the two compartments in the absence of ascorbate. The relatively slow initial increase of counts in the inside compartment as compared to Fig. 2 probably indicates that the re-dissolution of ferrichrome in the aqueous phase from the organic phase is the rate limiting step in the model.

Transport of ferrichrome A

When a dilute aqueous solution of deferriferrichrome A was placed in the outside compartment and $^{59}FeCl_3$ added, the metal was observed to be rapidly transported (Fig. 4). The transfer to the inside compartment was virtually quantitative by the end of 8 h. When the outside compartment is buffered at pH 7, the transport of ferrichrome A is completely abolished (Fig. 4). At this pH, the three carboxyl groups of ferrichrome A are ionized. As in the case of iron–citrate, the charged ferrichrome A has too limited a solubility in the organic phase to be an effective carrier.

Iron transfer between siderochrome ligands

Although it is known that the iron of ferrichrome is rapidly exchangeable [8], it has never been established that a direct transfer of the metal can take place between different siderochrome ligands. In order to test this possibility, [^{59}Fe]ferrichrome A (18 μM) was allowed to stand at room temperature with deferriferrichrome (180 μM) in 0.01 M phosphate, pH 7. Iron incorporated into ferrichrome was determined by

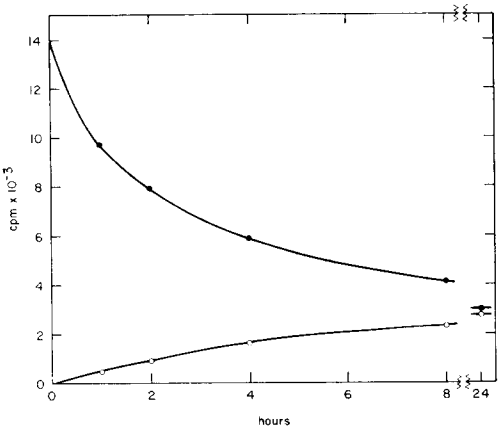

Fig. 3. Ferrichrome mediated diffusion of iron in the absence of a trapping mechanism. The experimental conditions are the same as for Fig. 2 except that ascorbate was omitted from the inner compartment, which was adjusted to pH 4.5 with acetic acid. (●) counts in outer compartment. (○) counts in inner compartment.

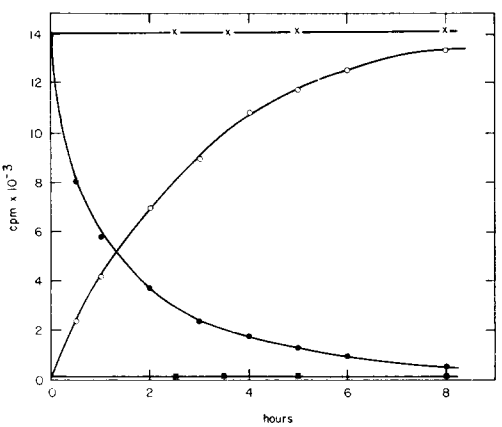

Fig. 4. Ferrichrome A as a ferric ionophore. The experimental conditions are the same as for Fig. 2 except that the outer compartment contained 0.2 mM deferriferrichrome A instead of deferriferrichrome. (●) counts in outer compartment. (○) counts in inner compartment. The experiment was repeated with the addition of 0.1 M potassium phosphate, pH 7.0, to the outer compartment. (×) counts in outer compartment. (■) counts in inner compartment.

passage of the solution through a DEAE column [8]. Passage of a control solution of labeled ferrichrome A showed that no counts passed through the column, i.e. all counts were originally in ferrichrome A. At the end of 48 h, 16 % of the counts were found in ferrichrome. Since the amount of free soluble iron at pH 7 in the presence of deferriferrichrome must be virtually zero, this experiment demonstrates that a direct transfer of the metal between siderochrome ligands is kinetically possible. The fact that ferrichrome A binds iron at least 100 times more tightly than ferrichrome explains why only a fraction of the metal is transferred even with a 10-fold excess of deferriferrichrome.

DISCUSSION

The reduction of the iron of siderochromes is the accepted mechanism for iron release. The results of this paper show that in the presence of an efficient reducing system and ferrous trapping chelator, siderochrome iron can be quantitatively transported between two aqueous phases across a lipid barrier. Because of the very low redox potential of siderochrome iron, it may be that in vivo the ligand is hydrolyzed prior to iron reduction. An enzyme has been isolated from *Escherichia coli* which hydrolyzes enterochelin, a siderochrome of the polyphenolic class [9]. The hydrolysis product, dihydroxybenzoylserine, is a much weaker chelator of ferric ion than is enterochelin itself. An in vitro system has been described in which release of iron by this enzyme makes the metal available for heme synthesis by the enzyme, ferrochelatase [10]. Reduction of the iron was accomplished by excess reduced glutathione. However, all efforts in our laboratory to demonstrate an enzyme in extracts of *U. sphaerogena* capable of hydrolyzing ferrichrome have been unsuccessful, in spite of the fact that large amounts of iron are handled by ferrichrome in this organism. Nevertheless, extracts of *U. sphaerogena* are capable of reducing ferrichrome iron [11]. Similarly, extracts of *Mycobacterium smegmantis* reduce the ferric chelate of mycobactin, a siderochrome very different in structure from ferrichrome [12]. In both cases, NAD serves as reductant.

It is interesting that conditions can be found under which ferrichrome, but not ferrichrome A, can function as an ionophore in our model system. Ferrichrome A is not an iron carrier in vivo [2]. It should be noted, however, that in our model system this difference in the two siderochromes is due to unfavorable solubility characteristics of the charged ferrichrome A ligand, whereas our in vivo experiments indicate that conformational differences play an important role. The reduction of ferrichrome A is extremely difficult in vitro, but our model system demonstrates that even as mild a reductant as ascorbic acid is effective, provided that an efficient ferrous trapping agent is present.

Why does an organism such as *U. sphaerogena* excrete large amounts of deferriferrichrome A when ferrichrome A is ineffective as a ferric ionophore? Experiments described in this paper show that transfer of iron from ferrichrome A to the ferrichrome ligand can occur in vitro. Such an exchange at the outer cell membrane could be driven to completion by subsequent transport of ferrichrome and trapping of iron by heme synthesis. A significant flux of iron from ferrichrome A to heme could thus be achieved. The acidic properties of ferrichrome A, combined with its extraordinarily high iron binding constant, make this substance ideal for sequestering iron from the environment.

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