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Transport of the iron ionophore ferrichrome in *Escherichia coli* K-12 and *Salmonella typhimurium* LT2

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1. INTRODUCTION

Iron(III) is actively transported as a ferrichrome complex into the cells of *Escherichia coli* and *Salmonella typhimurium* [1,2]. We have shown that in *E. coli* the ligand is modified and excreted as the iron-free form into the medium [3]. The modified ligand could be converted back into ferrichrome by mild acid hydrolysis. 1 mol of acetate was released per mol of ferrichrome; therefore it was proposed that an N-hydroxyl group, one of the six binding sites of iron(III) in ferrichrome [4], was acetylated. This could explain the low affinity of the modified ligand for iron(III). It was likely that reduction before modification took place. We suggested that the inactivation of ferrichrome prevented the intracellular withdrawal of iron by the ligand from sites of synthesis and function of the many iron containing proteins. We now describe a cell-free system that modifies ferrichrome in the same way as transporting cells. Reduction was a prerequisite for modification to occur. Both reduction and modification were membrane-bound processes. It is therefore likely that ferrichrome does not have to enter the cytoplasm in order to function as an iron(III) ionophore. To test this conclusion, we converted an analogue of ferrichrome (ferrichrysin) into a disuccinate derivative. The doubly charged derivative was nearly as effective an iron(III) ionophore as the uncharged ferrichrysin.

2. MATERIALS AND METHODS

The experiments were performed with *E. coli* K-12 AB2847 *aroB tsx thi malA* and with *S. typhimurium* LT2 *aroB* which are unable to synthesize their own iron ionophore (enterochelin) due to a mutation in the *aroB* gene that codes for the dehydroquinate synthetase. Cells were grown in tryptone-yeast extract medium as described before [5]. Membranes were prepared from cells in the late logarithmic growth phase. Cells of a 1 l culture were harvested, washed with 300 ml of a 0.1 M triethanolamine solution, adjusted to pH 7 with HCl. They were resuspended in 15 ml of the same buffer and passed twice through a French pressure cell. Remaining cells were removed by centrifugation for 15 min at $5000 \times g$. The membranes were collected by centrifugation for 2 h at $180000 \times g$. The sediment was washed once with the triethanolamine buffer and then used for the modification studies in 1 ml of the buffer. The cytoplasmic membrane was separated from the outer membrane according to the procedure of Osborn et al. [6].

Ferrichrysin (10 mg) was coupled with succinic

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anhydride (30 mg) in 1 ml absolute pyridine to yield the disuccinate ester as described previously for the preparation of ferricrocinyl monosuccinate [5].

To determine the modification of ferrichrome 0.1 ml of the cell homogenate (or of the membrane fraction, or the membrane-free cell homogenate) was incubated with a 10 μM final concentration of tritiated ferrichrome in 0.25 ml total volume of the triethanolamine buffer for 3 h at 37°C.

The following procedures were performed according to previously described methods: chromatographic analysis of the synthesis [5] and the modified products [3]; extraction of ferrichrome and the modified product from the reaction mixture with Servachrome XAD-2; quantitative determination of acetate with an enzymatic assay; labeling of ferrichrome with tritium; exchange of nonradioactive against radioactive iron(III) in ferrichrysin and ferrichrysinyl disuccinate [3]; transport assay [5].

Ferrichrome and ferrichrysin were obtained from H.-P. Fiedler and H. Zähner, at this Institute. All other reagents used were of the highest purity available commmercially.

3. RESULTS

3.1. Cell-free modification of ferrichrome

Cell-free modification of ferrichrome occurred in the membrane fraction obtained from *E. coli* and *S. typhimurium*, and required the addition of NADH or NADPH. The membrane fraction converted 50%–60% of the added ferrichrome whereas the membrane-free cell homogenate was inactive. The yield of the modified product increased from zero, without added NADH, to 0.65 nmol/mg protein/h with 10 mM NADH, and then decreased slightly at higher NADH concentrations. When NADH was replaced by NADPH the yield decreased to 82% with 20 mM NADP, and to 58% with 10 mM NADPH, as compared with the yield obtained with 10 mM NADH. Ascorbate (5–20 mM) was inactive. The membrane fraction modified the iron-free form of ferrichrome without addition of NADH.

Table 1

Hydrolysis products of [^{3}H]ferrichrome derivative obtained by the membrane fraction of *E. coli*

Substrate	Acetate/ferrichrome/ derivative/ (nmol/nmol)
Ferrichrome	0
Ferrichrome-derivative	14.5/15
Ferrichrome-derivative	9.9/7.5
Ferrichrome-derivative	5.5/5

Attempts were made to determine the substrate requirement for the modification reaction in the crude membrane fraction of *E. coli*. Addition of acetyl phosphate (20 mM) increased the yield of modified ferrichrome from 0.3 to 0.55 nmol/mg protein/h. Addition of acetate (10 mM) decreased the yield by 50%. When acetyl phosphate was regenerated by the supply of acetyl kinase and ATP the original yield was recovered.

The modifying activity was obtained in soluble form when 5 mM EDTA was present during homogenization of the *E. coli* cells, with the French pressure cell. It was also solubilized when the membrane fraction was suspended in the 0.1 M triethanolamine buffer which contained 5 mM EDTA. Ca^{2+} and Mg^{2+} (both 10 mM) were added to the 180000$\times g$ supernatant fraction prior to the assay since EDTA inhibited the modification reaction. After treatment of the supernatant fraction with alkaline phosphatase (50 μg) for 30 min at 37°C to hydrolyse intrinsic acetyl phosphate, modification of ferrichrome became dependent on the addition of acetyl phosphate (20 mM).

Addition of acetyl CoA, ATP, PEP or c-AMP (10 mM) to the membrane fraction, or to cells made permeable by treatment with toluene (7), decreased the rate of ferrichrome modification by 20–50%. None of the mutants deficient in ferrichrome transport, bearing mutations in the *fhuA*, *fhuB* or *tonB* genes [8], showed an altered modifying activity as compared with the parent strain *E. coli* K-12 AB2847.

The modified ferrichrome obtained with the cell-free systems had the same R_f-value after chromatography on silica gel thin-layer plates as that

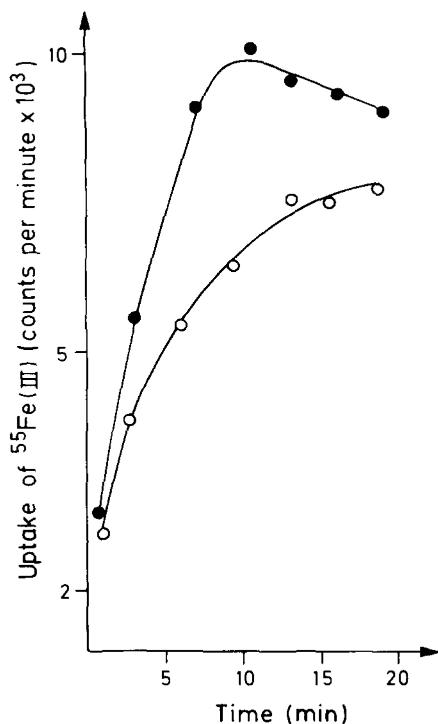

Fig. 1. Uptake of iron(III) as ferrichrysin (●) and as ferrichrysinyl disuccinate (○) complex into cells of *E. coli* K-12 AB2847. Cells were pregrown in tryptone yeast extract medium to a density of 5×10^8 cells/ml. They were harvested, washed once with M9 salt medium [1] which contained 0.4% glucose and 0.1 mM nitrilotriacetate. They were resuspended in the same medium to the orginal cell density. Transport was started at 37°C by addition of ^{55}Fe(III) ferrichrysin or ^{55}Fe(III) ferrichrysinyl disuccinate of equal specific activity. The solutions contained 10 times higher concentrations of the ligands than of ^{55}Fe(III). At the times indicated samples of 1 ml were filtered, the filters dried, and the radioactivity was determined in a liquid scintillation counter.

obtained after transport of ferrichrome into whole cells [3]. It was recovered from a reaction mixture, containing 7 ml of membranes, 10 μM ferrichrome, 10 mM NADH, and 20 mM acetyl phosphate, by adsorption to Servachrome XAD-2. It was further purified by thin-layer and column chromatography on Bio-Gel P-2 [3]. Acetate was determined after mild acid hydrolysis which did not release acetate from ferrichrome. The results are shown in Table 1. About 1 mol of acetate was obtained per mol of ferrichrome derivative.

The outer membrane and cytoplasmic membrane were separated by isopycnic centrifugation to localize the modifying system. Both the outer (H-band), and the cytoplasmic membrane (L-band) showed reproducibly similar activities, about 1.5 nmol product/mg protein/h. However, the M-band, a mixture of both membranes, was at least twice as active as the individual membranes. Mixing of the separated membranes did not result in a higher activity than that obtained with the single membranes.

3.2. Transport or iron(III) by ferrichrysinyl disuccinate

In ferrichrysin two of the glycine residues of ferrichrome are replaced by serine residues [9]. Both of the serine hydroxyl groups were esterified with succinate. Only one red compound was observed after silica gel thin layer chromatography with chloroform–methanol–water (65:24:4, v/v). The R_f values obtained after two experiments were 0.49 and 0.52. Comparison with the R_f values of the reference compounds ferricrocin (1.03), ferrichrysin (1.0), ferricrocinyl monosuccinate (0.74) demonstrated that the product obtained was the most highly charged. For this reason it must be the disuccinate and not the monosuccinate or the cyclic monosuccinate derivative. This conclusion was supported by electrophoresis studies, at pH 7, where the ferrichrysin derivative showed the highest mobility among these compounds.

Despite its two negative charges ferrichrysinyl disuccinate was an effective iron ionophore. In several experiments the rate of iron transport into cells of *E. coli* K-12 AB2847 was at least half the rate of that obtained with ferrichrysin or ferrichrome. An example is presented in Fig. 1.

4. DISCUSSION

Transport of iron(III) as a ferrichrome complex into cells of *E. coli* and *S. typhimurium* has several unusual features which set it apart from the mechanisms of other transport systems, for example those for amino acids and sugars. Free iron(III), at a concentration of 10^{-12} μM in equilibrium with the polymeric ferric hydroxy aquo complex under

aerobic conditions at pH 7, is complexed very efficiently by the iron-free form of ferrichrome. The formation constant is in the order of 10^{29} [10]. Ferrichrome is then bound to an outer membrane protein, coded by the *fhuA* (formerly *tonA*) gene and then transported into the cell under the control of two additional genes, *fhuB* and *tonB* [8]. The very insoluble iron(III) is thus extracted from the medium, concentrated at the cell surface and then translocated further through the outer membrane, which forms a permeability barrier for the free diffusion of molecules of the size of ferrichrome with a molecular mass of 740 [11]. The question then arises whether ferrichrome has to pass across the cytoplasmic membrane into the cytoplasm to deliver the iron, or whether the iron is dissociated from the ligand already within the cytoplasmic membrane. The amount of ligand found associated with cells was far below that of iron [3]. The rate of modification and excretion of the ligand corresponded with the rate of transport [3]. This showed that modification and transport were tightly coupled processes. In this paper we demonstrate that modification took place in the membrane fraction which strongly argues in favor of iron release from the complex within the cytoplasmic membrane. According to current models on the transport of solutes across the cytoplasmic membrane it would be difficult to comprehend how a neutral molecule, ferrichrome, ferricrocin or ferrichrysin, a negatively charged derivative, ferricrocinyl monosuccinate [5], and a doubly negatively charged molecule, ferrichrysinyl disuccinate, are all transported by the same system through the cytoplasmic membrane. The finding that the artificial chromium complex of deferri-ferrichrome, where the metal stays in the complex, remains associated with cells and may be in the cytoplasm [2] does not contradict our interpretation. It may only mean that without release of metal ion, and without modification of the ligand, the kinetically stable chromium complex leaks from the cytoplasmic membrane into the cytoplasm. The cell-free system has shown for the first time that reduction is a prerequisite of ferrichrome modification. The finding of 1 mol of acetate/mol of ferrichrome shows that the cell-free system mimicked the modification reaction coupled with ferrichrome transport. The result suggest that the enzyme system for the release of iron requires NADH as cofactor. The data are compatible with the conclusion that iron(III) is released by reduction to iron(II), and that the free iron binding site is immediately blocked by covalent introduction of an acetyl group.

The data do not reveal the substrate of the presumed acetylation. Acetyl phosphate is a likely candidate but the system is still too crude to allow definitive conclusions. The soluble system obtained with EDTA may allow the purification of the components necessary for reduction and modification. It should then be possible to obtain a clearer response to added substrates. Antibodies against the purified enzymes in question may also aid in localizing the reduction and modification systems. The requirement for NADH or another reducing cosubstrate clearly favor the cytoplasmic membrane. The finding of the reducing and modifying activities in both membranes may result from the rather loose attachment to the cytoplasmic membrane and their distribution between the membranes during the cell breakage and centrifugation steps. It is possible that the finding of the highest activity in the M-band reflects the preferential location of the enzyme system in specialized regions, most likely at adhesion zones [12] of the two membranes.

We obtained the same uptake curves for ^{55}Fe(III) and [^{3}H]ferrichrome in *Salmonella typhimurium* LT2 *aroB* as in *E. coli* K-12 AB2847, and we obtained the same ferrichrome derivative with membranes in the presence of added NADH. Therefore, there is no reason to assume different mechanisms for ferrichrome uptake in the two organisms as discussed previously [2].

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