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Arginyl Residues Are Involved in the Transport of Fe^{2+} through the Plasma Membrane of the Mammalian Reticulocyte

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Abstract. Sealed reticulocyte ghosts were treated with reagents that modify a variety of amino acid residues. Only ninhydrin and phenylglyoxal, both modifiers of arginyl residues, produced inhibition of the initial rate of $^{59}Fe^{2+}$ uptake. The inhibition (i) was dependent on the concentration of ninhydrin or phenylglyoxal, (ii) increased from pH 7 to 9, a feature of the modification of arginine by ninhydrin or phenylglyoxal, and (iii) was blocked when Fe^{2+} was present during the modification step. A23187, an effective membrane Fe^{2+} transporter, diminished the inhibitory effect of ninhydrin and phenylglyoxal, indicative that the transport of iron through the membrane, and not a secondary process, was selectively inhibited. We conclude that the iron transporter from the plasma membrane of erythroid cells has one or more arginyl residues in a segment accessible to ninhydrin or phenylglyoxal, and that this residue is involved in the transmembrane transport of iron.

Key words: Iron transport — Reticulocyte-sealed ghosts — Plasma membrane — Arginine — Ninhydrin — Phenylglyoxal

Introduction

Iron uptake by mammalian cells is a multi-step process which includes the internalization of the transferrin-transferrin receptor complex in endocytic vesicles, the labilization of iron from transferrin mediated by vesicle acidification, and the sequential reduction and transport of iron into the cytosol (*reviewed by* Crichton & Ward, 1992). Membrane-associated iron transport processes have been reported in early endocytic vesicles (Núñez et al., 1990; Escobar, Gaete & Núñez, 1992; Watkins et al., 1992) and in a variety of eucaryotic cell lines, including yeast (Egyed, 1988; Morgan, 1988; Sturrock et al., 1990; Wright & Lake, 1990; Kaplan, Jordan & Sturrock, 1991; Eide et al., 1992). In previous work, we established that sealed right-side-out reticulocyte ghosts transport and accumulate iron offered as Fe^{2+} ascorbate (Núñez et al., 1992). In the present study, we chemically modified amino acid residues exposed to the extracellular face of sealed right-side-out reticulocyte ghosts and examined the effect of these modifications on the kinetic parameters of iron transport. The overall results indicate that an arginyl residue is specifically involved in the initial steps of iron transport through the plasma membrane.

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Materials and Methods

Sealed Reticulocyte Ghosts

Reticulocyte ghosts were prepared by differential and gradient centrifugation as described (Núñez et al., 1992). Purified ghosts were sealed by incubation for 30 min at 37°C in MOPS-Tris-buffered saline (MOPS-Tris saline, 20 mM MOPS-Tris pH 7.0, 50 mM NaCl, 50 mM KCl).

^{59}Fe Uptake

^{59}Fe uptake was done at 4°C. It was started by the addition of 10 μM ^{59}Fe, 200 μM ascorbate in MOPS-Tris saline to 20–40 μg of sealed reticulocyte ghosts. At specified times, aliquots of the assay medium (corresponding to about 2 μg of protein) were filtered through Millipore filters (GSWP 0.22 μm) pretreated with MOPS-Tris saline, supplemented with 0.5% polyethylene glycol to decrease the nonspecific adsorption of ^{59}Fe to the filters. The filters were quickly washed

three times with cold MOPS-Tris saline, and the radioactivity trapped in the filters was measured in a Philips gamma counter. When the effects of A23187 or valinomycin were tested, the ghosts were equilibrated for 5 min with 0.6 μM A23187 or 1 μM valinomycin before proceeding with ^{59}Fe uptake.

Chemical Modification of the Sealed Reticulocyte Ghosts

Arginyl residues were modified with ninhydrin or phenylglyoxal. Sealed reticulocyte ghosts (200–500 μg/ml) were equilibrated in 50 mM NaCl, 50 mM KCl, 10 mM sodium phosphate, pH 9.0 or in 50 mM NaCl, 50 mM KCl, 10 mM sodium bicarbonate, pH 9.0, respectively. Ninhydrin, dissolved in phosphate saline, or phenylglyoxal, dissolved in ethanol, were added, and the mixture was incubated for 60 min at 37°C. The reactants were eliminated by centrifugation and the modified ghosts were equilibrated in MOPS-Tris saline and assayed for iron uptake. Control ghosts went through the whole procedure but without ninhydrin or phenylglyoxal. The maximal ethanol concentration achieved was 1% which, in control experiments, was shown to have no effect on iron transport. The modification of arginyl residues was also done with 10 μM to 1 mM *p*-hydroxyphenylglyoxal (Yamasaki, Vega & Feeney, 1980), 0.1 to 5 mM camphorquinone (Pande, Pelzig & Glass, 1980), and 0.1 to 5 mM cyclohexanedione (Suckau, Mak & Przybylski, 1992) in 0.12 M Na borate, pH 9.0. Histidine residues were modified with 1 to 10 mM diethyl pyrocarbonate (Zhang, Tsai & Kulmacz, 1992). Carboxylic residues were modified with 1 μM to 1 mM iodoacetic acid (Takahashi, Stein & Moore, 1967); with 1 μM to 1 mM N-ethyl-5-phenylisoxasolium-3'-sulfonate (Dinur, Kantrowitz & Hajdu, 1981); with 1 μM to 10 mM diazoacethylnorleucine methyl ester (Nakayama et al., 1981); with 1 μM to 1 mM 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (Chen & Tang, 1972); with 1 μM to 1 mM 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (Yamada et al., 1981); with 1 μM to 1 mM 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (Laszlo, Millner & Dilley, 1982), and with 10 to 100 μM *N,N'*-dicyclohexyl-carbodiimide (Michel & Oesterhelt, 1980). Cysteine residues were modified with 0.5 to 2 mM *N*-ethylmaleimide (Ijzerman & Melman, 1992), and 10 μM to 1 mM Nbs_2 (Nakayama et al., 1992). Amino groups were modified with 10 μM to 5 mM 2,4,6-trinitrobencene-1-sulfonate (Nakayama et al., 1992), 1 μM to 1 mM 2,4-dinitrofluorobencene (Schroeder & LeGette, 1953), and 10 μM to 1 mM iminothiolane (Traut et al., 1973). We also tested the effect of the tryptophan residue modifier BNPS-Skatole (10 μM to 5 mM, Hunziker, Hughes & Wilson, 1980).

Effect of pH on the Modification of Sealed Reticulocyte Ghosts by Ninhydrin and Phenylglyoxal

Sealed reticulocyte ghosts were equilibrated in 50 mM NaCl, 50 mM KCl, 10 mM sodium phosphate at pH 6.0 to 11.0 and modified with 10 μM ninhydrin or 50 μM phenylglyoxal. The modified ghosts were washed three times by centrifugation with MOPS-Tris saline and their iron uptake capacity was assayed as described before.

The Effect of Fe^{2+} During the Modification of Sealed Reticulocyte Ghosts by Ninhydrin and Phenylglyoxal

Sealed reticulocyte ghosts were equilibrated in phosphate saline, pH 9.0, and supplemented with 10 μM Fe^{2+} ascorbate. Ninhydrin or phenylglyoxal (100 μM) was then added and the mixtures incubated for 1 hr at 37°C. Control ghosts went through the procedure with either 100 μM ascorbate or nothing added. The modified ghosts were washed with MOPS-Tris saline and assayed for ^{59}Fe uptake as before.

Reagents

$^{59}FeCl_3$ in 0.5 M HCl was from DuPont (Du Pont Research Products, Boston, MA). 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate, cyclohexanedione, camphorquinone, diazoacethylnorleucine methyl ester, diethyl pyrocarbonate, *N,N'*-dicyclohexyl carbodiimide, 2,4-dinitrofluorobencene, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride, 1,2-epoxy-3-(*p*-nitrophenoxy)-propane, phenylglyoxal, iodoacetic acid, 2-iminothiolane, 5,5'-ditiobis-2-nitrobenzoato, *N*-ethylmaleimide, ninhydrin, *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, valinomycin were obtained from Sigma (St. Louis, MO); 3-bromo-3-methyl-2-[(2-nitrophenyl)thio]-3H-indole, *p*-hydroxyphenylglyoxal and 2,4,6-trinitrobencene-1-sulfonate were obtained from Pierce (Rockford, IL); A23187 was obtained from Calbiochem (San Diego, CA); Chelex-100 was from Bio-Rad (Richmond, CA); cellulose nitrate filters were obtained from Millipore (Bedford, MA), and from MFS (Micro Filtration Systems, CA). Solutions were prepared with distilled, deionized water, treated further with Chelex-100 (Bio-Rad) to remove traces of divalent metals. Similarly, stock solutions of monovalent salts and buffers were routinely filtered through Chelex-100.

Data Analysis

The experimental data from kinetic experiments were fitted to pseudo-first order rate equations using the Enzfitter program (Elsevier-Biosoft, Cambridge, UK).

Abbreviations

BNPS, 3-bromo-3-methyl-2-[(2-nitrophenyl)thio]-3H-indole; CDIS, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate; DCCD, *N,N'*-dicyclohexyl-carbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)-propane; EPS, *N*-ethyl-5-phenylisoxazolium-3'-sulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid); Tris, Tris(hydroxymethyl)-aminomethane.

Results

Chemical Modification of the Iron Transporter

Sealed reticulocyte ghosts were treated with a variety of amino acid modifiers. The effect of these modifiers on the kinetics of iron transport was investigated (Table 1). Of all the reagents tested, only ninhydrin and phenylglyoxal inhibited the initial rate of iron transport. These reagents are well-established modifiers of arginyl residues (Chaplin, 1976; Lundblad & Noyes, 1984; Strévey et al., 1992). In contrast, reagents that modify histidine, carboxyl, cysteine, amino or tryptophan groups had no noticeable effect. Treatment with can-

Table 1. Effect on the initial rate of iron transport produced by the chemical modification of sealed reticulocyte ghosts

Reagent	Amino acid modified	Effect on V_i
Ninhydrin; phenylglyoxal	Arginine	Inhibition
Diethyl pyrocarbonate	Histidine	None
Iodoacetic acid; EPS; diazo-acetyl norleucine methyl ester; EPNP; EDC; CDIS; DCCD; pHFG	Carboxylic	None
Camphorquinone; cyclohexane-dione; *p*-hydroxyphenyl-glyoxal	Arginine	None
N-ethylmaleimide; 5,5′-dithiobis-2-nitrobenzoate	Cysteine	None
2,4,6-trinitrobencene-1-sulfonate; 2,4-dinitrofluorobencene; 2-iminothiolane	Amino	None
BNPS	Tryptophan, tyrosine	None

Sealed reticulocyte ghosts were treated with different chemical modifiers (*see* Materials and Methods) and subjected to $^{59}Fe^{2+}$ uptake kinetics from which the initial rate of uptake was determined. The tests were repeated at least three times with the same results.

phorquinone, cyclohexanedione and *p*-hydroxyphenylglyoxal, which also modify arginyl residues (Pande et al., 1980; Yamasaki et al., 1980; Suckau et al., 1992), did not produce inhibition of the initial rate of iron transport.

It was further established that the effect of ninhydrin and of phenylglyoxal was concentration dependent (Fig. 1): 50% inhibition was reached at 10 μM ninhydrin (Fig. 1*a*) or at 75 μM phenylglyoxal (Fig. 1*b*).

The Effect of pH on the Modification of Sealed Reticulocyte Ghosts with Ninhydrin or Phenylglyoxal

Modification of sealed reticulocyte ghosts with ninhydrin or phenylglyoxal in the pH range 6 to 11, resulted in a pH-dependent modification of the initial rates of iron uptake (Fig. 2). No inhibition was observed at pH 6.5–6.6, while 50% inhibition was observed at pH 7.2–7.3 and maximal inhibition at pH 8–11. A paradoxical effect was observed at pH 6.0, where the initial rates of iron transport were actually higher in the modified ghosts than in the control. The effect was similar both for ninhydrin and for phenylglyoxal.

Fe^{2+} Protection

When the modification of arginyl residues by ninhydrin or phenylglyoxal was carried out in the presence of 10 μM Fe^{2+}, the inhibitory effect of ninhydrin and phenylglyoxal on the rate of iron transport was abolished (Fig. 3), suggesting that an iron binding site in the transporter was indeed a target of ninhydrin and phenylglyoxal.

Effect of A23187 and Valinomycin on the ^{59}Fe Uptake by Ninhydrin and Phenylglyoxal-Modified Ghosts

The sealed reticulocyte ghosts both transport and accumulate Fe^{2+}, being transport rate-limiting (Núñez et al., 1992). It could be possible that the decrease in the rate of Fe^{2+} uptake produced by ninhydrin or phenylglyoxal is secondary to an alteration in the accumulation step. If the modification by ninhydrin or phenylglyoxal affected primarily the iron transporter, the decrease in the iron uptake rate produced by ninhydrin and phenylglyoxal should be overcome by A23187, an artificial iron transporter (Young & Gomperts, 1977; Núñez, Cole & Glass, 1983). In contrast, if it affected a support system, the addition of the ionophore should not have any effect. The effect of A23187 on iron uptake in ninhydrin-modified sealed reticulocyte ghosts is shown in Table 2. A23187 produced a discrete increase in the initial rate of transport by sealed ghosts, and it increased to control levels the initial rate of transport by ninhydrin-treated ghosts. Therefore, ninhydrin and phenylglyoxal affected the transporter.

Another cause for a decreased rate of Fe^{2+} uptake could be the inhibition of the system that dissipates the putative electrical gradient generated by the inflow of iron. Valinomycin, an ionophore that under the assay conditions should dissipate this electrical gradient, did not reverse the effect of ninhydrin (Table 2), an indi-

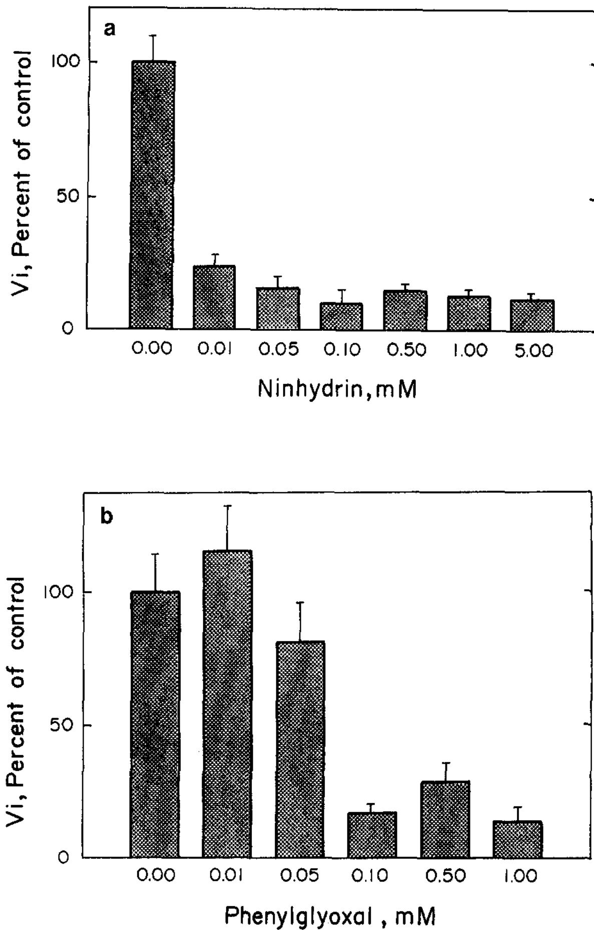

Fig. 1. Effect of ninhydrin and phenylglyoxal on initial rates of Fe^{2+} uptake. Sealed reticulocyte ghosts were reacted with ninhydrin or phenylglyoxal at the concentrations indicated, and the uptake of ^{59}Fe was assayed as described under Materials and Methods. Shown are mean ± SD of three experiments of the initial rates as percent of control for the modification with ninhydrin (*a*), and phenylglyoxal (*b*).

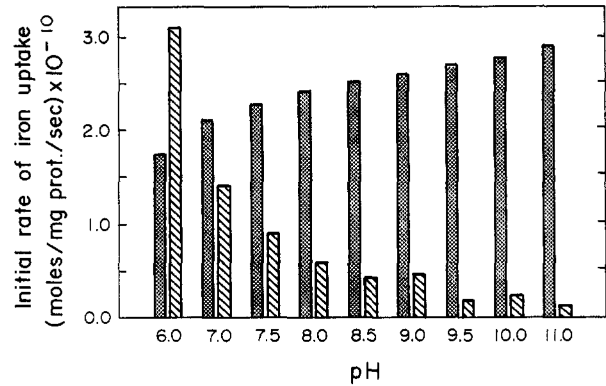

Fig. 2. The effect of pH on the modification of sealed reticulocyte ghosts by ninhydrin. Sealed reticulocyte ghosts were equilibrated in 50 mM NaCl, 50 mM KCl, 10 mM sodium phosphate at pHs ranging from 6.0 to 11.0, modified with 10 μM ninhydrin or 50 μM phenylglyoxal. Shown are the initial rates of iron uptake as a function of the pH in the modification step from one of three experiments. Hatched bars, ghosts modified with 10 μM ninhydrin; stippled bars, control ghosts. The data obtained for phenylglyoxal-modified ghosts were similar (*not shown*). Shown is one of two experiments. The data variability between the experiments was <18%.

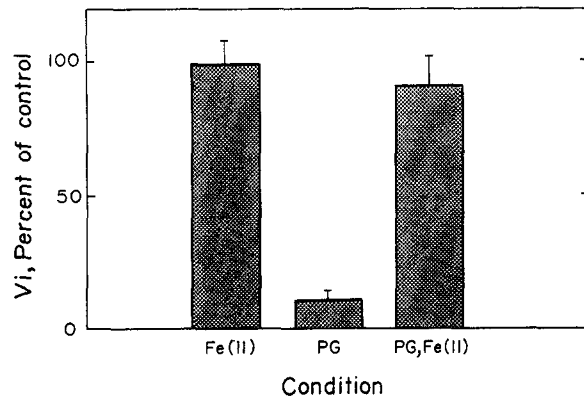

Fig. 3. The effect of Fe^{2+} on the modification of sealed reticulocyte ghosts by phenylglyoxal. Sealed reticulocyte ghosts were equilibrated in 50 mM NaCl, 50 mM KCl, 10 mM sodium phosphate (pH 9.0), with or without 10 μM Fe^{2+} ascorbate. Phenylglyoxal (0.1 mM) was then added, and the mixtures were incubated for 1 hr at 37°C. Control samples were incubated without the modifiers and with or without 200 μM ascorbate. The ghosts were then washed and assayed for ^{59}Fe uptake. Shown is the effect of the addition of Fe^{2+} during the modification by phenylglyoxal (PG). Similar effects were observed with 0.1 mM ninhydrin (*not shown*). Shown is the mean ± SD of three experiments.

cation that the reagent did not affect the electrical gradient dissipation system.

Discussion

Several reports indicate that a high-affinity, carrier-mediated, iron transport system is present in the membrane of a number of cells (Egyed, 1988; Morgan, 1988; Sturrock et al., 1990; Wright & Lake, 1990; Kaplan, Jordan & Sturrock, 1991; Eide et al., 1992). In contrast to transferrins, whose primary, secondary and tertiary structures have been elucidated (*reviewed in* Aisen, Leibman & Zweier, 1978; Crichton & Ward, 1992), the structure of the iron transporter is unknown. In this context, this work is the first approach of identifying amino acid residues involved in the transmembrane transport of iron. Since modifications were performed in sealed reticulocyte ghosts, the residues affected were mainly those acceded from the extracellular medium. This should correspond to the luminal side in the early endocytic vesicle.

Of all the modifiers tested, only ninhydrin and phenylglyoxal, both modifiers of arginyl residues, inhibited the initial rate of iron uptake. This inhibition was overcome by A23187 but not by valinomycin, an indication that the iron transport process, and not the

Table 2. Effect of A23187 and valinomycin on the initial rate of iron uptake by control and ninhydrin-treated ghosts

Treatment	Initial rate ± SD (n) (mol Fe/mg protein/sec)	$P <$
(A)		
Control	$1.50 \pm 0.49 \times 10^{-10}$ (7)	
Control + A23187	$2.76 \pm 0.55 \times 10^{-10}$ (6)	0.002
Control + valinomycin	$1.37 \pm 0.69 \times 10^{-10}$ (6)	NS
(B)		
Ninhydrin	$0.48 \pm 0.22 \times 10^{-10}$ (7)	0.001
Ninhydrin + A23187	$1.67 \pm 0.64 \times 10^{-10}$ (6)	NS
Ninhydrin + valinomycin	$0.78 \pm 0.32 \times 10^{-10}$ (8)	0.01

Sealed ghosts were treated with or without 0.1 mM ninhydrin, and subjected to $^{59}Fe^{2+}$ uptake kinetics with or without 1 μM A23187 or 1 μM valinomycin. Shown are the mean ± standard deviation and, in parentheses, the number of experiments. The Student t-test for differences between two means was applied for control (A) and ninhydrin-treated (B) ghosts; NS, not significantly different to control, $P > 0.05$.

possibly simultaneous electrical gradient dissipation process, was being primarily affected. Additionally, the inhibition produced by ninhydrin and phenylglyoxal was blocked by Fe^{2+} added during the modification step. Therefore, the modified arginyl residue should be in the proximity of a Fe^{2+} binding site involved in the transport of this ion.

The reaction of α-dicarboxyl compounds as phenylglyoxal and ninhydrin with arginyl residues is a result of the nucleophilic addition of the neighboring carbonyl groups to the guanidine group in arginine forming a double carbinolamine adduct. The first C-N bond formed is stabilized by the electron-withdrawing property of the vicinal carbonyl group (Patthy & Thész, 1980). Although camphorquinone, cyclohexanedione and hydroxyphenylglyoxal work through the same nucleophilic addition, they did not inhibit the rate of iron uptake. The reason may be a differential reactivity of these reagents towards the arginyl residue critical for iron transport. For example, the reactivity of transferrin is markedly higher towards butanedione than towards phenylglyoxal (Rogers, Borresen & Feeney, 1978) while phenylglyoxal is several-fold more reactive than glyoxal with ribonuclease A (Takahashi, 1968). Since the modifying reagents differ in size and polarity, one of the major causes for the lack of inhibition may be their accessibility to the reaction site: while phenylglyoxal was an extremely effective inhibitor, the more polar hydroxyphenylglyoxal was not. The above suggests that the modified site could be in a hydrophobic pocket.

A distinctive feature of arginyl residue modification is its pH dependence, exhibiting more extensive modification at alkaline pHs (Patthy & Thész, 1980; Yamasaki et al., 1980; Strévey et al., 1992). The experiments reported here show that the effect of ninhydrin or phenylglyoxal was maximal when the modification was done at pH 8–10, a strong suggestion that the modified residue was in the unprotonated form (Patthy & Thész, 1980). A paradoxical effect was observed when the modification was done at pHs 6.0, where the modified ghosts presented a higher rate of iron uptake than controls. It is possible that at this pH a residue different to arginine was modified. As ninhydrin and phenylglyoxal are electrophilic reagents (Chaplin, 1976; Tedeschi et al., 1992), the target residue should have available electrons at pH 6.0 as, for example, histidine. Importantly, such a residue should exert a negative control on the rate of iron uptake.

Because of its positive charge at physiological pHs, arginyl residues in proteins are considered anion-binding sites (Patthy & Thész, 1980). In lactoferrin, a carbonate functioning as a bidentate ligand bridges the interaction between arginine 121 and iron (Anderson et al., 1989), while in transferrin two arginyl residues, Arg 121 and Arg 210, are involved in the binding site for iron (Anderson et al., 1990). Since at physiological pH arginyl residues are charged positively, it is likely that a putative interaction between arginine and iron in the transporter could be mediated by a bidentate ligand such as carbonate.

We conclude that the iron transporter from the plasma membrane of erythroid cells has at least one arginyl residue in a segment accessible from the extracellular medium, and that this residue is involved in the transmembrane transport of iron. Moreover, an electronegative residue, reactive with ninhydrin and phenylglyoxal at pH 6.0, could exert a negative control on the rate of iron uptake through the transporter.

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